MEDICAL INTELLIGENCE UNIT 15

Prem Seth

Adenoviruses: Basic Biology to Gene Therapy

R.G. LANDES COMPANY

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Des Moines, Iowa, U.S.A.
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PREFACE

Ever since their discovery adenoviruses have proven to be a tremendous asset to biologists. Through the study of the adenoviruses, we have learned not only about the virus structures, mechanisms of viral replication, but also about eukaryotic gene expression, alternative splicing, regulation of cell cycle progression, and apoptosis. In the last five years, there has been an explosion in the use of adenoviruses as vectors for gene transfer to a variety of mammalian cells. Adenoviral vectors are also being tested in Phase 1 clinical trials for cystic fibrosis and for many kinds of cancers. These recent developments in utilizing adenoviral vectors for gene therapy have rejuvenated an interest in the basic science of adenovirus research. More importantly, it has generated a necessity for a single volume that covers both the biology of adenoviruses as well as our progress in the use of adenoviruses as vectors for gene therapy. This book was written precisely to fulfill such a need.

The book is divided into six sections to review all the essential topics. The first section describes the historic discovery and classification of adenoviruses. This section also reviews the protein and the genomic structures of adenoviruses. The second section describes the various steps involved in adenovirus replication in host cells. These steps begin with the entry of the adenovirus into the cell, and include the early gene expression, DNA replication, late gene expression, and adenovirus assembly. This section also describes the role of adenoviral endoproteases during viral infection. The third section describes the principles and methods of adenoviral vector development, and the preclinical evaluation of adenoviral vectors for the gene therapy of various diseases. Some of the target diseases discussed are cancer, cardiovascular diseases, neurological disorders, and muscular diseases. Other chapters in this section describe the use of adenoviral vectors in lipoprotein research, to correct enzyme deficiencies, and for vaccine development. The fourth section describes various approaches to generate targetable adenoviral vectors. These include the development of adenoviral chimeras with retroviruses and adeno-associated viruses, transcriptional and promoter targeting through the adenoviral vectors, and the use of adenoviruses replication-restricted for cancer. The final chapter in this section discusses how the relative resistance of hematopoietic cells to adenoviral infection can be exploited for selectively killing tumor cells during bone marrow purging. The fifth section discusses many of the safety issues involved in the use of adenoviral vectors for gene therapy. These include potential oncogenicity and transformation by adenoviruses, homologous recombination between the adenovirus and the host genome, and adenovirus-induced diseases. The last three chapters in this section describe how adenoviruses subvert the

normal immune response, the role of innate immunity in adenoviral infection, and the host immune responses against the adenoviruses and some of the strategies currently being employed to circumvent these problems. The final section describes the current status of ongoing clinical protocols using recombinant adenoviruses. One chapter describes a clinical protocol for cystic fibrosis, and the remaining chapters discuss the clinical trials for cancer using tumor suppressor genes and suicide genes.

Due to the complexity and the range of the topics that had to be covered, this book is written with the help of many outstanding scientists who specialize in varied aspects of adenovirus research. Each chapter in this volume presents state of the art description of past accomplishments, current status, future directions and the prospects of the particular theme of adenovirus research. This book should be useful to both researchers and clinicians interested in using adenoviral vectors for basic research and for gene therapy. Junior investigators, particularly graduate students and post-doctoral fellows in the medical discipline should also find this book a valuable reading resource.

Clearly, this book would not have been possible without the contribution of each author to whom I am very grateful. In spite of their busy schedule, all authors contributed their chapters in a timely manner, and wrote succint and focused chapters. I would also like to acknowledge my wife Reva, our parents, brothers, sisters, nieces and nephews for their constant support and encouragement to do this project. I am very much thankful to Priya and Kajal for their love and affection. I am grateful to Dr. Ira Pastan for providing mentorship during the earlier part of my career. I am also thankful to many friends and colleagues for providing numerous intellectual discussions over the years. Finally I would like to thank the publisher Dr. R.G. Landes, for giving me the opportunity to carry out this exciting project.

Like any other difficult project, this book is also likely to have some deficiencies, and perhaps some very important topics are not adequately covered, for which I apologize. I also express my regrets to numerous scientists whose work could not be represented in this volume.

Prem Seth
Des Moines, Iowa, U.S.A.

Chapter 1

Discovery and Classification of Adenoviruses

Harold S. Ginsberg

Discovery of Adenoviruses

In 1953, Wallace Rowe was then a postdoctoral fellow at the National Institutes of Health with Robert Huebner; they were working, with their colleagues, to isolate the "virus of the common cold." Toward this goal, Dr. Rowe was using explants of adenoids and tonsils grown in cell culture. He noted, in some cultures of adenoid cells, that they had rounded and clumped (note that the cells did not lyse, and that adenoviruses do not produce a "lytic" infection either in vitro or in vivo). Being a very smart young virologist, he decided to determine whether this cytopathic effect was due to a viral infection of the cells. He readily showed that he could serially pass the causative agent and that it was undoubedly a virus that had been latent in the adenoid cells.[1]

In 1954, Maurice Hilleman, who was then in the U.S. Army, investigated an epidemic of acute febrile respiratory disease thought to be influenza in a company of recruits at Fort Leonard Wood. Dr. Hilleman could not, however, isolate an influenza virus from any of the respiratory tract specimens, although he and Werner isolated a virus in cultured human tracheal cells. Dr. Hilleman was certain that if the etiologic agent was the influenza virus, he could isolate it and identify it; therefore, he considered it to be some other agent, perhaps a new one.[2] During World II, a new, acute respiratory disease in recruits had been recognized and studied extensively by the Commission on Acute Respiratory Diseases, of which Colonel John Dingle was the Director. The Commission had done human volunteer studies demonstrating that it was a transmissible acute viral disease. Dr. Hilleman, therefore, came to visit Dr. Dingle to ask if he would determine whether his virus might be the etiologic agent of the acute respiratory disease (ARD) in the recruits. By that time, in 1954, Dr. Dingle was Professor and Chairman of the Department of Preventive Medicine at Western Reserve University (now Case-Western Reserve University) School of Medicine in Cleveland, Ohio.

I was an Associate Professor at Western Reserve at the time and in a fortunate position. I was interested in the field of latent infections resulting from a virus, pneumonia virus of mice (now known to be a mouse respiratory syncytium virus), discovered while I was a post-doctoral fellow at the then Rockefeller Institute (now University) after leaving the Army. Dr. Robert Huebner came to Western Reserve to present a seminar and came to my laboratory to visit. He told me of Wally Rowe's viral isolation from adenoid cultures, and immediately recognized that it was a latent or persistent agent in adenoids. I asked Dr. Huebner if they would send me the virus after they had published their results. He immediately said, "You

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do not have to wait until then," and he telephoned Dr. Rowe and told him to send me the virus—can you imagine that happening at this time in history?

Dr. Hilleman shortly thereafter brought the agent, which he considered to be a virus, to Western Reserve, and asked Dr. Dingle whether he would test the specimens gathered from the ARD human volunteer experiments at Fort Bragg to see if the agent he had isolated was the etiologic agent of the acute respiratory disease in Armed Forces recruits—he knew that if it were the influenza virus, he would have isolated it. Dr. Dingle asked me if I would carry out studies to determine whether the virus that Hilleman and Werner had isolated was the virus causing ARD, and of course I was delighted and excited to do so. I soon showed that the viruses that both groups had isolated could be replicated in a continous respiratory, epithelial cell line that I had growing in the laboratory. We then demonstrated, using complement-fixation and neutralization tests, that the Hilleman and Werner virus was, indeed, the etiologic agent of Acute Respiratory Disease of Recruits.[3] It was then demonstrated, using a complement-fixation analysis, that the Hilleman and Werner virus and the Wallace Rowe et al virus were related, although they were clearly different viruses according to neutralization titrations. Subsequent studies have shown that all adenoviruses are immunologically related through a common antigen on the hexon protein detected using complement-fixation titrations.[4] The Hilleman and Werner etiologic agent of Acute Respiratory Disease of Recruits (ARD) was subsequently classified as type 4 adenovirus, which appears to be the major etiologic agent of ARD, although types 3 and 7 have also been isolated, albeit only occasionally, from cases of this disease.[5] The virologists that originally isolated these new viruses initially called them adenoid degeneration virus, adenoid-pharyngeal virus, and acute respiratory disease virus until Drs. Enders, Bell, Dingle et al in 1956 recommended the presently accepted name adenoviruses[6] in accord with the original cells in which the first virus was isolated.

Trentin and his coworkers in 1956 made the exciting discovery that type 12 adenovirus produced malignant tumors when inoculated into newborn hamsters,[7] which was the initial finding that a human virus was oncogenic. It must be noted, however, that there is no evidence that the type 12 or any of the other adenoviruses produce malignancies in humans.

One of the most important findings in adenovirus history was Sharp and colleagues' discovery of mRNA splicing.[8] This critical finding has impacted throughout the field of mRNA synthesis.

Classification

To date, adenoviruses have been classified into two genera: Mastadenoviruses and Aviadenoviruses, which have not yet been as completely studied as the Mastadenoviruses. A third genus has been proposed for viruses that infect calves. These viruses are similar to adenoviruses, but a number of differences from classical adenoviruses have led the International Viral Nomenclature Committee to be unwilling to accept this genus to date. The Mastadenovirus genus contains 49 distinct viruses related according to a common complement-fixation antigen located on the major capsid protein, the hexon.[9] The Mastadenoviruses include human, simian, bovine, porcine, canine, ovine, and opossum viruses. The Aviadenoviruses , however, infect only birds.

Human adenoviruses are divided into 49 specific types according to neutralization assays (Table 1.1). The major antigen responsible for each specific serotype is a surface component of the hexon capsid protein,[10] not the tip of the fiber which is the component responsible for attachment to susceptible cells for infection. Antibodies will of course neutralize the virus if there are sufficient anti-fiber antibodies to attach to every one of the twelve fiber tips—certainly not satisfactory for an effective vaccine. The human adenoviruses are classified into six subgroups, primarily on the basis of the the percentage of the guanine-cytosine in

Table 1.1. Classification of human adenoviruses

Subgroups	Hemagglutination groups	Serotypes	%G-C DNA
A IV	Little or no agglutination	12,18,31	48-49
B I	Complete agglutination in rat RBC	3,7,11,14,16,21, 34,35	50-52
C III	Partial agglutination in rat RBC	1,2,5,6	57-59
D II	Complete agglutination in rat RBC	8,9,10,13,15,17, 19,20,22-30,33, 36-39,42-47	57-61
D?	Complete agglutination in rat RBC	48,49	?
E III	Partial agglutination in rat RBC	4	57-59
F III	Partial agglutination in rat RBC	40,41	57-59

their DNAs (Table 1.1). To a lesser extent, they can be classified into four groups according to their characteristics of hemagglutination (Table 1.1). The most recently isolated types 48 and 49[11] have been identified on the basis of neutralization assays and shown to belong to the human Mastadenovirus genus according to its common complement-fixing hexon antigen. They belong to subgroup D on the basis of hemagglutination assays, but their guanine-cytosine content in DNA has not been determined (Table 1.1). It should be noted, however, that restriction endonuclease analyses have also indicated that they are members of subgroup D.

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Chapter 2

Adenovirus Capsid Proteins

John J. Rux and Roger M. Burnett

Since Rowe et al[1] first isolated adenovirus from human adenoid cells, more than 100 different species have been identified from various mammals. The family is characterized by the distinctive architecture of the virion, or virus particle, which is formed from a well defined set of structural proteins. Human adenoviruses, which form the most studied group, cause a variety of diseases ranging in severity from respiratory infections and conjunctivitis to the severe enteric dysentery that is a leading cause of death in Third World infants.[2] Most human adults have experienced the mild respiratory infections due to the prototypical type 2 adenovirus (Ad2) or the related Ad5.

In this chapter, the focus will be on describing the adenovirus capsid in terms of its overall architecture and its individual proteins. The relationship between the structural and biological properties of adenovirus will be discussed.

Virion Architecture

The adenovirus capsid proteins combine to form an icosahedrally-shaped shell (Fig. 2.1A) that surrounds the DNA-protein core of the virion. Early electron micrographs of negatively stained virions revealed that the capsid contains 252 surface projections, called capsomeres, and that long fibers extend from each of its 12 vertices. The capsomeres were classified as pentons or hexons depending on whether they are surrounded by five or six neighboring capsomeres. Analysis of the virus by SDS-PAGE revealed that it contains eleven structural proteins designated II to XII in the order of decreasing apparent molecular mass (Fig. 2.1B). Table 2.1 summarizes what is currently known about adenovirus structural proteins for the Ad2 virion, while indicating their variation in size within other types.

The 20 triangular facets of the capsid (Fig. 2.1A,B) are each formed from 12 copies of hexon (polypeptide II). Pentons, a complex of penton base (polypeptide III) and fiber (polypeptide IV), are located at each of the fivefold vertices. The diameter of the sphere enclosing the fivefold vertices is 914 Å, three times larger that that for poliovirus.[3] The particle mass of Ad2 is at least 150 x 10^6 Da, of which 22.6 x 10^6 Da is DNA.[4]

The first clues to the capsid architecture were obtained from studying the dissociation pattern of the virion and capsid components. Virions disrupted under mild conditions initially lose the vertex pentons (Fig. 2.2), then the adjacent peripentonal hexons, and finally the remaining planar groups-of-nine hexons (GONs), leaving only the core. The GONs dissociate in a non-random pattern that can be explained by the presence of a minor capsid protein, polypeptide IX, that binds between hexons and acts as a capsid "cement."[5]

The current model of the virion (Figs. 2.1, 2.2) is derived from a three dimensional image obtained using electron microscopy (EM) and image reconstruction, which provides a complete view of the virion at 35 Å resolution.[3] The EM reconstruction (Fig. 2.1) confirmed that hexons pack as a small two dimensional array on each facet (Fig. 2.2), but showed that

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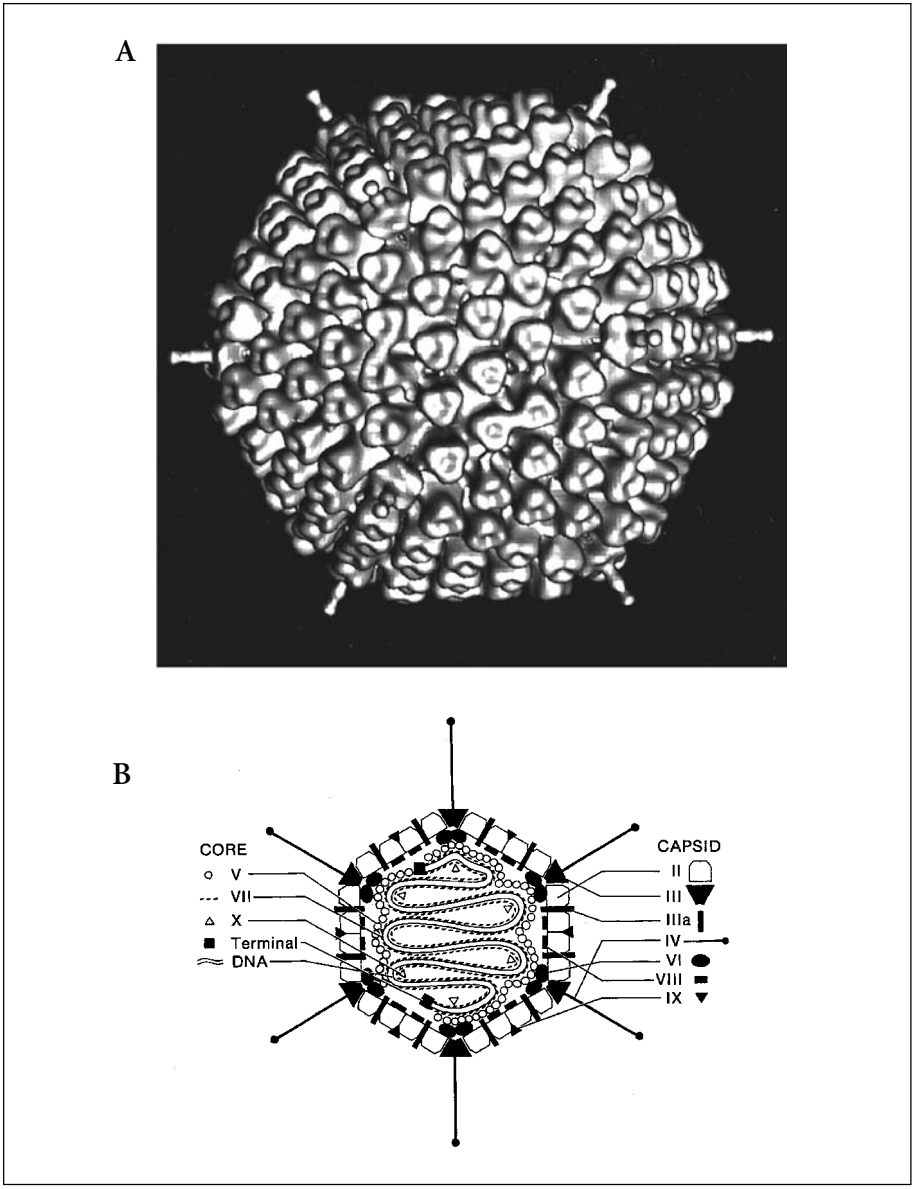

Fig. 2.1. Adenovirus virion. (A) The three-dimensional image reconstruction of the icosahedral capsid is viewed along the threefold axis. The major coat protein hexon has a triangular top with three towers. Fibers protrude from the penton bases at each fivefold vertex, but only the first third of the shaft is imaged as the remainder is washed out by the averaging method. Note that the capsid is rounded to provide a continuous protein shell (cf. the model in Fig. 2.2). (B) A stylized section summarizing current structural knowledge of the polypeptide components and the viral DNA. No single real section through the icosahedral virion would contain all these components. Reprinted with permission from Stewart PL, Burnett RM. Jpn J Appl Phys 1993; 32:1342-1347. ©1993 Japanese Journal of Applied Physics.

the capsid is significantly rounded to bring the hexons along the edges into close contact. Thus, close-packing of hexons occurs throughout the capsid despite the completely different chemical nature of the hexon-hexon interfaces at the edge and within the facet (Fig. 2.2). It was possible to extend the EM imaging by creating a three dimensional "difference map."[6] This was formed by subtracting a capsid simulated from the 2.9 Å resolution crystal structure of Ad2 hexon[7] from the EM reconstruction. This map not only provided a clearer view of the penton base and fiber, and the radial position of polypeptide IX, but also permitted the assignment of two additional minor proteins, polypeptides IIIa and VI.

Major Coat Proteins

Hexon (Polypeptide II)

Hexon is the most abundant of the structural proteins, accounting for approximately 80% of the capsid mass. There are 240 copies of the homotrimeric hexon molecule in the adenovirus capsid. The Ad2 hexon polypetide chain contains 967 amino acids, each with a molecular mass of 109,077 daltons (including the acetylated N-terminus). This is the longest of the known hexon sequences, which range from 919-967 residues (100-109 kDa) (Table 2.1). The three dimensional structure of Ad2 hexon has been determined by X-ray crystallography.[7] The hexon subunit (Fig. 2.3) has two similar β-barrel domains in its base. In the trimer, these domains form each corner of a hollow pseudo-hexagon. Electron micrographs of isolated hexons show this characteristic morphology. The β-barrels in hexon have the same folding topology as found in the coat proteins of almost all spherical viruses,[8] and in other proteins such as tumor necrosis factor. In adenovirus, the β-barrel axes are normal to the capsid surface. This orientation is similar to that in tumor necrosis factor, but contrasts with the mostly in-plane orientation of the β-barrels in other virus capsids.

The top of the hexon molecule, in contrast to the base, has a triangular shape with three "towers." These are formed from three long loops that splay out from the β-barrels (Fig. 2.3). Remarkably, each subunit contributes a different one of its three loops to form each tower domain. Thus, each tower is composed of three different loops, one from each subunit. Moreover, at the base of the molecule, an N-terminal arm from each subunit passes underneath the neighboring subunits. The molecule is therefore not only composed of entwined chains, but the cyclic symmetry causes the subunits to clasp one another, both at the top and the bottom. The result is that the trimer is locked together so that a subunit cannot be extricated without disrupting both tertiary and quaternary molecular structure (Fig. 2.4). These features make the trimer highly resistant to proteolysis, and so stable that it retains its physical and immunological characteristics even after exposure to 8 M urea.[9] As incorporation of 240 very stable trimers into the virion will clearly be less error-prone than the addition of 720 monomers, hexon's construction is an important factor in the accurate assembly and ultimate stability of the virion.

Hexon is almost hollow, as it has a large cavity in the base and a depression between the towers. This unusual "tubular" shape increases the solvent-accessible surface area and so reduces the hydrophobic contribution to molecular stability. However, this negative effect on stability is more than compensated for by the very large inter-subunit contact area that is buried upon trimer formation. As is common with multimeric proteins, each subunit interface surface has scattered hydrophobic patches that are complementary to patches on the neighboring surface. This feature ensures that the subunit is at least partially soluble and guides its accurate alignment with its neighbor. Due to its unusual topology, an isolated hexon monomer would be highly unstable, which suggests why transient complex formation with the adenovirus 100K protein is a prerequisite for correct folding of the hexon trimer.

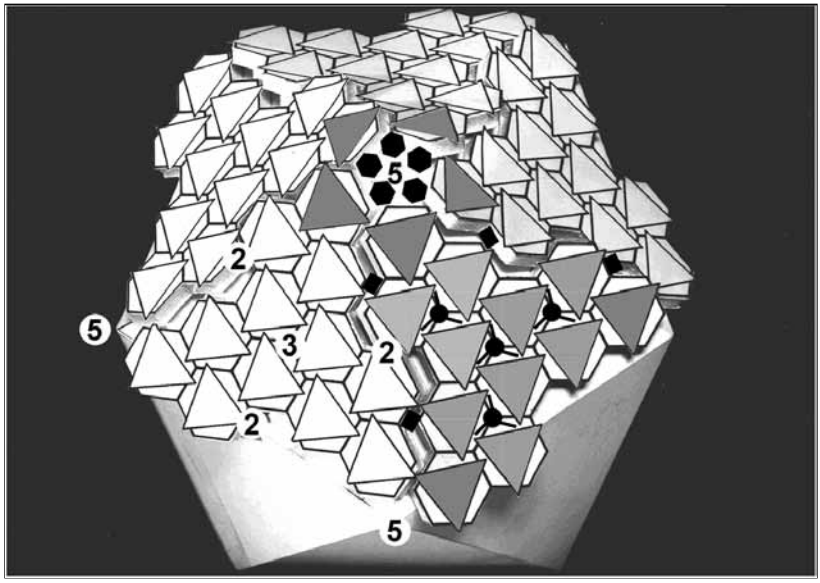

Fig. 2.2. Adenovirus capsid model. Five of the 20 planar facets are shown superimposed on an ideal icosahedron. Each facet contains 12 hexons, which are represented as a triangular top superimposed on an hexagonal base. The positions of the icosahedral symmetry axes are indicated on the lower left facet. The penton complex, which lies at each of the 12 vertices, is not shown in the model. The positions of the minor proteins are indicated on the lower right facet. Polypeptide IX (solid circle with three arms) binds as a trimer in four symmetry-related locations within the facet. It lies in a cavity formed between the towers of three different hexon molecules and cements the central nine hexons in a facet into the GON (highlighted with a pattern). Two copies of the monomeric polypeptide IIIa (solid square) penetrate each edge to rivet two facets together. A ring of five hexamers of polypeptide VI (solid hexagon) lies underneath five peripentonal hexons (shaded gray) to attach them to the core and provide stability at the vertices. Upon dissociation, the penton complex is lost, followed by the peripentonal hexons, which are not cemented into the facet. The GONs then dissociate as stable groups. Reprinted with permission from Burnett RM. The structure of adenovirus. In: Chiu W, Burnett RM, Garcia RL, eds. Structural biology of viruses. New York: Oxford University Press, 1997:209-238. ©1997 Oxford University Press, Inc.

The hexon gene is being employed as an excellent probe in clinical assays to detect adenovirus. As these employ DNA hybridization and polymerase chain reaction techniques, the hexon sequence database is rapidly expanding and is becoming important in understanding adenovirus evolution. Studies that map hexon sequences from other types onto the Ad2 structure suggest that their structures are not very different from that of Ad2. Within the individual subgroups C (Ad2 and Ad5) and F (Ad40 and 41), hexons have around 90% similarity. Most of the non-conserved residues are located in the tower regions at the top of the molecule, while the base is highly conserved. As deletions are found in all types with respect to Ad2, this species may be early in adenovirus evolution.

The immunological properties of adenovirus, such as the positions of the group- and type-specific epitopes, are not nearly as well determined as those for viruses like influenza virus and poliovirus that pose more of a problem to human health. Hexon contains both

Table 2.1. Adenovirus structural proteins

Polypeptide	Molecular Mass of Ad2 Monomer (Da)[a]	Number of Residues in Ad2 Monomer[a]	Polypeptide Length Range[b]	Biochemical Copy Number of Monomer[a]	Copy Number of Protein in Current Model
II (hexon)	109,077	967	919 - 967	720 ± 7	240 Trimers
III (penton Base)	63,296	571	497 - 571	56 ± 1	12 Pentamers
IIIa					
external portion	41,689	376[d]			
internal portion	21,846	194[d]			
Total	63,535[c]	570[c]	582 - 585	68 ± 2[c]	60 Monomers
IV (fiber)	61,960	582	319 - 587	35 ± 1	12 Trimers
V (core)	41,631	368	347 - 369	157 ± 1	____[e]
Terminal[f] (core)	~55,000	~500	606 - 653	2	____
VI	22,118[c]	206[c]	250 - 267	342 ± 4[c]	60 Hexamers
VII (core)	19,412	174	193 - 198	833 ± 19	____
VIII	15,390[c]	140[c]	227 - 233	127 ± 3[c]	____
IX	14,339	139	132 - 144	247 ± 2	80 Trimers
μ[g] (core)	~4,000	~36	70 - 79	~104[g]	____

Adapted from Stewart PL, Burnett RM. Curr Top Microbiol Immunol 1995; 199(1):25-38. [a]The molecular masses, residue numbers and biochemically determined copy numbers for the monomers are for human adenovirus type 2 according to van Oostrum and Burnett[4] but have been updated using the adenovirus protease cleavage sites given by Anderson;[24] [b]The range in length for the full-length (uncleaved) polypeptide was compiled for all the human adenovirus proteins listed in the Swiss-Prot database; [c]Proteins for which the adenovirus protease cleavage sites of Anderson[24] were used; [d]estimated division; [e]Dashes indicate proteins that have not yet been imaged; [f]Estimates by Rekosh;[25] [g]Estimates by Hosokawa and Sung.[26]

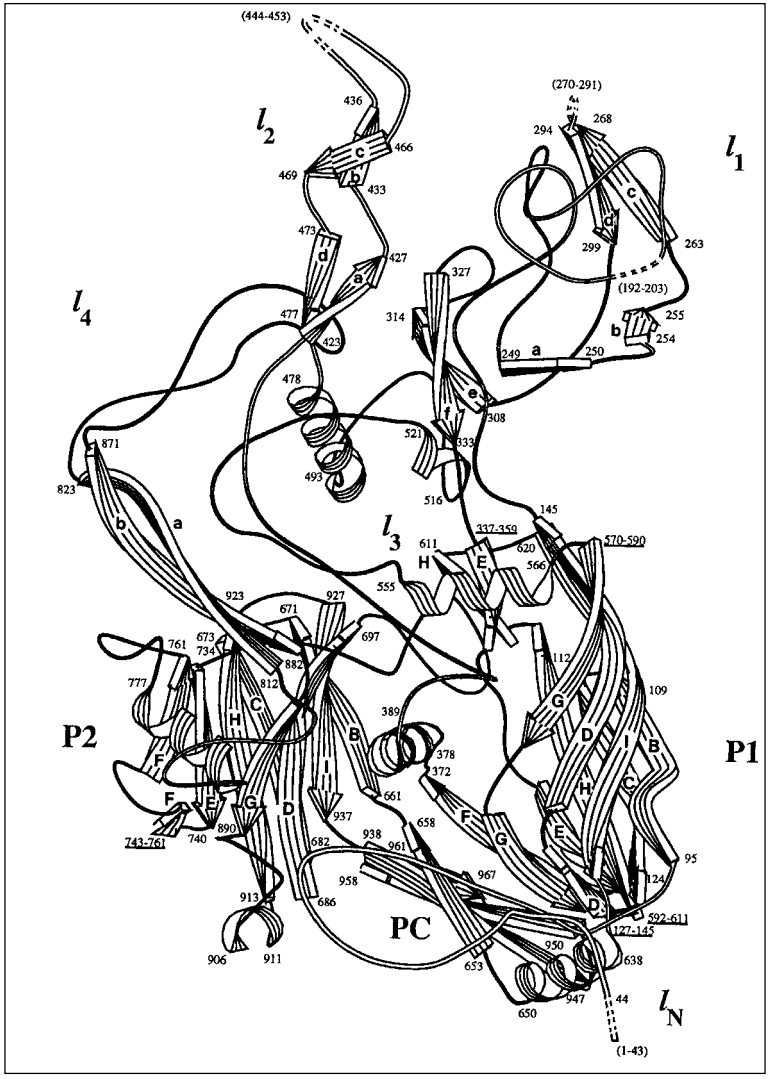

Fig. 2.3. Hexon subunit. A ribbon representation of the Ad2 molecule viewed in a direction perpendicular to the molecular threefold axis and from inside the molecule. In the virion, the hexon tops form the outer surface of each facet (see Fig. 2.1A). The top is formed from loops arising from two eight-stranded β-barrels in the base, P1 and P2. These are connected and stabilized below by the pedestal connector, PC, and above by loop l_3 from P2. The top is formed from loops l_1 and l_2 from P1, and loop l_4 from P2. The sequence numbers indicate the beginning and end of the α-helices and β-strands. The latter are labeled with capital letters in the base and lower case letters in the loops. Strands in which discontinuities occur are indicated by underlining their sequence numbers. The dashed traces indicate four stretches that were not defined in the crystallographic model at 2.9 Å resolution. Reprinted with permission from Athappilly FK, Murali R, Rux JJ, Cai Z, Burnett RM. J Mol Biol 1994; 242:430-455. ©1994 Academic Press Limited.

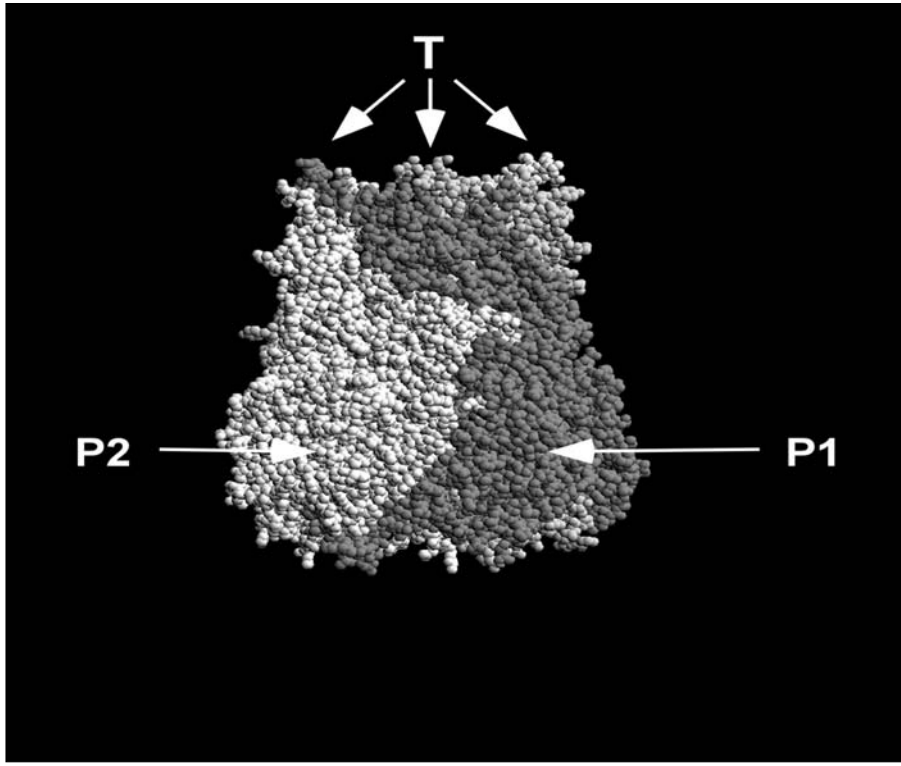

Fig. 2.4. Hexon trimer. The Ad2 hexon trimer is shown as a space-filling model with the individual subunits in different shades of gray. The view is from the side, normal to the threefold axis, with the interface between two subunits at the front. The subunits are curved vertically so that each clasps its neighbor. At the top of the molecule, each tower domain (T) is formed from three loops (l_1, l_2, and l_4), one from each subunit. The base of the molecule is formed from two eight-stranded β-barrel domains (P1, P2) in each subunit. The molecule was rendered with the program O.

group- and type-specific determinants, but these have not, in general, been correlated with the primary sequence or three dimensional structure. The best-characterized[10] are type-specific sites in Ad2 and Ad5 (subgroup C). They are located in regions of high sequence variability in the *l1* and *l2* loops (Fig. 2.3), which form the top of the molecule and the outer surface of the virion. These regions in hexon are sufficiently mobile to render their electron density invisible in the crystal structure at 2.9 Å resolution, although they could be seen at the lower resolution (35 Å) of the EM image.[6]

Penton Base (Polypeptide III)

The penton complex at the vertex is formed from penton base, a pentamer of polypeptide III (56-63 kDa), and fiber, which is a trimer (see below). Thus, an intriguing symmetry mismatch occurs within the penton complex. Early EM studies indicated that penton base

has a spade-like shape, with a polygonal cross-section that sometimes revealed a hole. Later EM studies on the penton complex show that fiber is embedded in the central ~30 Å diameter core of the hollow penton base. This information was used to define the molecular boundaries for Ad2 penton in the difference image[6] and so delineate the two separate proteins in the complex. The penton base has a height of 124 Å, and maximum and minimum diameters of 112 and 50 Å. There are five small protrusions, roughly 22 Å in diameter, on the top that contain an Arg-Gly-Asp (RGD) recognition sequence for an integrin receptor that mediates internalization.[11]

A recent reconstruction of an Ad3 penton dodecahedron structure,[12] with and without fiber, more clearly establishes the penton base boundary and reveals that the penton base undergoes a conformational change upon fiber binding. In addition, the penton base is hollow and so the fiber is thought to bind to the exterior of the Ad3 penton base. This conclusion differs from the "pole and socket" model of binding proposed in the Ad2 EM studies.[6] It should be noted that the low resolution of EM makes the exact assignment of molecular boundaries quite difficult for proteins not clearly separated from their neighbors.

Fiber (Polypeptide IV)

The fiber, a trimer of polypeptide IV (35-62 kDa), consists of a long "shaft" that is often kinked, terminated by a "knob." Fiber was the second structural protein to be crystallized,[13] but a structure determination has been elusive as the crystals are not well ordered,[14] presumably due to the length of the molecule. This problem was eliminated in the structure determination of the C-terminal knob at the end of the fiber, which was accomplished with recombinant type 5 protein (Fig. 2.5.)[15] The structure suggested a position for the primary receptor-binding site and provided unambiguous proof that the fiber is trimeric. The construction of the knob is similar to that of hexon, with β-barrels forming the core of each subunit, and the remainder formed from turns and loops connecting the individual β-strands. As in hexon, the β-barrels have eight strands, but their folding topology is different, and is unlike that for any other known structure.

The primary receptor-binding site is not precisely known, but has been assigned to the upper surface of the knob,[15] while the shaft emerges from below. The top has a deep central depression and three radial valleys that contain conserved residues. This situation is reminiscent of the "Canyon Hypothesis" developed for rhinovirus,[16] where a conserved binding site is protected from antibody binding by the steric hindrance provided by the surrounding non-conserved residues. Thus, Xia et al[15] postulated that there are two possible binding modes. The first would be with the cellular receptor[17] binding to the central depression, presumably through trimeric interactions. In the second, up to three dimeric receptors would bind to the valleys. The second mode would explain the knob's very low dissociation constant.

The N-terminal shaft region of the fiber contains repeating sequences of approximately 15 residues. The number of repeats is characteristic of the adenovirus type, as it defines the length of its shaft. The shaft is both very rigid and very narrow, consistent with a triple helical shaft model.[18] However, direct experimental evidence to support this model has been difficult to obtain. Although the recombinant knob protein used to determine the structure[15] includes 15 residues of the 22nd repeating unit of the Ad5 shaft domain, the first 7 are disordered in the crystal structure. Likewise, although a short portion of the thin, 37 Å diameter, fiber was visible in the Ad2 EM reconstruction,[3] the icosahedral averaging method imposes five-fold symmetry on the three-fold shaft and destroys the image. Thus, although the structure of the shaft is of great interest, detailed information is still not available.

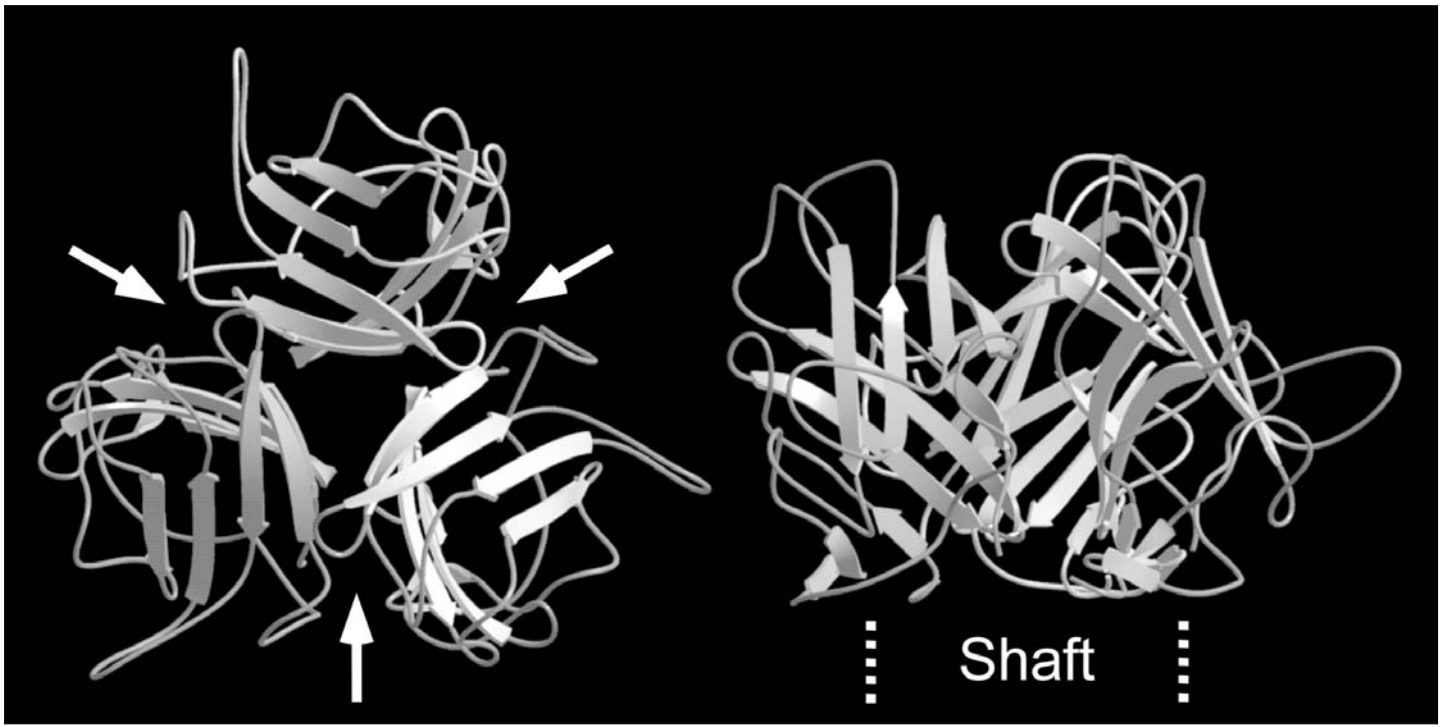

Fig. 2.5. Fiber knob. The structure of the Ad5 knob at the end of the trimeric fiber. Each subunit contains an eight-stranded β-barrel (strands shown as ribbons) that is topologically unrelated to that of hexon. (A) The view is down the threefold molecular axis, towards the virus. The trimeric knob resembles a three-bladed propeller, with a central depression between the β-barrels from which three valleys (arrows) radiate out along the subunit interfaces. The receptor-binding site is presumed to lie in these surface depressions. (B) The knob is rotated about the horizontal so that it is viewed from the side. The shaft would be attached at the bottom (dotted lines). The molecule was rendered with the program Setor.

Minor Coat Proteins

It has long been a puzzle why adenovirus, and other complex viruses, contain so many minor proteins. An early proposal was that polypeptides IIIa, VI, VIII and IX (Table 2.1), may act as capsid cement. Direct evidence that polypeptide IX stabilizes the virion, but is not required, is given by the assembly of mutants lacking the protein into virions that are more thermolabile than wild type[19] and cannot package full length DNA.[20]

Although the minor proteins are difficult to discern in EM images alone, the difference images[6] revealed capsid locations for all but polypeptide VIII. Their binding sites suggest the specific role that each protein plays in stabilizing the capsid. Polypeptide IX cements hexon-hexon contacts within the center of a facet; polypeptide IIIa spans the capsid to "rivet" arrays of hexons on adjacent facets; and polypeptide VI anchors the rings of peripentonal hexons inside the capsid, and connects the capsid to the core.

The existence of these cementing proteins can be rationalized as a mechanism to overcome the conflicting requirements of weak interactions to guide the accurate assembly of the virion, and the strong interactions to ensure its stability.[21] It seems likely that cementing proteins will be found in other large macromolecular assemblies.

Polypeptide IIIa

The three dimensional difference image[6] of Ad2 showed that polypeptide IIIa (64-65 kDa) is present as a large elongated monomeric component. Although its main bulk (~42 kDa) is on the outer surface of the virion where it contacts four different hexons, it tapers to span the capsid and reach the inside. Its role appears to be that of a rivet whose function is to hold the capsid facets together.

Polypeptide VI

Polypeptide VI (27-29 kDa full length, ~22 kDa cleaved) has been assigned[6] to a position on the inner capsid surface. It anchors the rings of peripentonal hexons and connects the highly ordered capsid to the less ordered core region. The molecule has a trimeric shape, with three 29 Å diameter lobes separated by 46 Å, and connects the bases of two adjacent peripentonal hexons. As the volume of each lobe is consistent with it being a monomer, but the stoichiometry indicates that it should be a dimer (Table 2.1), it has been suggested that half of each polypeptide is disordered.[6] As the N-terminus of polypeptide VI is basic, it may interact with the internal nucleic acid. Crystallographic studies have shown that similar domains in other viral capsid proteins are frequently disordered.

Polypeptide VIII

Polypeptide VIII is very small (~25 kDa full length, ~14 kDa cleaved) and could not be identified in the Ad2 difference maps.[6] A previous assignment to the inside of the capsid[22] is supported by the fact that there was no unassigned external density in the difference map.

Polypeptide XI

Early EM work that focused on the GONs has led to polypeptide IX (14-15 kDa) being the best characterized of the minor proteins. The origin of the GONs was originally unclear, and initially was attributed to the 60 peripentonal hexons being somehow different from the 180 hexons within GONs. Later, the non-random dissociation pattern of GONs, the suggestion that polypeptide IX could act as a capsid "cement," and the determination that 12 copies of polypeptide IX bind to each facet, led to a model of the GON[4] in which 4 polypeptide IX trimers bind to 9 hexons.

Two and three dimensional difference imaging confirmed the four trimer model, accurately defined the monomer dimensions, and confirmed that polypeptide IX lies on the

outer surface. Each monomer extends from a local three-fold just above the hexon base along almost the entire length of a hexon-hexon interface. Polypeptide IX is not observed at sites adjacent to the peripentonal hexons. Although deletion mutants lacking the polypeptide IX gene can still form virions, these are less stable and GONs are not formed upon dissociation.[19]

Analysis of the polypeptide IX primary sequence predicts that monomers are α-helical and that the trimer has a coiled-coil structure. The shape of the trimer can be explained by a model in which each monomer has two α-helical arms. Each arm interacts in a coiled-coil arrangement with an arm of a neighboring subunit to form a trimer with three coiled-coils extending out from its center. The model agrees with the observed shape of the trimer and the size of its arms, which are estimated to be 64 Å long and 18 Å in diameter at their midpoint.[6]

Future Directions

It is clear that many fundamental aspects of the capsid structure and its relationship to the processes of viral infection remain unknown. The crystallographic studies that have provided atomic resolution structures for hexon and the fiber knob must be extended to the other capsid proteins. The distribution of the minor capsid proteins, polypeptides IIIa and VI, that has been deduced from electron microscopy and image reconstruction has yet to be experimentally confirmed. Analysis of the interactions between the recently identified primary receptor and fiber knob,[17] and secondary cellular receptor and penton base,[23] may provide valuable insights into basic adenovirus biology as well as aid in the development of improved adenovirus vectors for human gene therapy.

Acknowledgments

This work would not be possible without the contributions of the many talented people that have contributed to the study of adenovirus throughout the years. We apologize for the many omissions necessitated by the brevity of this chapter. Funding for this work has been provided by the National Institute for Allergy and Infectious Diseases (AI 17270) and the National Science Foundation (MCB 95-07102).

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Chapter 3

Organization of the Adenoviral Genome

Jane Flint

The DNA genomes of adenoviruses have been the objects of intense scrutiny since the first representative of this virus family was isolated in the winter of 1953-54.[1] Both the oncogenicity of human serotypes, first recognized in 1962,[2] and the expression of viral genetic information via cellular RNA polymerases II and III,[3-5] spurred initial interest in the molecular biology of adenoviruses. Studies of these viruses have provided numerous insights into fundamental cellular processes, including the production of eukaryotic mRNA by splicing[6,7] and control of cell cycle progression by tumor suppressor proteins of the Rb family (for reviews see refs. 8 and 9). We now know a great deal about the organization of adenoviral genetic information, the nature and functions of viral gene products, and the mechanisms that ensure their orderly production during the infectious cycle, as well as the mechanisms by which specific viral proteins can alter the growth and proliferation of cells in which they are made. Such knowledge is the foundation for more recent efforts to exploit adenoviruses as vectors for both experimental and therapeutic purposes.

All adenovirus genomes are linear, double-stranded DNA molecules, with an inverted terminal repetition (ITR), and a covalently attached terminal protein (TP) at the 5′ end of each strand. These DNA molecules vary in length from some 34,000 (Ad12) to over 43,000 (chicken embryo lethal orphan (CELO) virus) bp,[10,11] and in such features as GC-content, the length of the inverted terminal repetition and degree of sequence conservation (for reviews see refs. 11,12 and 13). While the organization of the genome is generally well conserved among adenoviruses that infect mammals (*Mastadenoviridae*), the genomes of avian adenoviruses exhibit some radical differences. For example, the genomes of chicken adenoviruses lack not only genes encoding certain structural proteins such as core protein V, but also homologs of the majority of early genes of mammalian isolates.[11,14] Rather, they contain at both their left and right ends substantial blocks (5-15 kbp) of sequences with no homologs in the genomes of *Mastadenoviridae*.

Members of human adenovirus subgroup C, such as Ad2 and Ad5, have received the lion's share of attention, for the simple historical reason that they can be propagated readily in many established lines of cultured human cells. Consequently, the first adenovirus genome to be sequenced in its entirety was that of Ad2, a feat accomplished in 1984.[15-17] The resulting quantum leap in both information about this viral genome and its amenability to experimental manipulation reinforced the traditional focus on this model member of the family, and its very close relative Ad5. In subsequent sections, the organization of the adenoviral genome is therefore described in terms of the human subgroup C archetype.

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Organization of Coding Sequences

RNA Polymerase II Transcription Units

The Ad2 genome comprises 35,936 bp, with an ITR of 102 bp.[15-17] Its most striking feature is the high density of coding sequences, for over 40 proteins, yet the presence of only 8 RNA polymerase II transcription units (Fig. 3.1). The efficient utilization of genetic information is a hallmark of viral genomes, whose genetic "space" is limited, for example, by the size of nucleic acid molecules that can be accommodated in an icosahedral capsid of fixed dimensions. However, adenoviruses appear to have mastered the art of minimizing the genetic information that must be devoted to the *cis*-acting signals that define, and control expression of, sequences encoding viral proteins: The great majority of the adenoviral RNA polymerase II transcription units are polycistronic, with individual coding sequences expressed by means of alternative processing of primary transcripts (Fig. 3.2).

Adenoviral transcription units, which may be located in either strand of the genome, are not organized according to any readily discernible principle, such as the time at which they are active during the infectious cycle (Fig. 3.1). They were originally named according to whether their mRNA products accumulated during the early (E) or late (L) phases of infection,[18] but no systematic nomenclature was subsequently developed or applied (see Fig. 3.1). Furthermore, the temporal program of viral transcription is more sophisticated than simple division into just two periods preceding and following initiation of viral DNA synthesis: 4 classes of transcription unit can be defined, according to the criteria of when they are first active during the infectious cycle and the mechanisms by which their transcription is activated (for reviews see ref.19, chapters 5 and 7).

Immediate Early

The single immediate early E1A transcription unit is the first to be expressed, because it is efficiently transcribed by cellular proteins alone. A primary function of the two major E1A proteins, which are synthesized from differentially spliced mRNAs (Fig. 3.2A), is to optimize the intracellular milieu for reproduction of the virus, for example, to induce cells to enter S phase when they would not normally do so (for reviews see refs. 8, 19). One consequence of their mitogenic activity is stimulation of transcription from the viral early E2E promoter via members of the E2F family of cellular transcriptional regulators. The larger E1A protein contains a unique, conserved, internal segment of 46 amino acids, designated CR3[20] (Fig. 3.2A) that can stimulate transcription from essentially all of the many promoters that have been tested in transient assays (for reviews see refs. 19, 21, 22, 23).

Early

In infected cells, the activity of the early E1B, E2E, E3, E4 and ML promoters is stimulated by the CR3 domain of the larger E1A protein, via components of the cellular transcriptional machinery prior to the onset of viral DNA synthesis.[19,21-23] Although the E1A proteins are necessary for efficient transcription of viral early transcription units, they are not absolutely essential.[24-26] Thus, viruses whose E1A genes have been mutated or deleted, although crippled, can complete the infectious cycle following high multiplicity infection of HeLa cells,[26] and in some cell lines infected at low multiplicity produce substantial quantities of infectious virus.[27]

Intermediate

Viral DNA replication, which is necessary for further progression through the transcriptional program, begins when sufficient concentrations of viral replication proteins, encoded within the E2 transcription unit (Fig. 3.1), have accumulated in the infected cell

nucleus. The increased concentration of viral DNA templates appears to be sufficient to induce transcription of the intermediate pIX and IVa2 genes (Fig. 3.1), at least in part as a result of titration of cellular repressors.[28,29] Relief from promoter occlusion by actively elongating transcriptional complexes initiating at the upstream E1B promoter (Fig. 3.1) upon synthesis of new templates in the infected cell has also been implicated in activation of transcription of the *pIX* gene.[30]

Late

The IVa2 protein is the second adenoviral transcriptional activator: Its specific binding to two adjacent sequences within the first intron of the ML transcription unit (Fig. 3.3), as a dimer [31] and in association with a second, as yet unidentified, infected cell-specific protein,[32] accounts for the 20- to 30-fold stimulation of ML transcription characteristic of the late phase of infection.[33-35] A second E2 promoter, termed E2L (Fig. 3.1), is activated following viral DNA replication, and eventually accounts for at least 90% of E2 mRNA production.[36,37] How this promoter is turned on, and whether it should be classified as intermediate or late, are not known. Transcription of viral genes continues until impaired and eventually shut down as a consequence of the cytopathic effects of the virus.

RNA Polymerase II Transcriptional Control Signals

As would be expected, adenoviral transcriptional control regions closely resemble those of cellular genes transcribed by RNA polymerase II. Indeed, the Ad2 ML promoter was the first from which accurate initiation of transcription by this enzyme was achieved in vitro.[38,39] Thus, as illustrated for the viral E1B, E2E and ML promoters in Figure 3.3, viral transcription generally depends on a TATA sequence and a constellation of binding sites for cellular, sequence-specific activators upstream of the site of initiation. However, these viral promoters are usually more compact than their cellular counterparts. Early investigations of the organization of adenoviral promoters led to the discovery of several cellular activators of transcription, including USF,[40,41] E2F[42] and ATF[43] (Fig. 3.3). With the exception of that of the E1A gene, maximally efficient transcription from viral promoters by RNA polymerase II in infected cells requires viral proteins (E1A or IVa2 proteins, see above), even though these transcriptional control regions are quite active in in vitro systems. As the E1A proteins stimulate transcription indirectly, the viral promoters that respond to them contain no consensus, E1A-response sequence (for reviews see refs. 21-23). In contrast, late phase-specific stimulation of ML transcription by the IVa2 protein is achieved via specific DNA sequences located within the first intron of this transcription unit (Fig. 3.3) that cooperate with upstream promoter sequences recognized by cellular proteins.[31,33,34,35]

Conventional enhancer elements have been identified only in the E1A transcriptional control region. A number of different sequences with properties typical of enhancers were initially identified using transient assays (for a review see ref. 44). The contribution of these enhancers to E1A expression and virus reproduction in normal host cells that would be encountered in natural infections is not clear, because their roles have been examined, if at all, only in highly transformed HeLa cells. However, efficient E1A transcription from the viral chromosome in the latter cells requires a constitutively active enhancer that includes sequences repeated at positions -200 and -300 (Fig. 3.4) in this transcriptional control region.[45,46] A number of cellular proteins whose binding to sequences of this enhancer correlates with stimulation of E1A transcription have been identified,[47-49] as has a protein that represses enhancer function in undifferentiated rodent cells.[50] This region of the viral genome also contains an enhancer (enhancer II in Fig. 3.4) that stimulates transcription of all viral genes by RNA polymerase II, perhaps because it directs infecting viral genomes to intranuclear sites optimal for transcription.[45,46]

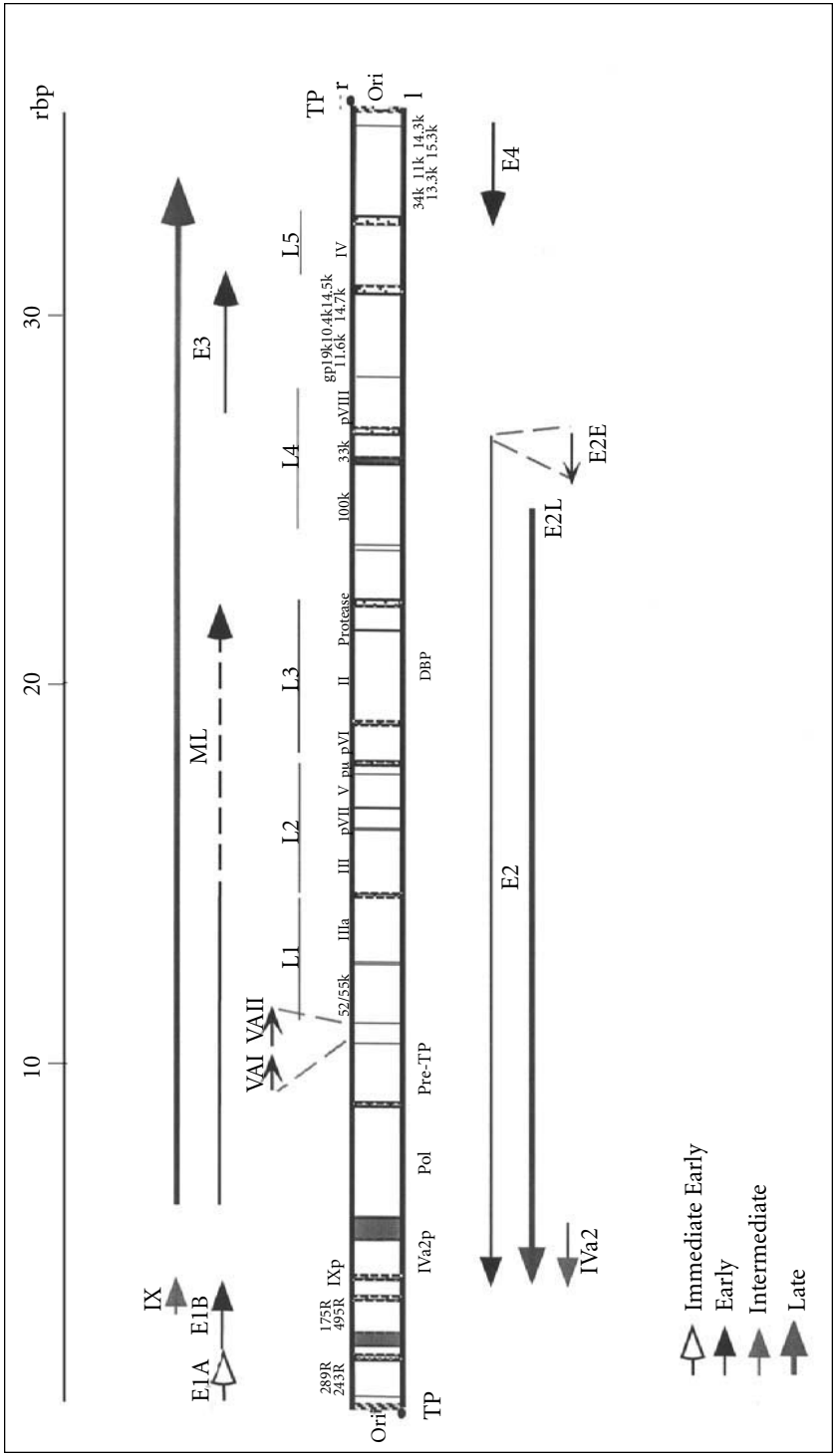


Fig. 3.1. See opposite page.

Fig. 3.1. (on opposite page). Transcription units and protein coding sequences of the Ad2 genome. The viral genome is represented by the pair of solid, horizontal lines at the center. The terminal protein (TP), origin of replication (Ori) and inverted terminal repetition (cross-hatched region) are shown at each end. The sequences encoding well characterized viral proteins are indicated by the white boxes within the genome, and labeled on the strand that carries the coding sequence. The many proteins encoded within the ML transcription unit are expressed via 5 families of mRNA species, designated L1 -L5, whose members share a common 3′ polyadenylation site (see Fig. 3.2B). Individual coding sequences for the E1A, E1B, E3 and E4 proteins are not delineated. Genomic segments that encode 2 proteins in different reading frames are indicated by the gray boxes, while inter-cistronic regions are stippled. Viral transcription units are represented by arrows, drawn in the direction of transcription. Those transcribed by RNA polymerase II (plain arrowheads) are shown from the site of initiation to the single (E1A, E1B, IX, IVa2 and E4) or most distal (ML, E2 and E3) site of polyadenylation of primary transcripts. As indicated, and discussed in the text, these transcription units are activated sequentially during productive infection. The 3′ segments of ML transcripts made during the early phase of infection are illustrated by a dashed line, to represent the termination of such transcription at multiple sites within the L2 and L3 regions of the ML transcription unit. The RNA polymerase III transcription units are depicted by barbed arrows.

The adenoviral signals that dictate termination of RNA polymerase II transcription are, in general, much less well understood than those controlling initiation. However, termination regions containing multiple copies of related sequences that can specify termination have been identified at the 3′ ends of the ML and E1B transcription units.[51,52] Furthermore, it is clear that this process can be regulated: During the early phase of infection, ML transcription never proceeds to the end of this long transcription unit, but rather terminates at multiple sites within the L2 and L3 segments (Fig. 3.1).[53] In contrast, the entire transcription unit is copied to and beyond the L5 poly(A)-addition site following the onset of viral DNA synthesis.[54] The mechanism responsible for this unusual transcriptional switch, which is dependent on viral DNA synthesis,[55] has not been elucidated. The ability of complexes transcribing the ML transcription unit during the late phase of infection to pass through an elongation block upstream of the second, l2 exon also appears to be regulated, by one or more viral late proteins of unknown identity.[56]

Protein Coding Sequences and Their Expression

The Ad2 genome encodes 12 virion proteins (see chapter 2) and on the order of 30 other proteins that have been characterized to greater or lesser degree (Fig. 3.1). Members of the latter group are made in each period of the infectious cycle, and, as discussed in subsequent chapters, fulfill a wide variety of functions. In many cases, the sequences encoding proteins that fulfill related functions are located within the same transcription unit, or subsegment thereof. For example, the coding sequences for all three viral replication proteins lie within the E2 transcription unit, while the proteins encoded in the E3 transcription unit counteract host defense mechanisms, and are not required for reproduction of the virus in cell in culture (see chapter 26). Similarly, within the ML transcription unit all core protein coding sequences are present in the L2 region (Fig. 3.2). How such organization of coding sequences by function might have evolved remains a puzzle.

Although we possess a reasonable understanding of the roles of the majority of viral proteins listed in Figure 3.1, it must be emphasized that a substantial number of additional viral proteins of unknown functions have been described. These include the L4, infected cell-specific 33 kDa protein (Fig. 3.1), the product of a third E1A mRNA (9S mRNA) that accumulates during the late phase of infection, two small E1B proteins related to the E1B 55 kDa protein and a protein largely encoded by a fourth upstream exon (the i leader, Fig.

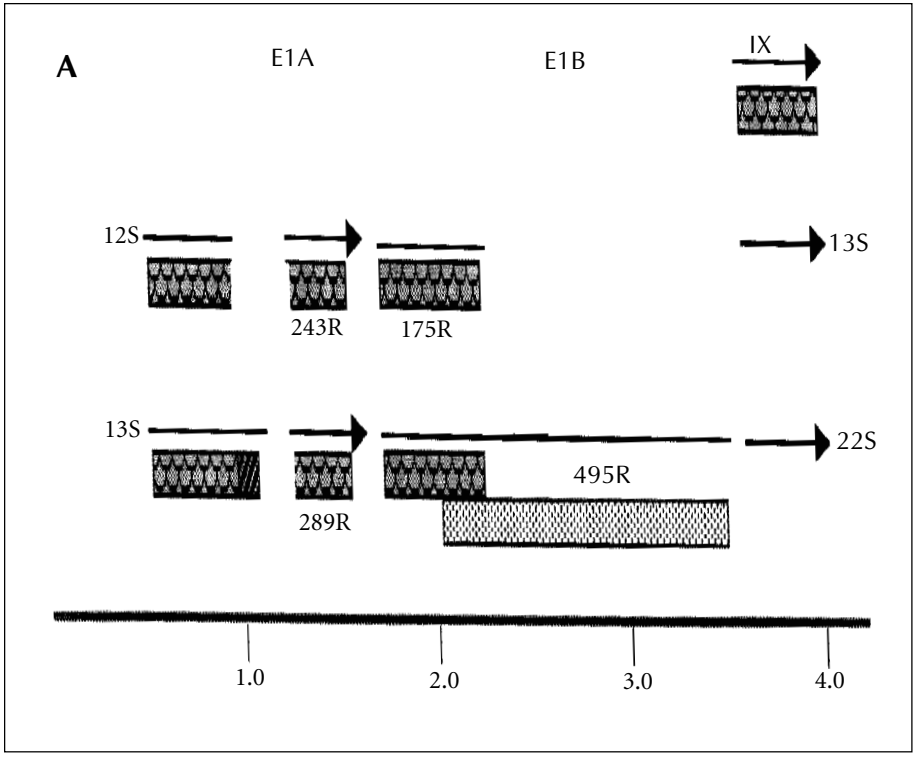
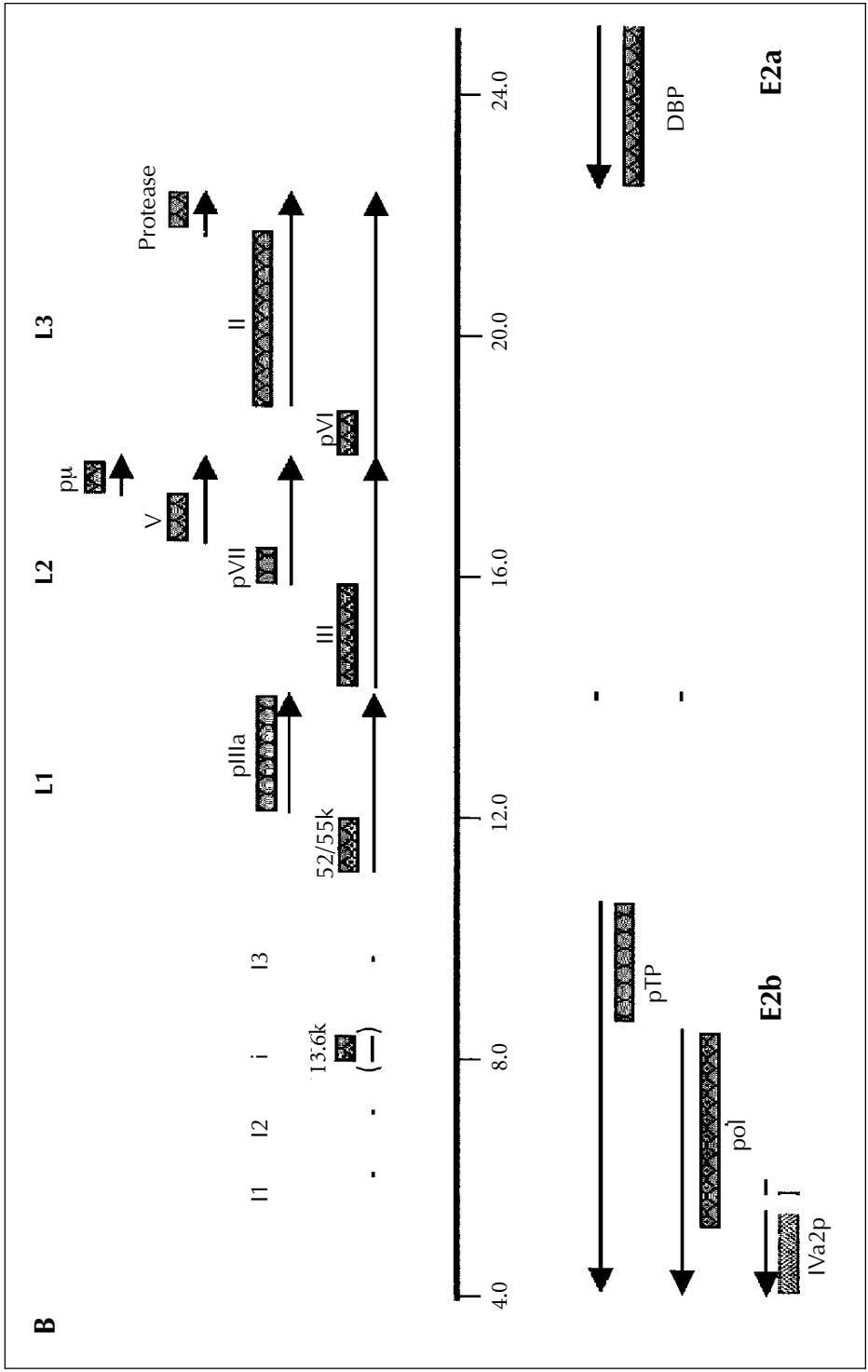


Fig. 3.2. Processing of some adenoviral mRNA species. In both parts of the figure, the Ad2 genome is represented by the solid horizontal line, with positions indicated in kbp. The horizontal arrows depict mRNA species, with the arrowhead at the site of polyadenylation and introns removed by splicing shown as gaps. The coding sequences translated from each mRNA are indicated by the boxes drawn In Fig 3.2A and 3.2B. (A) (above)mRNAs and proteins of the E1A, E1B and IX transcription units. As indicated, both E1A and E1B mRNAs are produced by alternative splicing of a singly polyadenylated primary transcript. The internal 46 amino acids unique to the 289R E1A protein are shown cross-hatched. The N-terminal portion of the sequence encoding the 495R E1B protein overlaps the sequence specifying the C-terminal portion of the 175R protein, but is translated in the -1 frame. (B) (see opposite page) mRNAs and proteins of ML, E2 and IVa2 transcription units. All ML and E2 mRNA species are produced from primary transcripts by alternative polyadenylation and alternative splicing. During the late phase of infection, the 5 poly(A)-addition sites that define the L1-L5 families of ML mRNAs are used with approximately equal frequency (for a review see ref. 44). However, E2 primary transcripts initiating at either the E2E or E2L promoters (see Fig. 3.1) are polyadenylated far more frequently at the site defining the single E2a mRNA than at the promoter-distal site for formation of the 2 E2b mRNAs. All mRNAs processed from ML primary transcripts carry at their 5′ termini the 3 small exons, designated l1, l2 and l3,that comprise the tripartite leader sequence.[6,7] As discussed in the text, during a specific period of the infectious cycle, a substantial proportion also include the i leader, shown in parentheses, which can encode a 13.6 kDa protein. Although the longer mRNAs in each ML family are polycistronic, only the 5′, cap site-proximal coding sequence is translated, as indicated.


B
L1
L2
L3
pIIIa
III
V
pμ
pVII
pVI
II
Protease
52/55k
13.6k
I1
I2
i
I3
IVa2p
pol
pTP
DBP
E2b
E2a
4.0
8.0
12.0
16.0
20.0
24.0

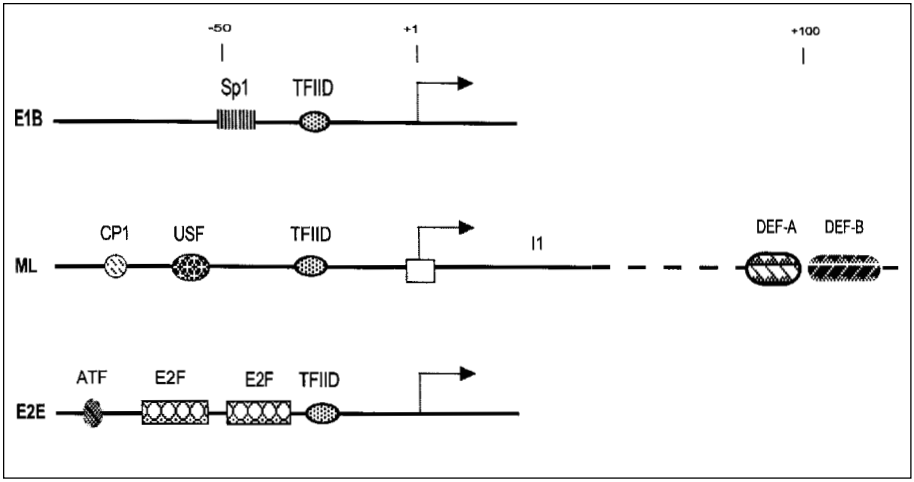


Fig. 3.3. Some Ad2 RNA polymerase II promoters are shown to scale, with the arrows indicating the sites of initiation of transcription (position +1). The upstream sequences recognized by the cellular general initiation protein TFIID and sequence-specific, cellular transcriptional activators are listed, as are the binding sites for the infected cell, late phase-specific proteins DEF-A and DEF-B in the first intron (dashed line) of the ML transcription unit. As discussed in the text, DEF-A is a dimer of the IVa2 protein, while both the IVa2 protein and an additional late phase-specific protein form DEF-B.

3.2B)[56] present on some 50% of ML mRNAs made before, or within a few hours following, the onset of the late phase of infection (for reviews see refs. 12,44). The Ad2 genome also contains some 20 other open reading frames with the potential to encode proteins of 100 or more amino acids, as well as numerous, smaller unidentified open reading frames.[12,15-17] The expression of the great majority of even the longer open reading frames in infected cells has not been examined, so it seems very likely that additional viral proteins necessary for successful adenovirus reproduction remain to be identified.

The primary transcripts synthesized from the majority of Ad2 transcription units are processed to multiple mRNAs via alternative splicing (for example, E1A and E1B mRNAs) or by alternative polyadenylation and splicing at one of several sites (for example, E2 and ML mRNAs) (Fig. 3.2). Although extreme in several adenoviral pre-mRNAs, such alternative processing mechanisms are typical of the production of many cellular mRNAs, and the viral signals specifying sites of poly(A)-addition and exon-intron boundaries are analogs of those present in cellular transcripts. Consequently, adenoviral mRNA production has served as an experimentally amenable model for this important cellular process ever since mRNA splicing was discovered through characterization of ML mRNAs.[6,7] Despite such extensive processing, a substantial proportion of the viral mRNAs, in particular the majority of ML mRNAs (Fig. 3.2B), are polycistronic. However, with the exception of the larger E1b mRNA (Fig. 3.2A), which can support synthesis of both the 175R and 495R E1B proteins, at least in vitro,[57] only the open reading frame closest to the 5' end of the mRNA is translated, in accordance with the general rules that govern initiation of translation in eukaryotic cells by the scanning mechanism.[58]

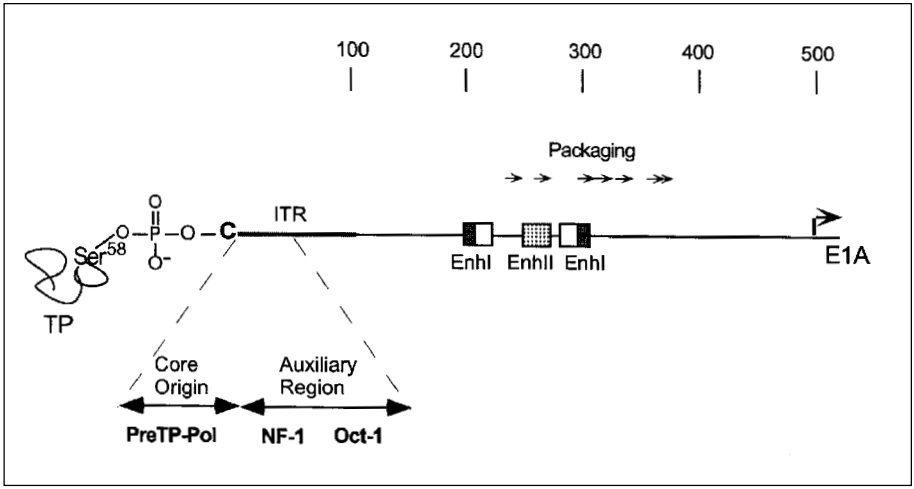


Fig. 3.4. Essential, *cis*-acting sequences at the left end of the Ad2 genome are shown to scale, in bp. The organization of the origin, and the viral and cellular proteins that recognize origin sequences, are shown in the expansion above. An identical copy of the origin and of the inverted terminal repetition (ITR) are present at the right end of the genome. The locations of functionally redundant, AT-rich sequences (arrows) that comprise the genomic packaging signal, and of the enhancers discussed in the text (EnhI and EnhII) are also indicated.

Small Viral RNAs Synthesized by RNA Polymerase III

The Ad2 genome also contains three RNA polymerase III transcription units, whose products are small *RNA* species. The *VA RNA* genes, which lie within an intron of the RNA polymerase II ML transcription unit (Fig. 3.1), posses typical intragenic promoters for this cellular enzyme, and are efficiently transcribed in infected cells (for reviews see refs. 59, 60). Indeed, *VA RNAI* accumulates to some 10^8 copies per cell, a very high concentration that is presumably necessary for its ability to counteract the activation of protein kinase R induced by infection, and allow translation of late mRNAs (for a review see ref. 69). In contrast to the ubiquitous *VA RNAI* gene, the genomes of many adenoviruses, including human serotypes 12,40 and 41, contain no *VA RNAII* gene. The contribution of this second *VA RNA*, when made in infected cells, is not known (for a discussion see ref. 62). However, Ad5 mutants that direct synthesis of neither *VA RNA* are more severely disabled than those with only the *VA RNAI* promoter disabled,[63] and examination of the distribution and conserved features of the *VA RNAII* gene suggest that its product fulfills a distinct, perhaps tissue-specific, function.[62] The third viral gene transcribed by RNA polymerase III is superimposed on the 5' end of the E2E RNA polymerase II transcription unit (Fig. 3.1, ref. 64). In Ad2-infected cells, RNAs of less than 100 nucleotides are synthesized from this transcription unit,[64] but the termination site for the smaller is not conserved among the genomes of all adenoviruses (Finnen R, Flint SJ, unpublished observations). In contrast to the *VA RNA*s, these E2E RNA polymerase III transcripts are present in infected cells at only very low concentrations.[64] Their function(s) are not yet known.

Other Important Features

Essential, Cis*-Acting Sequences*

In addition to protein or RNA coding sequences and the signals that allow their expression, the adenoviral genome contains several other types of sequence essential for successful virus reproduction. These include two identical copies of the origin of viral DNA replication, one located at each end of the genome (Fig. 3.1). Each origin (Fig. 3.4) comprises a conserved, minimal origin and adjacent sequences (the auxiliary region) that greatly increase the efficiency of viral DNA synthesis both in vitro and in infected cells (for reviews see refs. 65,66, chapter 6). The former is recognized by the viral DNA polymerase-preTP complex, an interaction that is facilitated by binding of cellular, sequence-specific transcriptional activators, such as NF-1, to adjacent sequences in the auxiliary region (see refs. 65,66, chapter 6). The origins are identical to one another, because they lie within the ITR (Fig. 3.4), a sequence that is also necessary for viral DNA synthesis. As discussed in chapter 6, replication from each origin proceeds continuously, so that one parental strand is copied while the second is displaced as single-stranded DNA coated with the E2 single-stranded DNA-binding protein. Annealing of the complementary ITR sequences of the displaced strand allows formation of a short, double-stranded stem to recreate an origin for initiation of replication of this strand. Thus, both parental strands are copied by a continuous mechanism, although not simultaneously.

A set of AT-rich and functionally redundant sequences located between the left-hand origin and the E1A transcription unit (Fig. 3.4) comprises the Ad2 packaging signal, essential for incorporation of newly replicated viral DNA genomes into capsids during virion assembly.[67-69] The inhibition of encapsidation of viral DNA observed when these sequences are deleted implies that they must be specifically recognized during virion assembly. However, the mechanism by which the packaging signal directs entry of viral DNA into assembling virions is not yet clear (see chapter 9). As illustrated in Figure 3.4, the sequences that comprise the packaging signal are interspersed among, or superimposed on, the viral enhancers described above.

The Terminal Protein

All infecting adenoviral genomes carry one copy of the terminal protein covalently attached to the 5′ ends of each strand (Fig. 3.1).[70] Although not part of the genome per se, this protein fulfills a number of important functions to influence both viral DNA replication and gene expression. The covalent linkage of TP to the DNA, via a phosphodiester bond from a serine to the terminal dCMP (in Ad2 and most other adenoviral genomes),[71,72] is created when the pre-TP made in infected cells primes viral DNA synthesis (for reviews see refs. 65,66, chapter 6). The mature TP is liberated from this precursor by the virion L3 protease during or following virion assembly (chapter 8). Its presence on the 5′ termini of genomic DNA molecules protects them from exonucleolytic attack, and from recognition by cellular, DNA end-binding proteins that might block access to the origins. The TP also significantly increases the activity of viral DNA molecules as templates for replication, probably by facilitating both binding of the preTP-DNA polymerase complex to the origin and unwinding of the origin during initiation (for a review see ref. 66). Presumably as a consequence of these activities, the infectivity of TP-DNA is much greater than that of deproteinized viral DNA.[73] The TP also directs infecting adenoviral genomes to the nuclear matirx, a function that appears to be required for maximally efficient transcription of viral genes.[74]

Sequences that Fulfill Multiple Functions

One important consequence of the production of a large number of viral mRNAs by alternative processing of a limited set of primary transcripts is that large segments of the genome contribute to more than a single transcription unit. This phenomenon is exemplified by the ML transcription unit, which extends from position 6039 to close to the right hand end of the r-strand of the genome (Fig. 3.1). Three additional transcription units, E3 (RNA polymerase II) and VA RNAI and VA RNAII (RNA polymerase III), lie within this same segment of the Ad2 genome. The coding and control sequences that comprise the VA RNA genes are contained entirely within an intron of the ML transcription unit (Fig. 3.1). In contrast, the promoter and 5′ end of the *E3* gene lie within the L4 segment of the ML transcription unit, such that a single r-strand sequence specifies the C-terminal 96 amino acids of the L4 protein pVIII, the 3′ untranslated region and poly(A)-addition site of all L4 mRNAs, and the control sequences and cap proximal transcribed sequence of the early E3 gene (Fig. 3.1). Similarly, near the left end of the viral genome, the pIX gene lies entirely within the 3′ untranslated region of the E1B transcription unit, and the pIX and E1B transcripts are polyadenylated at a common site (Figs. 3.1, 3.2A). Such complete or partial superimposition of transcription units is not restricted to those present in the r-strand of the viral genome. In the l-strand, the IVa2 transcription unit is completely included in the distal portion of the E2 transcription unit, within the coding sequence for the viral DNA polymerase (Figs. 3.1, 3.2B). Thus, the sequence that comprises the IVa2 promoter also encodes an internal segment of this enzyme. Furthermore, as noted previously, the E2E RNA polymerase III transcription unit is superimposed on the 5′ end of that transcribed by RNA polymerase II, an arrangement whose significance is not yet clear.

In several regions of the genome, both DNA strands carry information. In a large central portion of the genome, both the r- and l-strands are transcribed, as part of the ML and E2 transcription units, respectively (Figs. 3.1, 3.2B). The protein coding sequences expressed via these transcripts are interspersed in the two strands. However, the E2 promoters and adjacent, 5′ non-coding exons are specified by l-strand sequences whose complements form part of L4 protein coding sequences (Fig. 3.1). Conversely, the ML promoter and sequences that form the 5′ untranslated tripartite leader sequence common to ML mRNAs are also part of the l-strand coding sequences for the DNA polymerase or preterminal protein (Figs. 3.1, 3.2B). Thus, double duty of a single sequence both to specify part of a protein and to form signals that control mRNA production, or untranslated regions, is a common feature of the adenoviral genome. In contrast, the use of multiple translational reading frames within a single sequence is relatively rare (Figs. 3.1, 3.2).

Conclusion

The adenoviral genome provides a remarkable illustration of the general virological principle of efficient utilization of limited genetic information, in this case via dependence on the host cell's RNA processing machinery to produce multiple *mRNA* species from the transcripts of a limited number of transcription units. As summarized in preceding sections, we now possess a reasonably detailed picture of the mechanisms responsible for efficient and orderly expression of viral genes, as well as a good understanding of the functions of genomic sequences that direct viral DNA replication. On the other hand, many details of the processes that mediate and control viral gene expression remain to be elucidated, as do the molecular functions of a surprisingly large number of proteins specified by the viral genome. It is to be hoped that the current resurgence of interest in adenoviruses spurred by their potential as vectors with a wide variety of therapeutic applications will advance our basic understanding of the biology of these viruses, while allowing such potential to be realized.

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Chapter 4

Entry of Adenovirus into Cells

Prem Seth

With the advent of the electron microscope, it became possible to study the entry of adenoviruses into eukaryotic cells. Time course experiments of adenovirus uptake into the cells indicated a pathway of adenovirus entry which is similar to that followed by other ligands which enter the cells by receptor-mediated endocytosis.[1,2] Images taken at different time intervals showed adenoviruses bound to cell surface at initial time points. Soon thereafter, adenoviruses are detected in clathrin coated pits, and in endocytic vesicles termed endosomes. Adenoviruses escape from the endosomes into the cytosol, then traverse towards the nucleus using the microtubule system. The journey of adenovirus from the cell surface to the nucleus is completed in about 30 minutes, indicating a rapid rate of adenoviral uptake.[2]

While the binding of adenovirus to the cell surface is independent of temperatures in the range of 4-37°C, the internalization of the adenovirus into the cells is temperature-dependent, and is optimum at 37°C.[2,3] Known inhibitors of ATP-dependent transport processes also abolish adenovirus uptake into cells, suggesting that the entry of the adenovirus into cells is an active process.[4] The following are the key steps involved in adenovirus entry into the cells.

Binding of Adenovirus to the Cell Receptor, and its Entry into the Endosomes

It has been shown that adenovirus entry into cells is initiated by binding of the virus to its cell surface receptor. The fiber protein facilitates adenoviral binding to its receptor, as excess of purified fiber protein can compete with the binding of the adenovirus to its receptor[5,6] (Table 4.1). Using radiolabeled adenovirus, it has been shown that adenoviral binding to the cell surface follows saturation binding kinetics. Scatchard analysis has suggested that most epithelial cells have about 10,000 adenoviral receptors per cell. These experiments have also revealed that there could possibly exist two types of adenoviral receptors, a high affinity and a low affinity receptor.[7]

Attempts have been made to purify adenoviral receptors from the cell membranes by conventional methods including ion exchange chromatography, gel filtration, and affinity chromatography utilizing viral particles, pentons or fibers crosslinked with the inert surfaces.[8,9] To identify adenoviral receptors, other investigations involved the use of bispecific reagents to crosslink adenovirus with cell surface receptors. These approaches revealed the adenoviral receptor to be a glycoprotein of up to five subunits.[10] Earlier experiments, in which the effect of other viruses on the binding and infectability of adenoviruses were studied, indicated that adenoviral receptors are shared with coxsackie viruses.[11] Recently, adenoviral receptors have been identified by molecular cloning. These studies have also shown that the adenoviral receptors fall in the same category as those of coxsackie B3 viruses, and hence are

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Table 4.1. Adenoviral and cellular proteins involved in adenovirus entry

Entry Step	Protein(s) Involved	
	Viral	Cellular
Interaction with the receptor	Fiber knob	CAR protein (primary receptor)
	Penton base	Integrins αvβ3, αvβ5 (secondary receptors)
Endosome lysis	Penton base Viral-endoprotease	Proton pumps, Endosome membrane proteins
Movement across microtubules	Hexons	Microtubules, and microtubule-associated proteins
Nuclear entry	Preterminal protein Other proteins?	Nuclear pore complex

termed CAR (coxsackie and adenovirus receptor).[12,13] The exact binding sites of adenovirus fiber proteins with the receptors have also been mapped in recent years.[14-16]

Recent studies have indicated that the primary structure of adenoviral penton base possesses the tripeptide amino acid sequence RGD, which is known to bind the integrins. The ability of penton base to interact with integrins may explain some of the previously reported properties of adenoviruses to induce leakiness in the cell membrane, plasma membrane vesicles and in the artificial liposomes.[17-19] This can also explain the previously known property of adenovirus, and of penton base, to cause cell rounding and cell detachment, leading to early cytopathic effects.[20] Furthermore, it has been suggested that the binding of RGD amino acids with integrins of the $\alpha_V\beta_3$ and $\alpha_V\beta_5$ group may also be important for adenovirus entry into cells.[21] The binding of adenovirus to cells can be inhibited by excess of RGD peptide. Nevertheless, adenoviruses with mutations in RGD sequences are internalized into the cells, albeit at lower efficiency.[22] Therefore, the exact role of penton base-integrin interaction in the adenovirus entry process still needs to be further evaluated.

Adenoviral receptors are expressed on most cell types including epithelial, neural, fibroblast, and muscle cells.[23] The only known cell types deficient in adenoviral receptors are primary hematopoeitic cells, including $CD34^+$ stem cells.[24,25] However, adenoviruses can stay in the episomal state in some lymphoid cells.[10,20,26] While the mechanism of this latency is not clear, it raises the possibility of an alternate receptor or perhaps other means of adenovirus entry into these cell types.

The adenovirus, bound to its receptor, next moves to clathrin coated pits by random diffusion. At any given time, most cell types have about 1000 coated pits per cell.[27] In less than 5 minutes of the initial binding of adenoviruses to the cell surface, adenoviruses can be observed in endosomes. Endosomes are constitutively produced at an approximate rate of about 3000 per minute. Endosomes are generally believed to be formed by pinching off the

coated pits. They are very fragile and exhibit high ionic pump activities.[27] These properties of endosomal membranes are exploited by adenoviruses in ways which permit escape from the membrane-limited endosomes.

Adenovirus-Mediated Lysis of Endosome Membrane: Role of Low pH and Penton Base

It has been shown that adenoviruses escape the endosome by disrupting the endosome membrane.[2] The biochemical mechanisms of the endosome lysis have been extensively studied. The lysosomotropic agents such as chloroquine, which raise the pH of the endosomes, also inhibit the ability of adenovirus to disrupt the endosome membrane, suggesting that a low intraendosomal pH is important for vesicle disruption.[17] It has also been shown that while the preincubation of certain concentrations of anti-penton base antibody with adenovirus does not block the uptake of adenovirus into the endosomes, it can abolish the ability of the adenovirus to lyse the endosomes.[28] This suggests a role of the penton base in the process of endosome disruption. Additionally, it has been shown that a mildly acidic pH (pH 5.5) increases the binding of penton base with Triton X-114, suggesting that low pH can induce conformational changes in the penton base protein leading to an increase in the hydrophobicity of penton base.[29] Adenovirally mediated endosome lysis appears to involve an enzymatic component, as different treatments known to destroy enzymatic activities also abolish the endosomolytic activity associated with adenovirus.[30] Adenoviruses have also been shown to activate the Na^+, K^+-ATPase of the endosomes, resulting in increased ion fluxes across the endosome membrane.[4] Based on these findings, a model for adenovirally mediated lysis of endosome membrane has been proposed and is depicted in Figure 4.1. Interestingly, adenovirally mediated lysis of endosomes does not require intact adenoviral genomic DNA, as UV-inactivated adenovirus can be internalized into the endosomes, and is capable of endosome lysis.[7] However, the viral-associated proteases could be important for endosome lysis, as a temperature sensitive mutant of adenovirus-2 defective in the protease ts1 (P137L) is taken up into endosomes but is not able to lyse the endosome membrane. The role of this protease in adenovirus infection will be described further in chapter 8.

Vectorial Movement of the Adenovirus into the Nucleus

After escape from the endosomes, the adenovirus then moves towards the nucleus. There is much in vitro and in vivo evidence suggesting that this vectorial movement is facilitated by cellular microtubules. Earlier experiments had shown the presence of crystal-like complexes of virion-specific proteins and microtubule inside the cells.[31] Drugs such as vinblastine and cytochalasin B, which disassemble microtubules and inhibit microfilament function, have been shown to block the entry of adenovirus into the cells, suggesting that microfilaments and microtubular structures play an important role in the entry of adenovirus into the cells.[32,33] Crosslinking of adenovirus bound to the cells also suggested the association of adenoviral proteins with vimentin and a tubulin in adenovirus-infected cells. During the migration of adenovirus across the microtubular system, virus-induced cellular proteases have been shown to cleave vimentin protein, one of the components of the intermediate filaments,[34] again suggesting an active involvement of the microtubule system in the entry of adenovirus into the cells.

During the transport of adenovirus from the cell membrane to the nucleus, the adenovirus sheds its proteins in a stepwise manner.[10] It is generally believed that the fiber protein of adenovirus is immediately dissociated after the virus enters the coated pits. Similarly, the protein IIIa is released soon after that, presumably to allow penton base to interact with the endosome membrane. After adenovirus escapes the endosomes, the

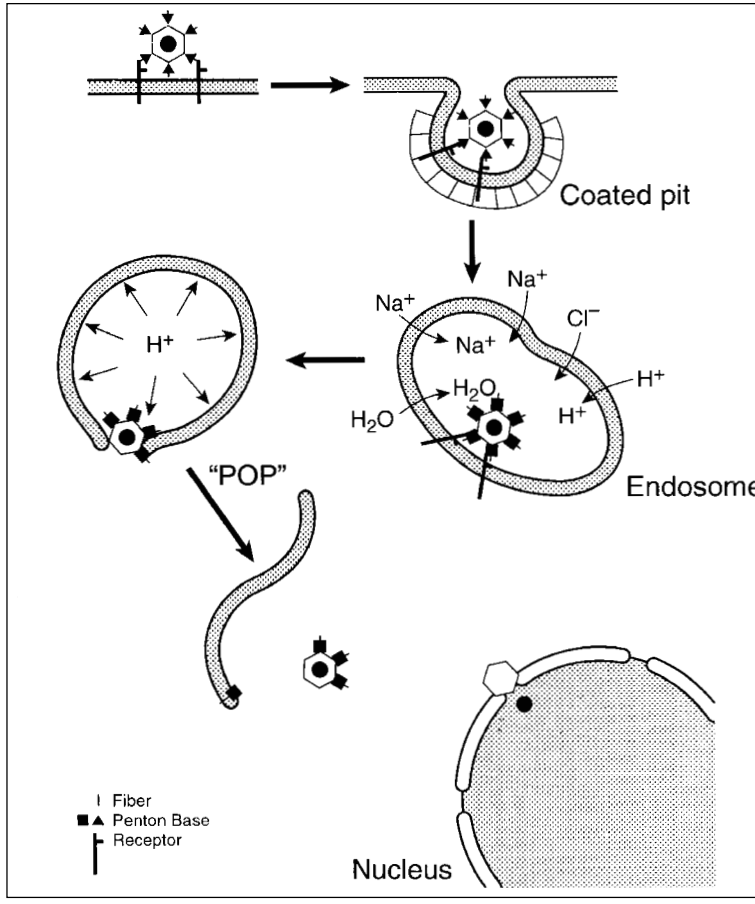

Fig.4.1. Model of adenovirus entry and vesicle disruption. Adenovirus binds to a cell surface receptor and moves into a coated pit. Soon thereafter, it appears in an endosome. Low pH of the endosome causes the penton base protein to undergo a conformational change (from ▲ to ■), as a result of which penton base acquires amphiphilic characteristics which enable it to interact with the membrane of the endosome. The endosome, which is distended as a result of osmotic pressure, ruptures at the point where adenovirus penetrates the membrane. See text for details (reproduced from ref. 40 with permission).

uncoating continues in the cytosol and penton base is degraded. Next, the proteins which bridge the DNA core to the viral capsid (proteins VIII and VI) are released from the virus, thus enabling the entry of DNA to the nucleus. The other adenovirus protein IX, and some hexons, are disassembled during the transport of adenovirus to the nucleus.[35] Presumably adenoviral-associated proteases are responsible for the degradation of at least some of these viral proteins.[36] Adenovirus bound to a subpopulation of hexons and core proteins μ, VII, IVa_2, V, and the terminal protein moves to the nucleus.[35]

Once the adenovirus gets near the nucleus, it ejects its genomic DNA into the nucleus through nuclear pores.[37] Based on the known mechanisms of the entry of other

macromolecules into the nuclei, it is predicted that some adenoviral proteins guide the entry of the genomic DNA into the nucleus using a stretch of amino acid sequences termed nuclear localization signals. Published work on known nuclear localization sequences suggested there might be a possibility of more than one type of consensus sequence.[38] One type of such signals is a cluster of basic amino acids flanked by proline or glycine as seen in SV40 large T antigen. Since the preterminal proteins of the adenovirus possess this type of nuclear localization signal,[10] it is likely to participate in releasing the adenoviral genome into the nucleus. It should also be noted that two of the adenoviral core proteins—DNA binding proteins and E1A proteins—also possess the nuclear localization signal.[10] Another potential signal consists of two short stretches of basic amino acids separated by a spacer. These sequences have been shown to be present in three other core proteins—μ, protein VII and protein V. The function of the nuclear localization signals in these core proteins in mediating DNA delivery to the nucleus is not clear.[10] The area of adenoviral entry to the cell nucleus clearly needs further research.

Once the adenovirus enters the nucleus, it places itself in the nuclear matrix[39] in such a manner that its core genome and terminal protein are accessible for initiating early gene expression and DNA replication, as described in the next few chapters.

Conclusion

Adenovirus represents one of the very few non-enveloped viruses whose entry process has been investigated in such detail. The various steps of viral entry, particularly the interaction of adenovirus with its receptor and the escape of adenoviruses from the endosomes, have been a subject of great interest in the field. Moreover, the ability of adenovirus to lyse the endosomes has been extensively exploited for the delivery of foreign DNA into the cells.[40] If the adenoviral protein responsible for the endosomal lysis can be identified, then eventually it might even be feasible to deliver the foreign genome into the cells using an isolated protein rather than intact adenoviral particles.

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Chapter 5

Early Gene Expression

Philip E. Branton

The major targets of human adenoviruses are terminally differentiated epithelial cells of the upper respiratory tract, gut and eye. Once the virus has entered the cell and its DNA uncoated appropriately, progression of the infectious cycle depends on the ability of the virus to solve several problems. One is the need to express early viral genes, and this function relies largely on a powerful transcriptional activator, the early region 1A (E1A) protein that amplifies expression of the *E1A* gene and that of all other early transcription units encoding functions necessary to drive viral replication. A second is the need to stimulate the infected cell to enter S-phase. Adenoviruses encode only a limited number of functions and must rely on host cell machinery to replicate viral DNA. E1A products serve this function as well, and are able to coax resting or differentiated cells to re-enter the cell cycle. As will be discussed, an unavoidable consequence of E1A expression is the activation of programmed cell death, and thus adenoviruses have evolved a number of strategies to avoid early apoptosis that would severely limit production of progeny virions. Nevertheless, adenoviruses use apoptosis later in infection to facilitate release of progeny, and thus must play a complex balancing act between induction and suppression of apoptosis to optimize infection. Other challenges include the need to convert infected cells into factories that mass produce viral products, and the necessity to counter attempts by the cell to block replication, including production of antiviral molecules like interferon and induction of immune and inflammatory responses. As we will see, adenoviruses encode a number of products that lessen or eliminate these effects. In this chapter we will review details of early adenovirus products and their role in viral replication.

Adenovirus Genes and Products

Figure 5.1 shows the transcription map of human adenovirus type 5 (Ad5). The sizes of adenovirus genomes vary between about 30 and 36 kb among the more than 40 human serotypes. Adenoviruses encode over 25 individual early products, as well as specialized VA RNA molecules and an array of late proteins that function largely in the process of particle formation and as structural components of mature progeny virions. By as early as one hour post-infection, proteins encoded by E1A can be detected, followed soon after by those of early regions 1B, 2, 3 and 4 (E1B, E2, E3 and E4) that are produced from transcription units regulated by individual promoters. Following synthesis of progeny viral DNA, the major late promoter becomes active, and ever increasing quantities of late proteins are produced, more or less until the death of the infected cell. Each viral product serves one or more precise functions in the advancement of the infectious cycle and the fabrication of progeny infectious virions.

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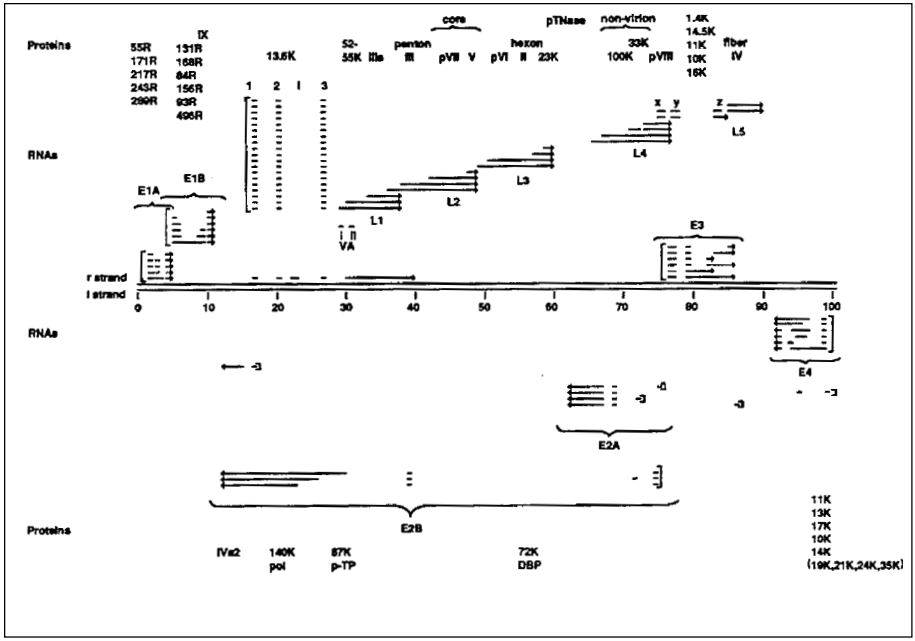

Fig. 5.1. Ad5 transcription and gene products. The early and late mRNAs produced by Ad5 are shown, along with identified and proposed protein products in relation to the genomic map units given in arbitrary units from 0 to 100. Transcription of E1, E3, VA RNA and late proteins is from the r-strand and thus from left to right. Transcription of E2 and E4 is from the l-strand and thus from right to left.

Early Region 1A (E1A)

E1A Products: Structure and Function

E1A products are the first to be synthesized and, at least in the case of human adenoviruses, they represent the major regulators of early events. Figure 5.2 shows the transcription map of the Ad5 E1A gene and its protein products. As with most adenovirus genes, E1A yields multiple polypeptides due to alternate mRNA splicing. E1A products vary somewhat among different serotypes, but as is the case with all viral proteins, a considerable degree of conservation of sequence and function exists. Each E1a mRNA encodes a unique protein; however, all share a common amino terminal sequence. With Ad5, the major early E1A transcripts are 13S and 12S mRNAs encoding proteins of 289 and 243 residues (289R and 243R). Each is comprised of two exons and, as the splice joining exons 1 and 2 of the 12S mRNA is in frame with the spliced 13S transcript, the encoded proteins are identical apart from a central 46 residue region present in the larger product. The 11S and 10S mRNAs encode a corresponding pair of proteins that, due to an additional splicing event in exon 1, lack residues 27 to 98 present in the 13S and 12S products. The 9S mRNA encodes a small E1A protein that shares only the first 26 residues with other E1A products, the remainder being unique, as a different reading frame is used. These three minor E1A mRNAs are expressed primarily at later stages of infection and the functions of their products are largely

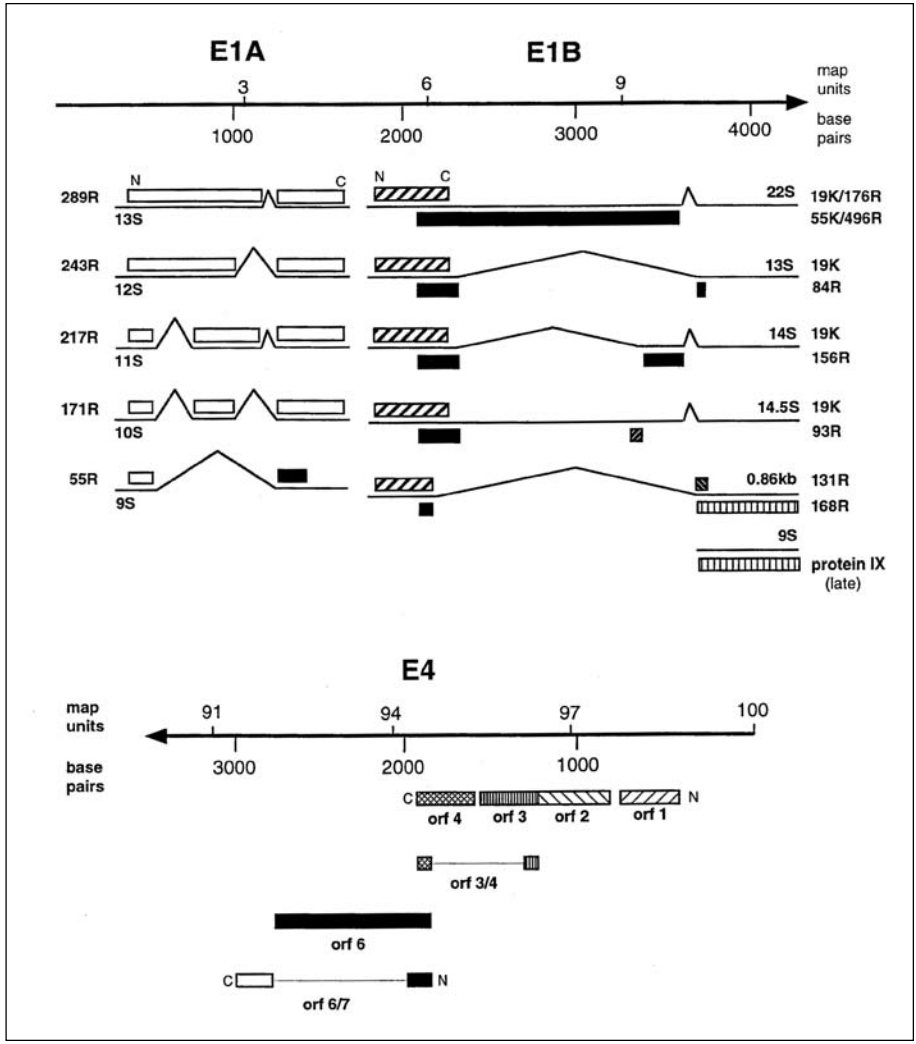

Fig.5.2. E1A, E1B and E4 transcripts and proteins. Detailed transcription maps and encoded proteins for E1A, E1B and E4 are shown in relation to genome map units and base pairs. Changes in relative reading frames as a result of splicing are indicated in the patterns of boxes defining protein products.

unknown. The roles of the major 13S and 12S products have been studied extensively and their importance is well documented. Both the 289R and 243R E1A proteins are nuclear; they are highly acidic and extensively phosphorylated; and they have short half lives of about 20 to 30 minutes. They are also composed of almost 20% proline that is dispersed throughout the molecule, suggesting that E1A proteins may be highly linear molecules containing functional domains in distinct units along the E1A sequence. This feature is probably the reason why E1A has been so amenable to genetic analysis, as deletion of portions of the protein coding sequence yields products that are still highly active for unaffected functions.

Mutational analyses by a number of investigators have identified a series of discreet E1A functional regions. All E1A products, except that of the 9S mRNA, contain a strong nuclear targeting sequence at the carboxy terminus. As will be discussed, an important role of the major E1A products is to activate transcription of early mRNAs and to stimulate DNA synthesis. Both of these functions appear to derive from complex formation with a provocative array of cellular proteins and the subsequent modification, inhibition or utilization of their biological activities. Binding of many of these proteins requires three regions that are highly conserved in virtually all human adenoviruses and in many adenoviruses of other animal species. As illustrated in Figure 5.3, these regions in Ad5 include conserved regions 1 and 2 (CR1 and CR2) located in Ad5 between residues 40 to 80, and 120 to 139, respectively, and CR3 located between residues 140 to 185. All three are encoded within exon 1 of the 289R product. CR1 and CR2 are present in both 289R and 243R; however, CR3 is unique to 289R, as it represents the 46 residue region eliminated by splicing of the 12S mRNA. In addition, a region at the amino terminus, which has not been extensively conserved, is also of great importance. The first E1A-binding proteins to be detected[1,2] were eventually identified as the products of the retinoblastoma (Rb) tumor suppressor gene, pRb, and related proteins p107 and p130 (reviewed in ref. 3), and a 300 kDa species, p300, now known to be a relative of the cyclic AMP responsive element binding (CREB) protein and a related but uncharacterized p400 species (reviewed in ref. 4). The Rb gene is commonly deleted or inactivated in a variety of human cancers, notably bilateral retinoblastoma, in which most cases are caused by a germline mutation in one Rb allele and the acquisition of a somatic mutation in the second. Figure 5.3 shows that binding of Rb family proteins occurs primarily through a conserved binding site Leu-X-Cys-X-Glu found in CR2; however, a minor but critical contact is also made with a portion of CR1 (reviewed in ref. 5). Binding of p300/CREB/p400 requires the amino terminus and a region of CR1. p300 and CBP possess endogenous histone acetyl transferase (HAT) activity and also bind an additional HAT enzyme (reviewed in ref. 6). CR3 interacts with a number of transcription factors, including the TATA-binding protein (TBP), a critical component of the basal transcription complex, and upstream factors such as ATF family members, Sp1 and c-Jun. A relatively uncharacterized 48 kDa species, termed CtBP, binds to a region encoded by exon 2, just adjacent to the carboxy terminal nuclear localization signal. The biological importance of such interactions will be discussed below.

Regulation of Gene Expression by E1A Products

The primary initial goal of E1A products is to activate viral gene expression (reviewed in ref. 7). The 289R E1A protein is a powerful transcriptional activator, and although it does not bind directly to DNA, it binds to a variety of transcription factors that target it to appropriate promoters. Much of the transactivation activity relies on CR3, which is the major site of interaction with transcription factors. Thus 289R is the principal regulator of viral gene expression, as the 243R E1A product lacks CR3. CR3 can be divided into two subdomains involved in transcriptional activation. The more amino terminal 'activation domain' contains a 'zinc finger' that is involved in binding to TBP, a component of the TFIID basal transcription complex. In addition, CR3 binds other transcription factors, including members of the ATF family, utilizing sequences at the carboxy terminus or 'promoter binding domain' of CR3 and several residues of the region encoded by the second exon that interact with basic leucine zipper (bZIP) domains of these proteins. It is believed that activation of transcription by E1A protein relies both on stabilization of the transcription complex (perhaps through concomitant binding of TBP and upstream factors), or in some cases by the activation of individual factors. For example, binding of ATF-2 is believed to result in the stimulation of its activation domain, perhaps via a conformational change. Thus, depending on the

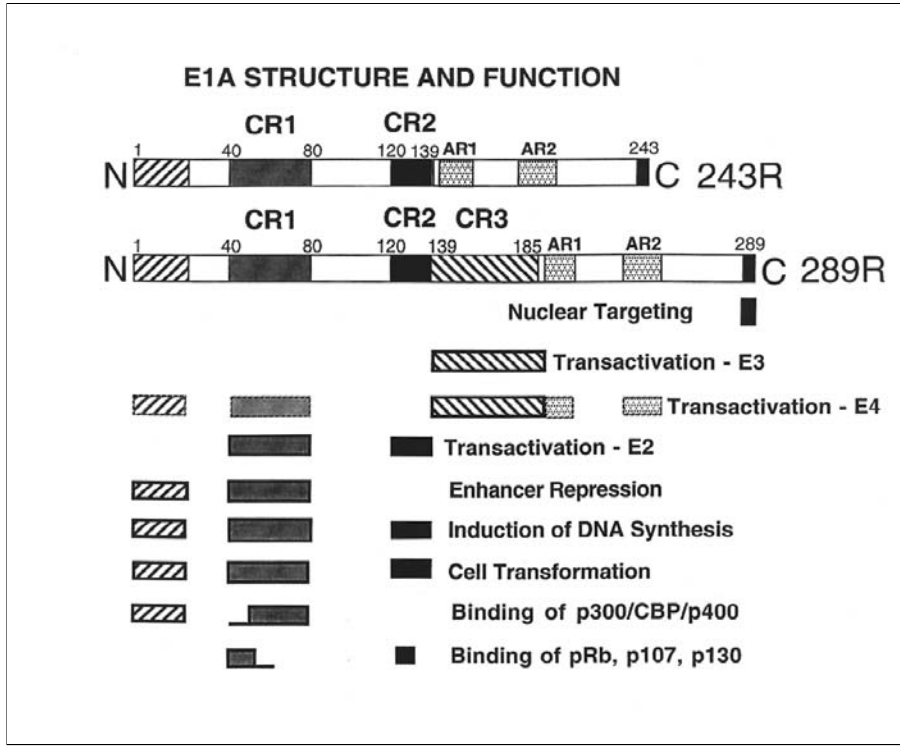

Fig. 5.3. Functional map of Ad5 E1A proteins. The coding sequence of the 289R and 243R E1A proteins is given, along with the regions involved in complex formation and functional activities of E1A products (see text).

specificity of interaction with particular transcription factors and the composition of viral (or cellular) promoters, E1A-289R is able to activate gene expression fairly selectively.

E1A products transactivate expression of E1A itself and of E3, and such activity relies largely on CR3. The CR3 region also contributes to increased expression of E1B and E2, although E1B is expressed fairly efficiently by read-through from the E1A promoter, and E2 relies greatly on a separate E1A-dependent mechanism of activation. The E2 promoter contains both ATF and E2F binding sites, the latter of which are of major importance. E2F was originally described as a factor regulating adenovirus E2 transcription; however, as discussed in more detail below, it soon became apparent that it is of general importance in expression of genes encoding DNA synthetic enzymes and regulators of the cell cycle (reviewed in ref. 8). E2F transcription factors exist as a family of heterodimers containing one of six E2F proteins bound to one of three DP molecules. A major function of the Rb tumor suppressor family is to bind to and inactivate E2F. E2F-1, E2F-2 and E2F-3 heterodimers bind to pRb; E2F-4 to pRb, p107 and p130; and E2F-5 to p130 only. E2F-6 is still under study at this writing. Binding E2F to Rb family members involves the 'large binding pocket' that overlaps the region required for E1A protein binding. E1A products interact with pRb, p107 and p130 via the major binding site in the CR2; however, interactions with CR1 release E2F heterodimers that then activate E2 expression. Such activation does not

require CR3, and therefore even the 243R E1A protein can stimulate E2 mRNA production quite efficiently. In the case of E4, gene expression requires the amino terminus as well as CR3, implying a requirement for one or more members of the p300/CBP family. Recently it has been shown that in addition to CR3, transactivation of all promoters by E1A protein relies significantly on two acidic regions within the second exon coding sequence known as auxiliary regions 1 and 2 (AR1 and AR2).[9] AR1 is of most importance, although AR2 can function as well if placed immediately adjacent to CR3. The precise biochemical role for AR1 and AR2 in transactivation are not yet clear.

E1A proteins also repress certain enhancers and thus downregulate expression of some genes. In most cases, this function requires the amino terminus and CR1, implicating complex formation with p300/CBP family members. A number of enhancers have been shown to be regulated in this way, including that in the E1A promoter itself. Thus E1A products both activate and repress E1A expression. p300/CBP appears to function in gene expression at least in part by acetylating histones and presumably other proteins, possibly those in the basal transcription complex, resulting in enhanced transcription. E1A-dependent repression may therefore result from sequestering of p300/CBP by E1A products.

A curious effect of expression of low levels of E1A products is the induction of epithelial-specific markers in a variety of cell types.[10] Thus, in some ways E1A could function as a tumor suppressor by stimulating epithelial differentiation and reducing cell proliferation. This effect could also play a role in the pathogenesis of adenoviruses by enhancing their ability to replicate in other cell types by providing epithelial products necessary for the infectious cycle. Although this process presumably results from changes in gene expression, the regions of E1A involved have not yet been determined.

Induction of DNA Synthesis by E1A Products

The natural targets of adenoviruses are terminally differentiated epithelial cells. As will be discussed below, adenoviruses encode only a few proteins involved in DNA synthesis and thus must stimulate infected cells to enter S-phase to make available the synthetic machinery required for viral DNA replication. E1A is a powerful mitogen, and genetic studies have shown that binding of either p300/CBP or Rb family proteins by E1A products is sufficient to stimulate DNA synthesis.[11,12] While it is still unclear exactly how complex formation with p300/CBP produces this effect, the importance of interactions with Rb family members is quite well understood. As discussed above, pRb, p107 and p130 regulate E2F activity, which in turn controls expression of genes encoding DNA synthetic enzymes and regulators of cell cycle progression.[3] In uninfected cells, regulation of E2F is controlled through the phosphorylation of Rb family members by cyclin-dependent kinases (Cdk), resulting in release of active E2F during G1 and promotion of S-phase. E1A products undo this carefully orchestrated control by binding directly to Rb proteins, thus freeing active E2F heterodimers to induce DNA synthetic enzymes and permit synthesis of progeny viral DNA. As described in more detail below, adenoviruses possess oncogenic potential, a property closely linked to the ability of E1A products to induce unscheduled DNA synthesis.

E1A Products and Apoptosis

Perhaps the major obstacle to successful completion of the lytic cycle and production of high yields of progeny virions is the cellular tumor suppressor p53 (reviewed in ref. 13 and 13a). p53 has been termed the 'guardian of genomic integrity' and its role, at least in part, is to protect multicellular organisms from individual aberrant cells that, if allowed to survive and enter S-phase, might pose a threat to the entire organism either because of their neoplastic potential or infection by microorganisms. p53 is a transcription factor containing a powerful acidic activation domain that induces gene expression following binding to specific

promoter sequences. Activation of some promoters relies on binding to p300/CBP that could enhance transcription by acetylating key factors, possibly including p53 itself. p53 also represses certain promoters by an as yet undefined mechanism. The presence of active p53 protein affects cells in two ways. First, p53 induces G1 arrest, thus preventing, for example, irradiated cells containing damaged DNA from entering what could be a catastrophic round of DNA replication, and affording the opportunity for DNA repair. One mechanism of this arrest involves p53-dependent transactivation of a gene encoding an inhibitor of Cdks, termed $p21^{WAF\text{-}1/Cip\text{-}1}$, that prevents phosphorylation and inactivation of Rb family members. p53 also induces cell death by apoptosis through a genetically controlled process triggered by the activation of degradative enzymes that generate the classic apoptotic phenotype characterized by shrinkage and rounding of cells following disruption of the cytoskeleton, condensation of chromatin into dense granular masses, cleavage of DNA to nucleosome-sized fragments, extensive cytoplasmic vacuolization and blebbing, and in the final stages, fragmentation of the cell into membrane-bound vesicles or apoptotic bodies that are rapidly engulfed by neighboring cells. It appears that p53-dependent apoptosis results from changes in expression of critical cellular genes through p53-induced transcription and/or repression. In this way, for the benefit of the whole organism, p53 eliminates a variety of aberrant cells, including those with genotoxic damage, imbalances in growth control (such as cancer cells containing activated oncogenes or inactivated tumor suppressor genes), or those challenged by virus infection. p53-dependent apoptosis is characterized by a rise in p53 levels and the presence of highly active p53 molecules. A great many laboratories have been studying the molecular basis of changes in p53 activity and, at this writing, some combination of phosphorylation and acetylation appear most likely.

It has been known for some time that E1A causes a rise in the level of p53, a result of an increase in stability of the usually very short lived p53 protein. Recent mapping studies have linked this rise in adenovirus-infected human cells to regions in E1A proteins involved in complex formation with either p300/CBP or Rb family proteins.[14] Thus, activation of p53 corresponds to the induction of unscheduled DNA synthesis by E1A proteins. Although other E1A-dependent events may play a role, it seems that the critical need of adenoviruses to induce entry into S-phase may result in activation of p53 and apoptosis. Such early cell death would severely limit virus production, as cells would die before significant viral progeny had been produced. Adenoviruses have therefore evolved at least five separate mechanisms to thwart this critical obstacle. The first relates directly to E1A proteins. As p53 usually relies on p300 as a cofactor in transcriptional activation, binding of E1A products to p300 might therefore reduce p53 activity. The other mechanisms, which will be discussed in detail below, rely partially or wholly on E1A-dependent transactivation of other early viral genes.

E1A as an Oncogene

Adenoviruses were the first human viruses shown to possess oncogenic potential. Although no study has yet linked adenoviruses with any human cancer, infection of newborn rodents by certain highly oncogenic serotypes (such as Ad12) leads to the formation of tumors. In addition, virtually all adenoviruses can generate transformed cells that, following injection into nude mice or other newborn rodents, are usually capable of forming tumors (reviewed in ref. 15). Oncogenic potential maps primarily to E1A, and specifically to regions of E1A products involved in complex formation with both p300/CBP and Rb family proteins. Unlike the stimulation of DNA synthesis that requires binding to only one of these classes of proteins, interactions with both families are essential for the creation of transformed cells. Thus, the capacity of E1A as an oncogene appears to derive from the constitutive induction of S-phase and the breakdown of normal control of cell cycle progression and growth arrest. Expression of E1A in rodent cells frequently leads to

the formation of transformed cells possessing growth characteristics associated with cancer cells; however, such cell transformation usually aborts quite rapidly due to extensive cell death. As discussed above, E1A is highly toxic because of the induction of p53-dependent apoptosis, and thus stable cell transformation by adenoviruses usually occurs only when one or more additional viral gene(s) are expressed. As we will see below, the role of these gene products is to protect cells from the lethal effects of E1A proteins. Studies on the mechanism of cell transformation by human adenoviruses over the past two decades have yielded enormous insights into regulatory processes of the cell cycle, tumor suppressor genes, apoptosis, and other phenomena associated with cancer.

In addition to inducing cell transformation, E1A products contain a region towards the carboxy terminus that appears to lessen the tumorigenic properties of E1A-transformed cells. Deletion of these sequences results in transformants that yield more tumorigenic and metastatic tumors.[16] The mechanism of this effect is not known.

It is interesting to note that many different DNA tumor viruses have evolved similar strategies to promote productive infection and cell transformation. The large T antigens of simian virus 40 (SV40) and polyomaviruses, and the E7 protein of human papilloma viruses (HPV) all bind to and inactivate the Rb family. SV40 large T antigen and the HPV E6 protein also inactivate p53, but by mechanisms that differ from adenovirus products, discussed below.

Two additional points should be considered concerning the oncogenic potential of human adenoviruses. First, does this capacity play any role in pathogenicity? It would be reasonable to conclude that oncogenicity is only a byproduct of the requirement to induce DNA synthesis during lytic infection; however, adenoviruses can maintain persistent or latent infections that might take advantage of this transforming capacity in some fashion. Second, why are human cells so highly resistant to transformation by adenoviruses? In the case of infection by whole virus, rodent cells are incapable of adenovirus replication and thus can survive to form transformants, whereas infected human cells are permissive and are killed. But transfection of human cells with DNA expressing E1A and cooperating genes rarely leads to stable transformation. In fact, E1A/E1B transformed 293 embryonic kidney cells[17] represent one of only a very few adenovirus transformed human cell lines. Although other explanations may exist, it is possible that E1A is more toxic in human than in rodent cells, making continued survival extremely difficult even in the presence of protective viral gene products. A thorough discussion of cell transformation by adenoviruses can be found in chapter 23.

Early Region 1B (E1B)

E1B Products

Figure 5.2 shows that E1B also produces multiple alternatively spliced mRNAs. Early in infection the major Ad5 E1B transcript is a 22S mRNA that encodes a 19 kDa polypeptide (176R/19K) and, using an internal translation initiation site, a 55 kDa species (496R/55K) that shares no sequence homology with 19K because it is encoded in a different reading frame. In the absence of other viral products (such as in E1A/E1B transformed cells), 55K is predominantly perinuclear; however, as will be discussed below, a unique shuttling system involving the E4 ORF6 product targets it to the nucleus in infected cells. The 19K E1B protein is associated with intermediate filaments and nuclear lamina, but it is also present in nuclear and cytoplasmic membranes, presumably targeted there by acylation that occurs at two or more sites. An alternatively spliced E1B 13S transcript is also produced early after infection, and, in addition to 19K, it encodes an 84 residue (84R) species composed of the 79 residue amino terminus of 55K fused to a polyproline unique carboxy terminus. Two

other E1B transcripts of 14S and 14.5S are produced somewhat later, again by alternative splicing, and encode 19K as well as proteins of 156R and 93R. The splice donor sites in these messages are identical to that of the 13S mRNA encoding 84R, and thus 156R and 93R also contain the 79 residue amino terminus of 55K; however, unique splice acceptor sites yield different carboxy termini. With 93R, the carboxy terminal 14 residues are unique, but with 156R the last 77 residues are identical to those of 55K, making 156R effectively a deletion mutant of 55K. An additional alternatively spliced 0.86 kb transcript has also been reported that contains a unique splice donor site, yielding a shorter modified version of the 19K polypeptide and a protein related at the amino terminus to 55K, but linked to the entire late protein IX sequence. Although much has been learned about 19K and 55K, essentially nothing is known about the roles of the other E1B products. They may represent products of unintentional splicing events; however, conservation of E1B splicing patterns in most adenovirus serotypes suggests that they may play some role later in infection, perhaps by expressing individual functional domains present in their more prominent relatives. The 84R species is produced in large quantities, 156R somewhat more modestly, and the others at very low levels.

E1B and Apoptosis

A primary function of both the 19K and 55K E1B polypeptides is to protect infected cells from apoptosis induced by E1A proteins and other processes associated with infection, thus keeping them alive long enough to manufacture large quantities of progeny virus. But as will be discussed below, regulation of apoptosis represents a complex balancing act, as induction of apoptosis plays a vital role in the ultimate death of infected cells and spread of progeny virions to new targets.

Inhibition of p53 by E1B-55K

The E1B-55K protein performs several critical functions, but none is of more importance early in infection than the inhibition of p53-dependent apoptosis and cell cycle arrest associated with activation of p53 by E1A products. The fact that expression of E1B proceeds relatively independently of E1A is an advantage, as it ensures that significant levels of 55K (and 19K) are present shortly after E1A proteins appear. 55K binds to the acidic activation domain of p53 and inhibits p53-mediated transcription. But this process involves more than inhibiting the p53 activation domain, as mutants of 55K exist that bind to p53 apparently normally, but that nevertheless fail to affect p53 activity. Studies in which 55K had been fused to the DNA binding domain of the Gal4 transcription factor indicated that 55K functions as a potent transcriptional repressor.[18] It is believed that inhibition of p53-dependent transcription results from the tethering by p53 of this repressor to promotors containing the p53 binding site, thus reducing gene expression below basal levels. In this way, the ability of p53 to induce arrest of the cell cycle and apoptosis is effectively quashed. Repression by 55K has recently been mapped to a region near the carboxy terminus that is present in both 55K and 156R.[19] Whether 55K is itself a repressor or serves to bind an as yet unidentified repressor is not known. Repression, but not other 55K functions, is regulated by phosphorylation at three carboxy terminal sites located at Ser-490, Ser-491 and Thr-495.[20] Inhibition of p53 by 55K represents an important tool for the virus to cope with the major roadblock p53 represents to viral replication. In the case of cell transformation, stable transformants have been shown to result from coexpression of E1A with either 55K or 19K. It seems clear that the role of 55K in this process is to prevent death of transformed cells from E1A-induced p53-dependent apoptosis and growth arrest.

Inhibition of Apoptosis by E1B-19K

Although p53 is a major regulator of apoptosis, other apoptotic pathways exist, such as those dependent on tumor necrosis factor (TNF) and Fas ligand, that lead to cell death independently of p53. Virus infection activates some of these p53-independent processes, and for this and other reasons discussed below, adenoviruses also rely on 19K in addition to 55K to sustain the lifespan of infected cells. 19K is a potent inhibitor of most apoptotic pathways and appears to act by a mechanism similar to that of the cellular Bcl-2 protein. As shown in Figure 5.4, apoptosis induced by many pathways, including those requiring p53, appears to be controlled by at least two molecular checkpoints.[21] One of these, and perhaps common to all apoptotic pathways, involves activation of a class of proteases termed caspases, that cleave and activate the enzymes that actually kill the cell. In some cases caspases can be activated directly, as is believed for TNF and Fas ligand, but frequently such activation depends on a signal, possibly involving release of cytochrome C, from an upstream checkpoint regulated by dimerization of members of a family of integral membrane proteins related to Bcl-2. Homodimerization of one of these, termed Bax, promotes activation of caspases. Apoptosis is prevented by Bcl-2 and similar proteins that heterodimerize with Bax, thus preventing formation of lethal Bax-Bax homodimers. Death is the default pathway and thus all cells remain alive through the presence of 'survival factors' and by the positive balance of death suppressors, like Bcl-2, relative to death promoters, like Bax. Dimerization results from the interaction of specific binding regions, termed Bcl-2 homology (BH) domains, found in all Bcl-2 family members. The only sequence similarity between Bcl-2 and E1B-19K is in two BH domains that allow 19K to bind to Bax, thus preventing activation of caspases and the onset of apoptosis. Bcl-2 family members are components of large membrane complexes located in mitochondria or the endoplasmic reticulum that many researchers believe constitute channels that regulate release of initiators of downstream caspase activation, perhaps including cytochrome C. Several cellular proteins have been shown to associate with 19K, termed 19K-interacting proteins (Nips), and many of these presumably play some role in the suppression of apoptosis by 19K. Studies have indicated that in some human cell types 19K can block apoptosis induced by TNF and Fas. These observations are interesting, as TNF and Fas activate caspases directly, and therefore apparently downstream of the Bax-Bcl-2 checkpoint. Recently it has been suggested that 19K, but not Bcl-2, binds to and inactivates FADD, an essential component of the Fas ligand/TNF signaling pathways that activate caspases. As will be discussed below, adenoviruses have other means to block the effects of TNF and Fas ligand. Infection of most human cells by adenovirus mutants defective in 19K yields the cyt/deg phenotype characterized by many apoptotic hallmarks, including degradation of DNA and cytolysis, and by poor virus yields.[22] 19K and Bcl-2 both support cell transformation by E1A, presumably by blocking apoptosis induced by E1A proteins.

Other Functions of 19K and 55K

Although prevention of apoptosis is of major importance, E1B proteins also serve other functions upon which adenovirus replication relies. 19K has been reported to affect transcription, but as many of these experiments employed cells transfected by plasmid DNA, these effects may be complicated by the ability of 19K to block apoptosis, a common result of the transfection process. Increases in transcription could therefore derive from the presence of higher levels of reporter DNA in elevated numbers of surviving cells. At present, the role of this potential activity remains to be established. In the case of 55K, at least three additional functions exist that are apparently independent of its transcriptional repression activity and that are critical to the progression of the infectious cycle. First, 55K functions in the shut-off of host cell protein synthesis, an effect that may be related to a second function,

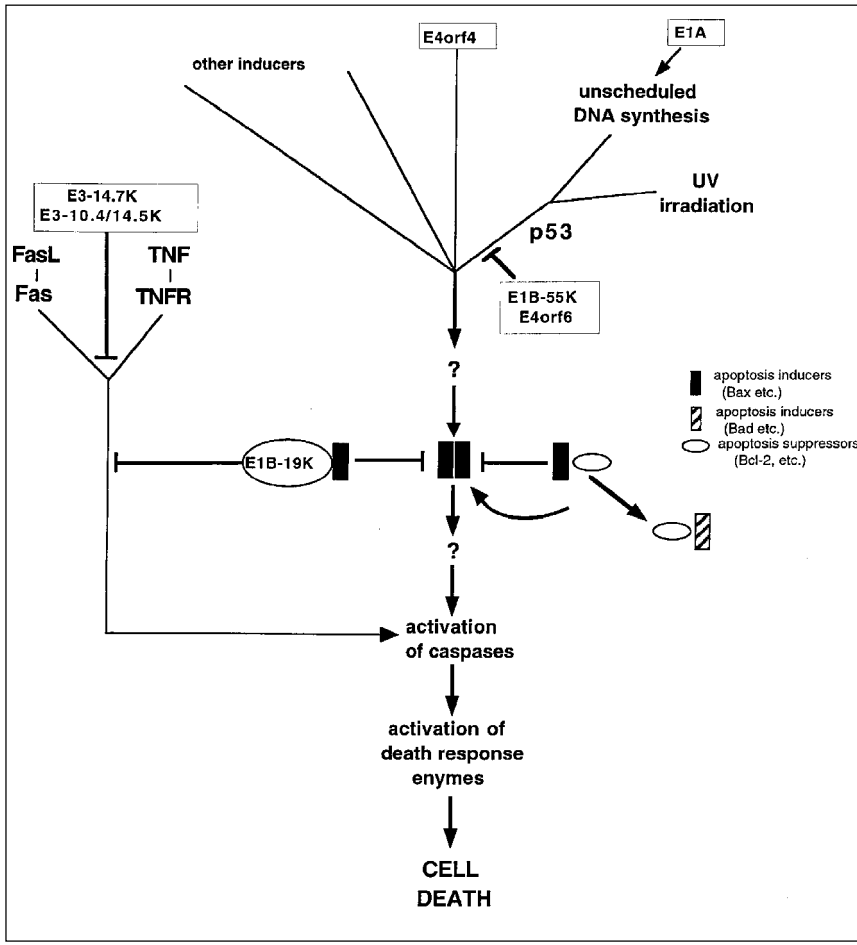

Fig. 5.4. Proposed pathways of apoptosis and involvement of Ad5 products. The proposed apoptosis pathway has been indicated, along with the point of action of Ad5 proteins. Cellular apoptosis promoters such as Bax (solid rectangles) or Bad (hatched rectangles) have been indicated, as have the Bcl-2 family of apoptosis suppressors (open ovals).

the selective stabilization, transport and translation of viral mRNAs. These activities assure high production of late viral structural proteins at the expense of cellular polypeptides. Such activities, which will be discussed below in more detail, require complex formation of 55K with a second viral protein, E4 ORF6. E4 ORF6-55K complexes are critical for the generation of high virus titers and, as we will see, they also provide an alternative means of controlling p53. Mutants defective in 55K replicate poorly and, with some serotypes such as Ad12, the block in the lytic cycle occurs even prior to viral DNA synthesis. One can speculate that this inhibition is due both to cell cycle arrest induced by p53 (against which 19K offers no protection), and to the additional 55K functions just described.

Early Region 2 (E2)

The E2 region of human adenoviruses is expressed from two regions of the viral genome, termed E2A and E2B, that by alternative splicing encode three proteins necessary for synthesis of viral DNA. Of major importance is the terminal binding protein (TP) that is essential to prime viral DNA synthesis. The 87 kDa pre-TP (that is ultimately cleaved to form the 55 kDa TP) is targeted to the nuclear matrix, where it binds both to 5' ends of viral DNA and, via a serine β-OH group and an ester linkage, to the α-phosphoryl group of dCMP, thus providing a primer for DNA synthesis catalyzed by the 140 kDa viral DNA polymerase also encoded within E2. The E2 72K (actually 59 kDa) single-strand DNA binding protein also helps promote a stage of DNA synthesis involving single-stranded viral DNA by enhancing processing and chain elongation catalyzed by viral polymerase. All of these functions are essential for virus replication and are discussed in detail in chapter 6.

VA RNA and Regulation of Protein Synthesis

Human adenoviruses produce one or two small RNAs of about 160 nucleotides that are transcribed by host cell RNA polymerase III and that play an important role in regulation of host cell protein synthesis and protection against interferon α and β. Interferon blocks viral replication through the activation of a protein kinase, PKR, that is stimulated by double-stranded RNA. PKR phosphorylates and inactivates translation factor eIF-2, thus inhibiting protein synthesis and continuance of viral replication. VA RNA blocks the interferon pathway by binding to PKR and preventing its activation. As VA RNA is present largely in viral translation centers, the effect is to permit translation of viral mRNAs, but, in other parts of the cell, translation of cellular mRNAs is reduced. Such host cell shut-off permits viral proteins to be synthesized at the expense of cellular products.

Adenoviruses have additional mechanisms of enhancing translation of viral mRNAs. Late in infection, translation factor eIF-4F becomes dephosphoryled and inactivated. eIF-4F is needed to relieve secondary structure at the 5' end of many mRNAs to facilitate their translation. Late viral mRNAs contain a tripartate leader sequence that is relatively free of secondary structure and thus does not depend on eIF-4F. A late 100 kDa viral protein also binds to RNA, leading to selective translation of viral mRNAs by a mechanism that is still being studied. Finally, as mentioned previously and discussed in detail below, the E1B-55K protein and E4 ORF6 function in the selective transport of viral mRNAs for translation in the cytoplasm. Thus, adenoviruses have multiple mechanisms of regulating the translation machinery, leading to escape from inhibition by interferon and selective enhancement of viral protein synthesis.

Early Region 3 (E3)

The E3 region encodes several mRNAs through alternative splicing. E3 can be completely deleted with little effect on adenovirus replication in cultured cells; however, it is critical for successful productive infection in humans, as many of its products are involved in protection of infected cells against host immune responses. E3 products are reasonably well conserved among various adenovirus serotypes, and Ad5 produces at least six proteins, some of which have been partially characterized. E3 products will be discussed only briefly in this chapter, but a detailed analysis can be found in chapter 26.

gp19K

The E3 gp19K glycoprotein is present largely in the endoplasmic reticulum, where it binds to certain class I histocompatibility antigens, preventing transport to the cell surface. Such inhibition allows infected cells to suppress lysis by class I-restricted, adenovirus-specific cytotoxic T lymphocytes, an obvious advantage to production and spread of viral progeny.

14.7K and the 10.5K/14.5K Complex

Expression of E1A proteins containing CR1 sensitizes cells to killing by inflammatory cytokines such as TNF and Fas ligand. Both the E3 14.7K protein, which is found in the cytosol, and E3 products of 10.5K and 14.5K, which associate as heterodimers in the plasma membrane, are effective inhibitors of TNF-induced effects. The 14.7K protein functions as a more general inhibitor than 10.5K/14.5K heterodimers that are effective in only some cell types. Both block phospholipase A2 (cPLA2)-induced release of arachidonic acid, which represents one important part of the TNF signaling pathway.[23] Binding of TNF to its receptor induces a calcium-dependent translocation of cPLA2 to the plasma membrane, resulting in the release of arachidonic acid to form prostaglandins and leukotrienes that amplify the inflammatory response. The increased effectiveness of 14.7K may result in part from its ability to inhibit the action of a range of cytokines, including interleukin-1β. The 10.5K/14.5K heterodimers function primarily on TNF. The mechanism of inhibition by these E3 proteins is still being worked out, but it is likely that 14.7K functions indirectly, perhaps by blocking proteolytic destruction of a cellular inhibitor. The 10.5K/14.5K heterodimers also block Fas-induced apoptosis. They were shown to stimulate endosome-mediated internalization and turnover of certain membrane receptors, including that for epidermal growth factor. Although TNF receptors do not appear to be affected, the 10.5K/14.5 complex efficiently induces loss of Fas from the cell surface, thus lessening cytolysis induced by release of Fas ligand from cytotoxic T lymphocytes.

It is therefore clear that armed with the E1B-19K protein and these three E3 products, adenovirus-infected cells are at least partially protected from the host inflammatory response.

E3-11.6K (Adenovirus Death Protein)

The E3 region encodes a protein of 11.6 kDa that appears not to function against the host response, but rather is implicated in the ultimate death of infected cells at the end of the replicative cycle. The mRNA transcript encoding this protein is synthesized at very low levels early in infection under the E3 promoter, but is produced in higher amounts late in infection. Death of cells infected by mutants defective in 11.6K is significantly delayed, and such cells generate high levels of viral progeny that accumulate in vast crystal arrays within the nucleus.[24] These results suggest that this E3 product, now termed the 'adenovirus death protein' (ADP), plays a role in promoting the final demise of infected cells. ADP is localized in Golgi and the nuclear membrane during late stages, but its mechanism of action is not understood at present. It is tempting to postulate that one target might be the E1B-19K protein with which it partially colocalizes. Inhibition of 19K (or Bcl-2-related proteins) could leave cells vulnerable to death by apoptosis. It may also act more directly to kill cells by another mechanism.

Early Region 4 (E4)

The E4 transcription unit is located at the extreme right end of the adenovirus genome and, as determined by identification of open reading frames (orf) and sequencing of cloned E4 cDNAs, encodes at least seven proteins. The functions of these proteins are only now being established, but E4 products clearly possess a wide and varied range of activities crucial to successful viral replication.[25] As will be discussed, one or more E4 products are cytotoxic and thus may be of importance for adenovirus gene therapy vectors as, even in the absence of E1A, the E4 promoter is expressed at low levels, and such E4 product(s) may represent a major source of inflammation in recipients. The E4 promoter is partially regulated by ATF sites, but expression depends highly on two sites that resemble but are not identical to ATF binding sites, and that bind a novel transcription factor termed E4F.

Interactions of E4F with E1A proteins differs from that of ATF, as the former lacks the usual bZIP region involved in binding to CR3. Activation of E4F-dependent transcription by E1A requires both the amino terminus and auxiliary region AR1 and possibly AR2 encoded by the second exon, and E1A-dependent phosphorylation of E4F.

E4orf1

Until recently, little was known about the 14.3 kDa E4orf1 product. Ad9 is known to induce mammary carcinomas in rats, and this function was mapped to E4orf1 and shown to be independent of E1A. The oncogenic potential of E4orf1 derives at least in part from its carboxy terminus, that binds a series of as yet unidentified cellular proteins. Although there is some variation in E4orf1 sequence among adenovirus serotypes, Ad5 E4orf1 also binds a subset of these same proteins, suggesting a common and probably important role for such complex formation.[26] Identification of these binding proteins may lead to the discovery of new pathways of oncogenesis in human cancer. The function of E4orf1 in the lytic cycle is unknown, but it is possible that it assists replication in certain types of epithelial cells, perhaps by promoting cell cycle progression.

E4orf2 and E4orf3/4

Little is known about either the E4orf2 and E4orf3/4 proteins. The former is a 14.6 kDa polypeptide encoded by a unique E4 transcript, whereas the latter results from a spliced mRNA encoding the amino terminus of the E4orf3 product fused to the carboxy terminus of E4orf4 to yield a 7.1 kDa product.

E4orf3

The 13.2 kDa E4orf3 protein is localized at least in part in the nuclear matrix, and E4orf3-defective mutants typically grow well as long as E4 ORF6 is present. As will be discussed below, E4 ORF6 plays a role in shuttling E1B-55K to and from the nucleus to promote selective transport of viral mRNAs and host shut-off. E4orf3 can partially complement E4 ORF6 defects and thus may contribute to this process, perhaps because of its nuclear localization. Both E4orf3 and E4 ORF6 are involved in the control of splicing of late viral transcripts by an as yet poorly understood process.

E4orf4

E4orf4 is a 14 kDa protein that plays a crucial role in replication and possibly viral DNA synthesis. Its only known function is to bind to the Bα subunit of the serine/threonine phosphatase PP2A.[27] In doing so, the trimeric form of PP2A (i.e., the A and B regulatory subunits in complex with the catalytic C subunit) may be activated. Targets of PP2A include mitogen-activated protein (MAP) kinases (and possibly other related kinases such as Cdks) that are inactivated following dephosphorylation of critical regulatory sites. Increased PP2A activity leads to decreased phosphorylation and inactivation of several transcription factors, including AP-1 and E4F, either directly or through the inactivation of such kinases.[9,28] E1A proteins also become hypophosphorylated at MAP kinase-dependent serine sites in and adjacent to CR3 that appear to be required uniquely for transactivation of E4 expression.[29] Thus, by decreasing both the activity of E4F and the ability of E1A-289R to transactivate E4 expression, E4orf4 functions as a negative regulator of E4 transcription. As was the case with E1A, which represses its own expression, the reason for such E4-specific autoregulation may be to reduce production of toxic products.

E4orf4 plays an important role in late mRNA switching (discussed in detail in chapter 7). It is possible that this function results from effects of E4orf4 on PP2A activity.

In addition to p53-dependent apoptosis discussed above, in the absence of E1B, infection of cells lacking p53 with virus expressing the 289R E1A protein also causes cell death exhibiting all of the hallmarks of apoptosis, indicating that 289R can induce p53-independent apoptosis.[30] No such response was observed with virus expressing only E1A-243R. This effect was shown to rely on the ability of the E1A-289R to transactivate E4, indicating that one or more E4 products is cytotoxic. Furthermore, in the presence of E1B, cell killing at the final stages of the infectious cycle is prevented or greatly delayed in the absence of E4 products.[31] Studies using E4 mutants implicated both E4 ORF6 and E4orf4 in this process; however, only E4orf4 induces apoptosis when expressed alone in the absence of other adenovirus products.[32] It has now been shown through mutational analyses that binding to PP2A is essential and sufficient for this cytotoxicity. E4orf4-dependent cell killing may play a role in the ultimate death of infected cells and spread of progeny virions by inducing p53-independent apoptosis. Many viruses have adopted the strategy of killing cells by apoptosis (reviewed in ref. 33), a process that is highly advantageous, as it diminishes the host inflammatory response and releases progeny virus in protected apoptotic vesicles that are taken up by neighboring cells for new rounds of replication. Mutants defective in E4orf4 typically cause rapid destruction of cell monolayers, as might be expected because E4orf4 is not available to downregulate E4expression. However, such infected cells remain intact for extended periods, presumably because E4orf4 is not present to induce apoptotic death. Although killing mechanismsinvolving additional early or late viral proteins may contribute to the death of infected cells, E4orf4-induced apoptosis may represent the major exit strategy. The E1B-19K protein blocks or delays E4orf4-induced p53-independent apoptosis. Thus, how do adenoviruses ultimately kill cells in order to release viral progeny? The mechanism is still not clear, but may involve the E3-11.6K ADP that partially colocalizes with 19K and thus may play a role in its inactivation late in the infectious cycle.

E4 ORF6/7

The 17.1 kDa E4 ORF6/7 protein is produced from a spliced mRNA that encodes the amino terminus of E4 ORF6 linked to a unique orf7 sequence. E4 ORF6/7 molecules form homodimers that contribute to viral DNA synthesis by ensuring production of high levels of E2 products. Each E4 ORF6/7 molecule in the homodimer binds transcription factor E2F. The structure of these complexes perfectly places E2F molecules at the two E2F binding sites in the E2 promoter to ensure a cooperative and stable association, and thus high levels of E2 transcription (reviewed in ref. 34). As the spacing and number of E2F sites in cellular promoters varies, E4 ORF6/7 enhances only E2 expression.

E4 ORF6

The 34 kDa E4 ORF6 protein serves several critical roles in adenovirus infection. It binds directly to both p53 and E1B-55K and regulates the activity of each. In the case of p53, these interactions involve a central region of E4 ORF6 and the carboxy terminus of p53 and result in the inhibition of p53 activity.[35,36] Such inhibition therefore represents the fourth mechanism that adenoviruses possess to inactivate p53. E4 ORF6 enhances E1A-mediated cell transformation, presumably via this effect on p53 and the resulting reduction of p53-dependent apoptosis. The fifth mechanism of p53 regulation involves both E4 ORF6 and E1B-55K, which form stable complexes through interactions between the amino terminus of E4 ORF6 and 55K.[36] In the presence of both of these virus products, E1A no longer stimulates a rise in p53 levels. The reason for this effect is that E4 ORF6 and 55K, possibly bound to p53 in a trimeric complex, stimulate the turnover of p53.[37,38] The mechanism of this effect is not known at this writing, but is reminiscent of the HPV E6 protein, which binds to p53 and targets it to the ubiquitin degradation pathway. It is likely that an E4

ORF6- or 55K-binding protein may serve a similar function. Therefore, through inactivation of p53 and induction of its degradation, adenovirus products deal effectively with the threat p53 poses to successful viral replication.

E4 ORF6-55K complexes also serve a direct role in viral replication. As mentioned previously, such complexes function in the selective transport of viral mRNAs and the shut-off of host cell protein synthesis. E4 ORF6 plays a vital role in these processes as a shuttle for 55K, which is found in the perinuclear region in the absence of this E4 product. E4 ORF6 possesses three targeting signals: a basic (i.e., arginine/lysine-rich) nuclear localization signal (NLS) towards its amino terminus; a nuclear export signal (NES) in the central region of E4 ORF6; and a nuclear retention signal (NRS) towards the carboxy terminus.[39] Thus, association with E4 ORF6 targets 55K to the nucleus via the NLS and NRS sequences. Recently, 55K has been shown to associate with a cellular RNA-binding protein.[40] Although this species does not seem to recognize specific RNA sequences, E4 ORF6-55K complexes appear to be localized in centers of viral transcription in the nucleus, leading to selective binding of adenovirus mRNAs. Such mRNA-55K-E4 ORF6 complexes are then shuttled to the cytoplasm via the E4 ORF6 NES sequence, resulting in selective export of viral transcripts and translation of late viral products. Mutants defective in E4 ORF6 replicate somewhat poorly, presumably at least in part due to the absence of such selective expression of viral proteins. As discussed above, E4orf3 can partially compensate for this function, presumably by a mechanism related to its presence in the nucleus of infected cells. It is still uncertain if the shut-off of host cell translation induced by E4 ORF6-55K complexes is a direct cause of selective export of viral mRNAs, or if it results from an additional function of 55K-E4 ORF6 complexes. In summary, E4 ORF6 and 55K function individually and in concert against p53, but they also serve a critical role in the production of high levels of virus products needed to generate large amounts of progeny virions.

Adenoviruses and Adenoviral Products as Therapeutic Agents

Human adenoviruses are the cause of annoying and sometimes serious infections. But they have been of great value as model systems to uncover the molecular basis of many cellular processes. They have been particularly important in cancer research, especially regarding the mechanism of cell cycle control, tumor suppressors, and apoptosis. But adenoviruses and their products have the potential to provide effective therapies against genetic disorders, cancer, and other diseases. As is discussed in chapters 10-17, they have already been adapted as vectors used for gene therapy or vaccines. The first generation of such vectors lacked E1 (E1A + E1B) and E3, to prevent virus replication and to allow space for insertion of transgenes, as adenoviruses have a defined size limit for packaging DNA molecules. One problem has been that such viruses eventually induce an inflammatory response, preventing prolonged gene therapy, and thus the efficacy of future adenovirus vectors might be improved by leaving E3 intact. They remain extremely efficient vehicles to introduce heterologous genes into human cells, and future generations of adenovirus vectors will surely be created in which existing problems are reduced or eliminated.

Adenovirus gene therapy vectors are designed to be defective for replication. But recently an ingenious approach has been taken that capitalizes on the ability of the virus to replicate in and kill cells selectively. If, for example, adenoviruses could be developed that replicate only in cancer cells, such cells could be eliminated selectively from cancer patients by replication and spread of virus. Current cancer therapies are limited in part by the ability of chemotherapeutic agents to kill cells lacking p53. This problem is serious, as over half of all human cancers survive by inactivating p53. A new approach has been initiated by Frank McCormick and Onyx to overcome this problem using an adenovirus mutant, termed ONYX-015, that fails to express the E1B-55K protein.[41] As 55K represents a major defense

against p53, it grows poorly in normal cells due in part to the activation of p53. But, in p53-null cancer cells the inhibitory effects of p53 pose no obstacle to viral replication. This virus showed promising results in Phase I clinical trials, and Phase II trials are underway at this writing. But clearly, ONYX-015 is only a first step. Elimination of the entire 55K product is deleterious to effective replication because, as discussed above, it serves other critical functions in the replicative cycle. In addition, as described earlier, adenoviruses have other mechanisms to regulate p53 and thus at least limited replication and killing of normal cells would be expected. Future versions of such therapeutic adenoviruses could therefore be developed to further optimize selective killing of cancer cells lacking p53. One could also envision using other genetic defects inherent in diseased cells as a means to allow selective killing by adenoviruses or other animal viruses. Many are skeptical about this approach, as a great number of difficulties still must be overcome, including the effective spread of virus to all affected cells, especially those in metastases, and the problems of host defense mechanisms. But selective killing of diseased cells by viruses is a fascinating new concept that deserves further study.

It is also possible that individual adenovirus proteins or mimetics based on them could offer new strategies for therapy. As discussed above, E3 products are effective immunosuppressors and may be useful for this purpose therapeutically. E1B proteins modulate p53 and apoptosis and thus could be effective in diseases characterized by excessive apoptotic cell death. E4orf4 and the E3-ADP play roles in cell death and might be employed in the selective killing of cancer cells that are otherwise resistant to apoptotic pathways. Thus, adenoviruses may provide important new reagents in the treatment of disease.

Conclusion

As summarized in Figure 5.5, shortly after infection of human epithelial cells, adenovirus E1A proteins are expressed, leading to the transactivation of other early viral genes. E1A products, through the formation of complexes with Rb or p300 family proteins also stimulate entry of cells into S-phase to permit synthesis of viral DNA using host cell DNA synthetic machinery. E1A proteins induce two types of apoptosis. One is p53-dependent and may relate directly to induction by E1A proteins of unscheduled DNA synthesis. The other is p53-independent and relates to E1A-mediated expression of E4orf4 and possibly other viral products. Adenoviruses have evolved several mechanisms to block or delay cell death to ensure efficient virus production. p53-dependent apoptosis is blocked directly by both 55K and E4 ORF6, which also act in combination to prevent accumulation of high levels of p53 by a separate mechanism. E1B-19K also blocks apoptosis, presumably by binding to death-inducing proteins like Bax. Later in infection, genes encoding viral structural proteins are expressed and the infected cell becomes full of progeny virions. Ultimately, the infected cell is killed, at least partially by apoptosis induced by E4orf4, resulting in the spread of virus to neighboring cells through endocytosis of apoptotic vesicles containing infectious virus. In the case of rodent cells, although early adenovirus genes are expressed and host cell DNA replication is induced, synthesis of viral DNA and late proteins does not occur and thus the infection is abortive. Stable integration of viral DNA into rodent cell chromosomes and permanent expression of E1A and E1B proteins can lead to the creation of stably transformed cells that, under the appropriate conditions, can form tumors in newborn or immunosuppressed rodents. Adenoviruses remain important model systems for the study of a variety of cellular processes and represent new vehicles to treat human diseases.

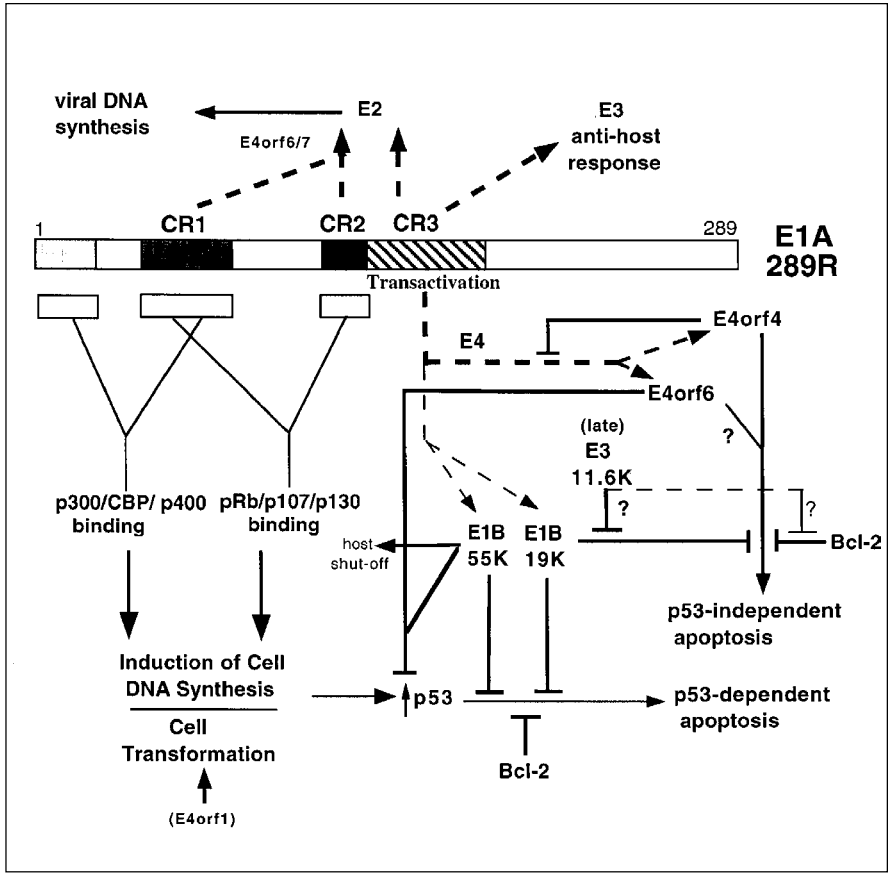

Fig. 5.5. Summary of early events in Ad5 infection. Arrows represent promotion of an activity, those with dashed lines involving transcriptional activation. Solid lines with bars indicate inhibition of an activity. In some cases, a ? denotes that the process is not understood.

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Chapter 6

Adenovirus DNA Replication

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After adenovirus (Ad) enters the cell by receptor-mediated endocytosis, the viral DNA is uncoated and transported to the nucleus. Beginning at 6-8 hours post-infection, DNA is efficiently replicated, generating high amounts of progeny molecules (10^5-10^6/cell). Development of a cell-free system[1] has contributed greatly to our understanding of viral DNA replication (for reviews see refs. 2,3). DNA replication results from an orderly interaction between viral proteins, cellular factors and template DNA at discrete sites within the nucleus that appear to be distinct from the transcription sites. DNA synthesis begins by a novel protein priming mechanism in which viral polymerase (AdPol) catalyzes the covalent linkage of the 5′-terminal nucleotide dCMP to the β-OH of a serine residue of the viral preterminal protein (pTP), which is the precursor of the terminal protein (TP). This initiation of DNA replication occurs at specific DNA sequences at the origin of replication in the presence of cellular transcription factors, nuclear factor I (NF-I) or CAAT transcription factor (CTF-1) and nuclear factor III (NF-III) or octomer-binding transcription factor (Oct-1). The pTP-dCMP complex formed in the initiation reaction serves as the primer for subsequent elongation catalyzed by AdPol via a strand displacement mechanism in the presence of the virus-encoded DNA-binding protein (DBP) and the host factor, nuclear factor II (NF-II), which is a type I DNA topoisomerase.

Viral Genome and the Origin of DNA Replication

The Ad genome contains a linear double-stranded DNA of about 35-36 kb with a 55 kDa protein (TP) covalently linked to the β-hydroxyl group of a serine via a phosphodiester bond at each 5′ end (for reviews see refs. 2,3). The genome is in a highly condensed form, associated with viral basic proteins V and VII in a nucleosome-like structure. The replication origins are located within the first 50 base pairs (bp) of inverted terminal repeats (ITR) of about 100 bp in length; the exact size depends upon the serotype. The minimal core origin that is conserved in various Ad serotypes is located between 9-18 bp and contains binding sites for pTP and AdPol. An auxiliary origin, located between 19 and 48 bp, contains recognition sites for cellular transcription factors NF-I and NF-III/Oct-1. In Ad2 (and Ad5), this auxiliary origin stimulates the basal core origin-mediated replication initiation about 200-fold. However, in Ad4, the terminal 18 bp is sufficient for efficient replication initiation in vitro and in vivo.[3] Although the auxiliary region of Ad4 ITR contains a binding site for NF-III/Oct-1, it lacks the NF-I binding site, and neither factor is required for Ad4 DNA replication.[3]

E2 Region and Its Regulation

The E2 transcription unit encodes proteins AdPol, pTP and the DBP, which participate in viral DNA replication (for a review see ref. 4). The E2 transcription unit consists of E2

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early promoter (E2A at genome coordinate 76), which is activated by E1A during early times after infection, and E2 late promoter (at genome coordinate 72), which is activated at intermediate times after infection through an unknown mechanism (for a review see ref. 5). DBP is encoded by the E2A region, which shares common RNA leader sequences near genome coordinates 75 and 68 with mRNAs for pTP and AdPol. pTP and AdPol are encoded by the E2B region of the viral genome and their mRNAs share a common exon at genome coordinate 39. These short exons are spliced to the main body of the open reading frames (ORF) for pTP and AdPol at genome coordinates 28.9 and 24.1, respectively.

Using linker scanning mutagenesis, four *cis*-acting elements of E2 early promoter were identified: TTAAGA (-22 to -30), recognized by human TATA box-binding protein (TBP); two E2F binding sites TTTCGCGC, inverted to each other (-35 to -63); and an activating transcription factor (ATF) binding site, TGACGTCA (-68 to -77).[4] In Ad-infected cells, the efficient transcription of the E2 early promoter is dependent on the viral E1A 13S protein[5] and the cellular transcription factor E2F, which functions as a heterodimer with DP1 (E2F/DP1) and binds to the two E2F-binding sites. In G0 and most of G1 phases of the cell cycle, E2F/DP1 exists as a transcriptionally inactive complex with the tumor suppressor retinoblastoma protein (Rb), which negatively regulates expression of a number of cellular genes involved in G1→S-phase transition. However, subsequent to adenovirus infection, E1A protein induces the dissociation of the Rb-E2F/DP1 complexes and stimulates transcriptional activation function of E2F/DP1, which in turn activates the E2 early promoter (for a review see ref. 6). The interaction of E2F/DP1 with the two E2F-binding sites is stabilized by the product of E4 transcription unit, the 19 kDa E4 ORF6/7 fusion protein, which binds as a dimer to two molecules of E2F/DP1. In addition to E2F/DP1, one member of the ATF family, ATF-2, is also involved in E1A-mediated activation of E2 and E4 promoters.[5] The basis for the delayed activation of the E2 late promoter in the viral lytic cycle is not known. Unlike the E2 early promoter, E2 late promoter is not transactivated by E1A.[4]

Viral Replication Proteins

DBP

DBP is a nuclear phosphoprotein that accumulates to high levels, up to 2 x 10^7 molecules/cell. DBP is composed of 473 to 529 amino acids, depending on the viral serotype, and is synthesized both early and late in the infectious cycle. It is a multifunctional protein that is involved in DNA replication, early and late gene expression, host range,[7] transformation, virion assembly and possibly recombination. It also participates in replication of adeno-associated viruses. The less conserved N-terminal domain, encompassing about one-third of the protein, is extensively phosphorylated and contains the nuclear localization signal. The highly conserved, non-phosphorylated C-terminal domain binds to DNA and participates in DNA replication.[8] The three dimensional crystal structure of the C-terminal domain[9] reveals that the protein is globular and the overall folding of the polypeptide backbone is stabilized by binding of two zinc atoms. The 17 residue C-terminal arm of one monomer hooks onto the adjacent monomer, resulting in the formation of an oligomeric protein chain.

DBP binds to single-stranded DNA with cooperativity and high affinity, thus protecting single-stranded DNA from nuclease attack. It exhibits a helix destabilizing property, which is required for unwinding double-stranded DNA in an ATP-independent manner during the elongation phase of DNA replication by strand displacement.[10-12] DBP also increases the rate of renaturation of the displaced complementary strands.[13] DBP enhances initiation of DNA replication by lowering the K_m for dCTP during the formation of the initiation complex pTP-dCMP and by enhancing the binding of NF-I to its recognition site in the

auxiliary origin.[14,15] It increases the processivity of AdPol[16] and modifies the sensitivity of AdPol to nucleotide analogs.[17] In addition to its role in viral DNA synthesis, DBP has been shown to affect the rate of transcription at several promoters and to be involved in mRNA stability[18] and virus assembly.[19]

pTP

pTP exists as a stable heterodimer with AdPol and participates in initiation of DNA replication. During replication initiation, AdPol catalyzes the covalent linkage of dCMP to serine-580 of pTP, and the resulting pTP-dCMP then serves as the primer for DNA synthesis. Unlike DBP, pTP is not synthesized in large amounts in adenovirus-infected cells. Cloning of cDNA for pTP and subsequent high level expression using vaccinia virus[20,21]and baculovirus systems[21,22] have facilitated a detailed characterization of pTP (for a review see ref. 23). During initiation of DNA replication, pTP binds to the core origin sequences in a phosphorylation-dependent manner.[24] Late in the infection cycle, pTP is processed to TP via an intermediate, iTP, by the action of the virus-encoded protease.[25] Hay and colleagues[25] have shown that pTP and iTP, but not TP, are capable of binding to AdPol, suggesting that residues critical for interaction with AdPol are located between iTP and TP cleavage sites. Among the three forms, only pTP recognizes origin DNA.

Several studies have shown that pTP also mediates attachment of the Ad genome to specific sites on the nuclear matrix.[26] Phosphorylation status of pTP is also important for attachment to the nuclear matrix.[27] Studies of pTP mutants have indicated an important role for pTP, potentially in the formation of replicative complexes at the nuclear matrix.[28] Recently, Angeletti and Engler[29] have reported that pTP binds to the pyrimidine biosynthesis enzymes known as CAD (for carbamyl phosphate synthetase, aspartate transcarbamylase and dihydroorotase) at the active sites of viral DNA replication on the nuclear matrix. They suggested that pTP-CAD interaction may serve to anchor the viral genome in the proximity of factors required for DNA synthesis.

The functional significance of covalent attachment of pTP/TP to the viral genome is not fully understood. Genome-linked TP also determines the subnuclear location of the viral DNA templates for transcription and replication.[26,28,30] Covalent attachment of pTP/TP has been suggested to protect viral DNA from exonucleases and facilitate unwinding of DNA duplex at the origin of replication. TP-bound origin sequences have been shown to adopt a different structure and stabilize binding of the AdPol-pTP heterodimer[31] to the origin. Consistent with this finding, in transfection experiments the infectivity of TP-DNA is orders of magnitude higher than the naked DNA.[32] The processing of pTP-DNA to TP-DNA is not required for viral DNA replication or virion assembly, because both processes occur in the temperature-sensitive Ad protease mutant H2ts1 at restrictive temperature.[33] However, this processing is required for infectivity of the virions, although the basis for this requirement is unknown. The processing of pTP is presumably to create a functional template for either early transcription or the first round of DNA replication.[25] pTP contains its nuclear localization signal (NLS) sequence within TP and is efficiently transported to the nucleus in a NLS-dependent manner.[34] pTP also facilitates the nuclear localization of AdPol as a complex.[34]

AdPol

AdPol is a 140 kDa phosphoprotein that catalyzes both the initiation and elongation steps of Ad DNA replication. Similar to pTP, functional characterization of AdPol has been possible using the high level heterologous expression systems (for a review see ref. 23). AdPol is a member of the family of DNA polymerases and shares five of six conserved regions with other members. Mutational studies have revealed that essential regions of AdPol are

scattered across the entire molecule and not limited to five regions of homology.[35] Similar to other DNA polymerases, AdPol also exhibits an intrinsic 3′→5′ proofreading exonuclease activity.[36,37] Unlike other eukaryotic DNA polymerases, AdPol is relatively inactive with RNA primers, and is less sensitive to aphidicolin. AdPol contains two potential zinc finger motifs that are important for its DNA binding and DNA replication initiation functions.[38]

In addition to forming a stable heterodimer with pTP, AdPol also physically interacts with NF-I, and this interaction targets the AdPol-pTP complex to the DNA replication origin.[39-41] During chain elongation, DBP is believed to stabilize the interaction of AdPol with the template DNA and increase its processivity. However, the proofreading exonuclease activity of AdPol is inhibited in the presence of DBP.[36] AdPol also associates with a histone H1 kinase that is capable of phosphorylating AdPol, although the functional significance of this interaction in DNA replication is not known.[42] Phosphorylation of AdPol occurs exclusively on serine residues, and serine-67 is the major site of phosphorylation.[43] Dephosphorylation studies have indicated that phosphorylation of AdPol is important for its replication initiation function.[43] Heterologous expression and biochemical characterization of A temperature-sensitive (ts36; leucine-391→phenylalanine) mutant AdPol was previously shown in vivo to be defective in viral DNA synthesis in human cells and transformation in rat cells.[44] Heterologous expression and biochemical characterization has revealed that the ts36 defect in in vitro initiation and elongation assays is dependent on the temperature (37°C or 32°C) at which the recombinant protein is expressed in HeLa cells.[45] In contrast to the wild type protein, ts36 AdPolexpressed at 37°C failed to recognize the viral DNA replication origin, but bound to a single-stranded DNA cellulose column with greater affinity, suggesting that the defect in the ts36 AdPol for DNA replication can be attributed to its altered DNA-binding properties.

Involvement of Other Viral Proteins

Although not required for DNA synthesis in the in vitro assays, other viral proteins contribute indirectly. As mentioned before, immediate early products of E1 and E4 genes contribute towards activation of E2 early promoter to ensure abundant expression of the viral DNA replication proteins (for a review see ref. 4 and references therein). The E1B 55 kDa and 19 kDa proteins block induction of apoptosis and prevent early degradation of both cellular and viral DNA in infected cells (for a review see ref. 46). Among the E4 gene products, ORF4 encodes a product that prevents viral DNA replication, while the products of ORFs 3 and 6 antagonize the effects of ORF4 on DNA synthesis[47] (see also this volume). It was suggested that since ORF4 and ORFs 3 and 6 have opposing effects on DNA accumulation, they participate in setting the level of DNA replication in Ad-infected cells. Recent studies have shown that ORF4 downregulates E2 expression and that in E4 ORF3-, 6- genetic background, levels of E2 expression are correlated with the accumulation of viral DNA.[47] However, themechanisms contributing to the stimulatory effects of ORF3 and ORF6 on replication 0remain to be explored.

Cellular Factors Required for Replication

NF-I

The cellular transcription factor NF-I binds as a dimer with high affinity to double-strand DNA with the sequence 5′-^{25}TGGC(N)$_6$GCCAA^{38}-3′ at the auxiliary replication origin, and this interaction is enhanced by DBP (for a review see ref. 2). NF-I also interacts with AdPol-pTP complex as the result of binding to AdPol, and recruits this complex to the core origin through protein-protein interaction.[39] The position of the NF-I binding site relative to the core origin is critical, as indicated by mutational analyses,[48] which

is consistent with the requirement for interaction of NF-I with AdPol-pTP. Deletion analysis has indicated that the region of NF-I that interacts with the AdPol-pTP complex is located between amino acids 68 and 150 of NF-I.[35,39] Interactions with NF-I leads to increased stability of the AdPol-pTP complex at the origin,[40] and the degree of stimulation ranges from 60 to 2-fold with increasing amounts of pTP-AdPol. Mutational analyses have indicated a highly conserved N-terminal domain of NF-I that participates in DNA binding, dimerization and DNA replication, and a less conserved C-terminal domain involved in transcriptional activation.

NF-III/Oct-1

NF-III/Oct-1 binds next to the NF-I binding site to the sequence 5′-^{39}TATGATAATGA^{49}-3′, in the auxiliary origin and stimulates replication initiation 3 to 7-fold in vitro (for a review see ref. 49). This NF-III/Oct-1-mediated stimulation also depends on the AdPol-pTP concentration and a DNA-independent interaction between its DNA binding domain and the AdPol-pTP complex.[48] DNA bending induced by NF-III/Oct-1 at the origin of DNA replication is thought to facilitate interactions between the components of the initiation complex.[50] However, analyses of the role of NF-III/Oct-1 in viral DNA replication in vivo have revealed that the deletion of the NF-III/Oct-1 site has no effect on replication.[51] In a later study, analyses of in vivo replication efficiencies of mutants with deletions of sequences between 44 and 107 bp containing NF-III/Oct-1, adjacent Sp1 and ATF sites and between 107and 195 bp, have revealed that there are redundant elements between 107 and 195 which could functionally substitute for the deletion of NF-III/Oct-1, Sp1, and ATF sites in the mutantcontaining a deletion between 44 and 107 bp.[52] The stimulatory effect of NF-III/Oct-1 is attributed to the DNA-binding POU domain, which consists of two subdomains: a POU-specific domain that recognizes 5′-^{39}TATGA^{43} and a POU homeodomain which binds to 5′-^{44}TAATGA^{49}-3′.[53,54] Moreover, the POU domain contacts the pTP in the PTP-AdPol complex, in contrast to NF-I which contacts the AdPol; together, these transcription factors enhance the affinity of the pTP-AdPol complex for the core origin. Importance of NF-III/Oct-1 for adenovirus replication is also revealed in a study in which insufficient levels of NF-III/Oct-1, and its low affinity for the origin of DNA replication, were correlated with abortive infection of BHK21 hamster cells by Ad12.[55]

Other Host Factors

Synthesis of full length Ad DNA by AdPol in vitro seems to require NF-II, a type I DNA topoisomerase.[56] For in vivo replication, the requirement for both topoisomerases I and II activity has been demonstrated using specific inhibitors.[57] Inhibition of topoisomerase I activity led to an immediate termination of Ad DNA replication, while inhibition of topoisomerase II blocked replication only after completion of approximately one additional round.[57] Additionally, a novel factor termed template activation factor-1 (TAF-1), isolated from uninfected HeLa cytoplasmic fractions, greatly stimulates DNA replication in a cell-free system utilizing Ad genome complexed with viral core (V and VII) proteins as a template.[58] The role of this factor in in vivo replication of the Ad genome is presently unknown.

Initiation and Elongation of DNA Replication

Assembly of a large preinitiation complex comprised of AdPol-pTP, DBP, NF-I and NF-III/Oct-1 at the origin precedes the initiation event (Fig. 6.1). The recognition site for NF-I within the Ad replication origin is one of the high affinity sites, and binding of NF-I to this sequence is facilitated by DBP.[14,15] Specific interactions of NF-I and NF-III/Oct-1 at the auxiliary region of the replication origin recruit and stabilize recognition of the core

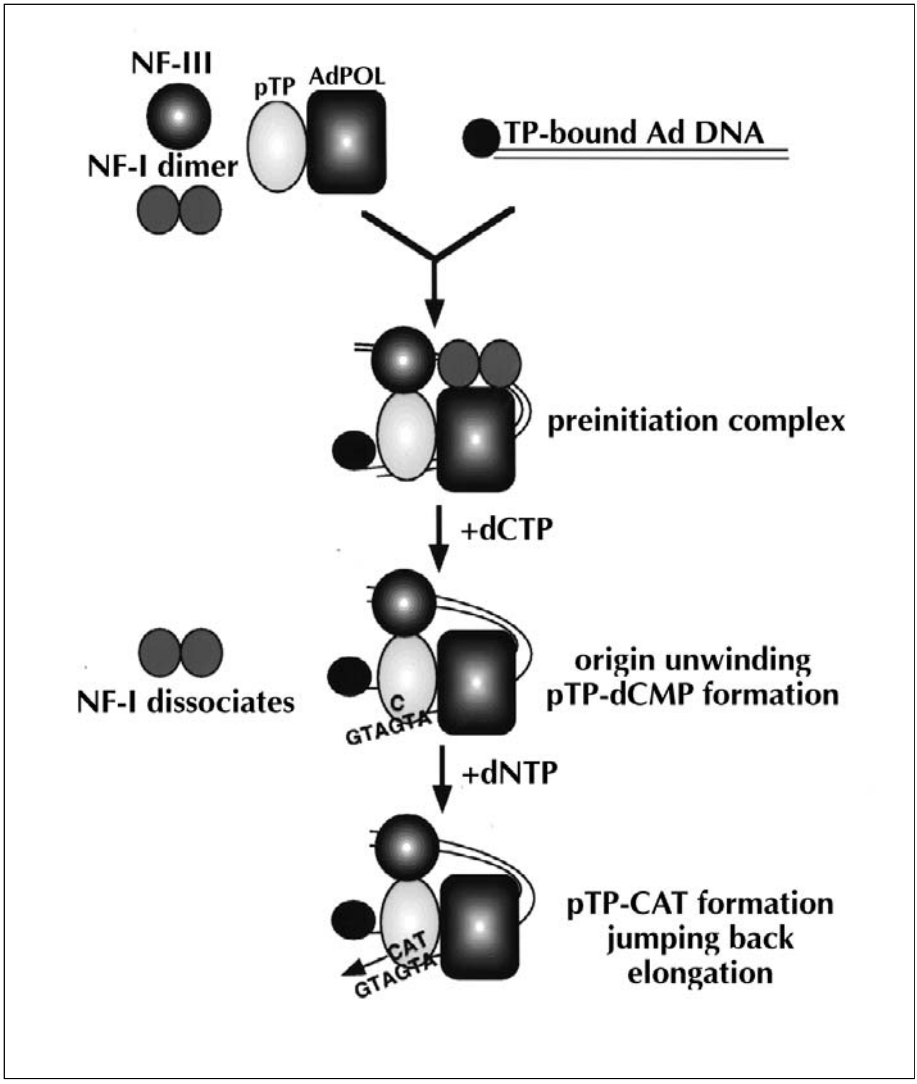

Fig. 6.1. Model for initiation of DNA replication. See text for details.

replication origin by the AdPol-pTP complex.[39-41,59,60] The binding of the AdPol-pTP complex to its recognition sequences is further enhanced by an interaction with genome-linked TP and between NF-I and AdPol[2,3] as well as between the POU domain of NF-III/Oct-1 and pTP.[48] The phosphorylation state of AdPol and pTP and/or the association between AdPol and a cellular cdc2-like kinase is likely to influence these protein-DNA and protein-protein interactions, although direct experimental evidence remains to be established.[23] After the assembly of the preinitiation complex, the origin unwinding is believed to take place, by a mechanism that is not clear. In vitro assays have indicated that unwinding takes place in an ATP-independent manner without the participation of helicase.[61] It has

been suggested that unwinding may be facilitated by DBP, which has the intrinsic ability to unwind DNA. After the unwinding, the AdPol-pTP complex positions in such a way that serine-580 is placed opposite to GTA at positions 4-6, instead of GTA at positions 1-3.[62] In the initiation reaction that requires Mg^{2+}, AdPol catalyzes the formation of a phosphodiester bond between the α-phosphoryl group of the incoming dCTP and the β-OH group of serine-580 in pTP.

Initiation of DNA replication is followed by formation of a pTP-trinucleotide intermediate, pTP-CAT, by AdPol using the complementary sequence GTA located at nucleotides 4-6 from the terminus as template.[62] At this stage of replication, the majority of the AdPol-pTP complexes are dissociated, and free AdPol then extends pTP-CAT when this intermediate "jumps back" to the terminus[62,63] and forms base pairs with nucleotides 1-3 from the terminus. This jumping-back mechanism is believed to play a role in correcting errors made during initiation. This mode of replication is conserved in ϕ29 phage DNA replication, which also utilizes a protein-primed mechanism.[64] Subsequent DNA elongation by strand displacement requires DBP in addition to AdPol. It is believed that when the replication fork moves towards the end of the molecule, AdPol dissociates and the displaced single-stranded DNA is released as a stable single-stranded DNA-DBP complex. The single-stranded DNA is believed to then function as a template by formation of a panhandle, restoring a double-stranded origin which can again be used as a template for the pTP-primed initiation reaction.[65] Alternatively, displaced single strands can be converted to double-stranded DNA by reannealing to complementary single-stranded DNA.[13]

Conclusion

Adenoviruses have served as valuable model systems to study transcriptional control, DNA replication, cellular transformation and apoptosis. There has been a tremendous progress in last 15-20 years in our understanding of molecular interactions that lead to Ad DNA replication. Much of this knowledge has been gained using in vitro reconstituted assays. However, within Ad-infected cells there may be additional regulatory mechanisms that contribute to efficient synthesis of viral DNA. As we learn more about the mechanisms that operate at the intracellular level, in addition to expanding our understanding of precise control of DNA replication within eukaryotic cells, these findings may enable us to design better vectors for gene therapy. In light of the recent interest in conditionally replicating adenoviruses as vectors for cancer therapy, a clear understanding of the adenoviral DNA replication might offer new avenues to design improved, selectively replicating vectors. Further insights into the mechanism by which viral DNA is efficiently transported to the nucleus, transcribed and replicated might be valuable in developing better viral and non-viral vectors for gene therapy.

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Chapter 7

Adenovirus Late Gene Expression

Julie Boyer and Gary Ketner

The late phase of an adenovirus infection begins with the onset of viral DNA replication. During the late phase, protein synthesis in adenovirus-infected cells is dominated by the production of large quantities of the adenovirus capsid proteins and of a few non-structural proteins required for capsid assembly, about 13 in all. Almost all of the proteins produced late in infection are the products of translation of 'late mRNAs' derived from the major late transcriptional unit (MLTU; see below for exceptions). The MLTU extends rightward from the major late promoter (MLP), located at genome position 17, almost to the end of the genome. The nearly exclusive expression of MLTU products late in infection is the result of profound changes in patterns of both viral and host gene expression that coincide with the beginning of viral DNA replication. On the viral genome, the rate of transcription from the MLP is dramatically upregulated from the low level observed earlier in infection, and transcriptional termination within the major late transcriptional unit is abolished. Transcription of most of the viral early genes is gradually reduced. Posttranscriptionally, splicing and polyadenylation site utilization in the primary transcriptional product of the MLTU changes, as do rates of transport and stability of viral RNAs. Transport and translation of most host mRNAs are concomitantly inhibited. As these events in gene expression are occurring, host cell DNA synthesis is inhibited in favor of synthesis of viral DNA. Cells thus become efficient machines for the production of virus particles for subsequent release.

Structure of the Late RNAs

The adenovirus late mRNAs occupy a singular position in the history of molecular biology. In the course of mapping the physical position of the gene for the viral late protein hexon, Berget, Moore, and Sharp determined that hexon mRNA was not colinear with the viral DNA, consisting instead of RNA sequences that, while contiguous in the RNA, are encoded by widely separated segments of the viral genome.[1] To explain its unexpected structure, Berget et al suggested that hexon mRNA was assembled by the intramolecular joining—splicing—of separated portions of a precursor RNA molecule. Thus splicing, now appreciated to be universal in eukaryotes, was demonstrated first for adenovirus hexon mRNA.

All MLTU-derived late mRNAs share the organization first demonstrated for hexon RNA (Fig. 7.1).[2] At their 3′ ends, each possesses a typical poly(A) tract. The roughly twenty distinct late mRNAs share five polyadenylation sites (map positions (mp) 39, 50, 62, 78, and 91), and late messages are grouped into five late families (L1-L5; Figure 7.1A) based on their site of polyadenylation. At their 5′ ends, viral late RNA molecules contain an untranslated leader, usually consisting of three exons with a total length of about 200 bases (the tripartite leader). In most late messages, the tripartite leader is joined directly to a large exon that

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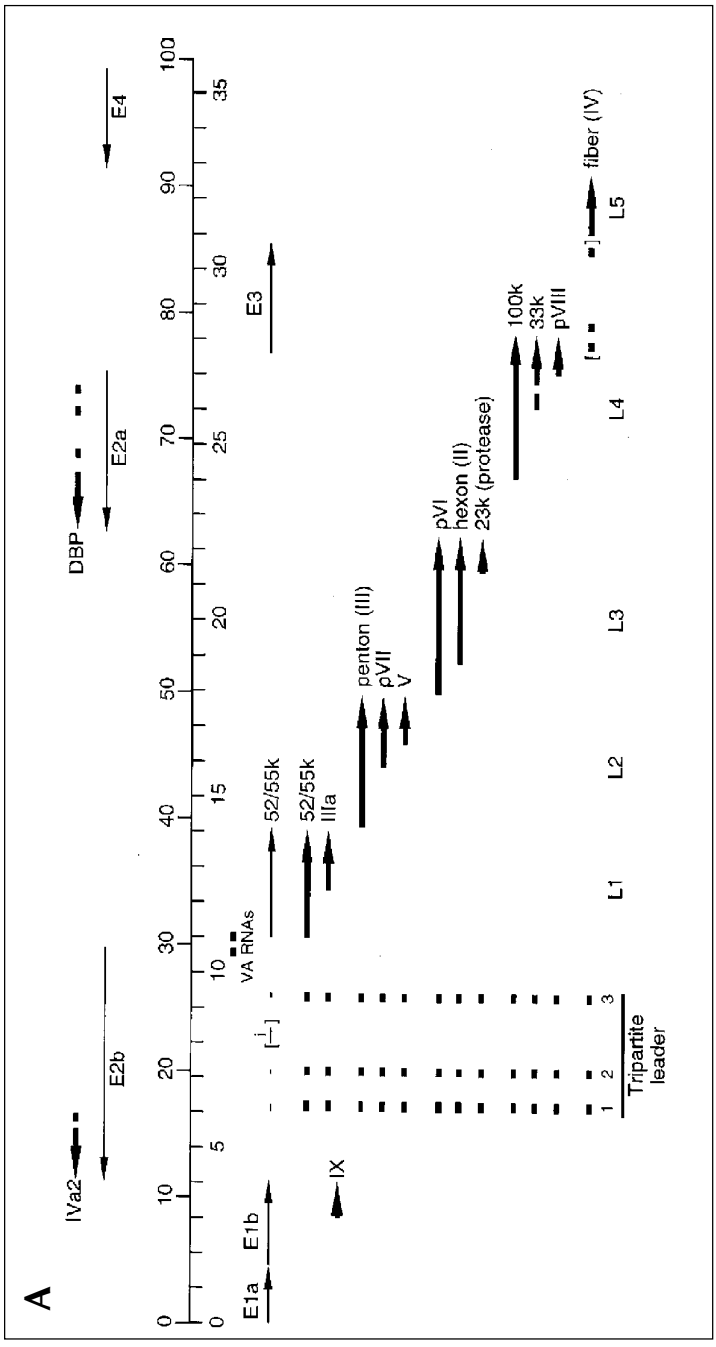
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E2b
E1a
E1b
IX
VA RNAs
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52/55k
IIIa
penton (III)
pVII
V
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E2a
pVI
hexon (II)
23k (protease)
E3
100k
33k
pVIII
E4
fiber (IV)
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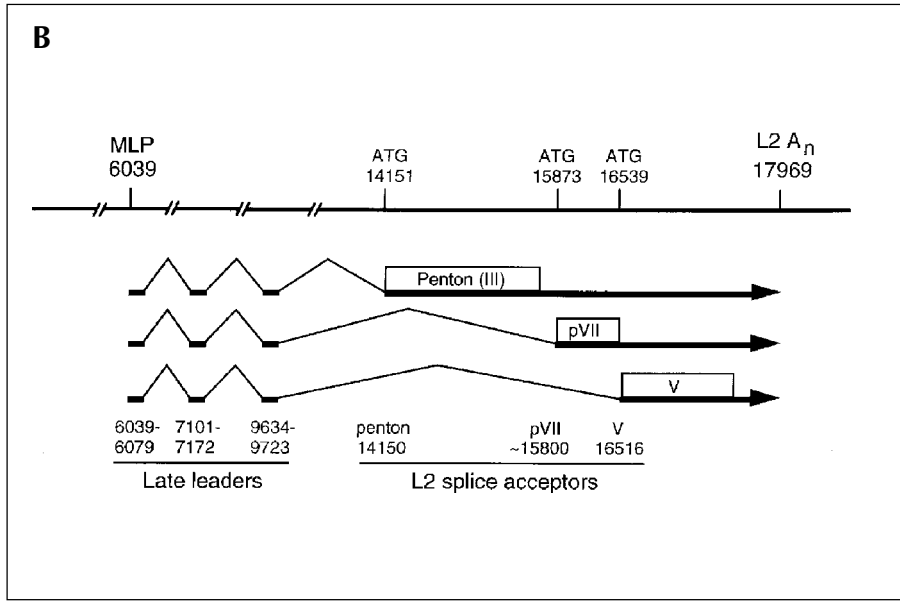

Fig. 7.1. Adenovirus 2 late mRNAs. (A)(see opposite page) Simplified transcription map of adenovirus 2 illustrating the organization of the MLTU. Thin lines indicate the primary transcripts of early regions E1A, E1B, E2A, E3, and E4. The region covered by stable cytoplasmic E2B mRNAs, and the spliced structure of the principal early mRNA from L1 are also shown. Thicker lines indicate exons of the major mRNAs made late in infection. mRNAs dervied from the MLTU (late regions L1 through L5) consist of the three components of the tripartite leader spliced to a body containing protein coding sequences. Spliced messages encoding DBP (from E2A) and IVa2 are also present late in infection, as is an unspliced mRNA encoding IX. The VA RNAs are polIII transcripts, also made during the late phase. The scale indicates position in map units (above the line) and kb (below). Arrowheads mark polyadenylation sites; brackets indicate 'optional' exons. The protein encoded by each late mRNA is indicated next to the message. The short leader segments shown are not drawn to scale. Panel (A), after Broker.[2] (B) Details of the structure of three mRNAs derived from one late region (L2). The location of the MLP, L2 polyadenylation site, and initiator codons for the three L2 proteins are indicated on the scale. The thick lines indicate the exons of the three L2 mRNAs, the tented thin lines introns. The location of the protein coding sequences and the product of each mRNA are indicated by the labeled boxes above each line. The positions are given in nucleotide number (for Ad2).

contains the protein coding sequences. Each of the splice acceptors used to join a coding exon to the tripartite leader is located immediately upstream of one of the late genes, and the position of the final splice acceptor thus determines the identity of the protein produced from each late mRNA. Among the members of a particular late family, up to five different acceptors can used for the splice that joins the tripartite leader to the coding exons. Members of a single late mRNA family use splice acceptors associated with a group of adjacent late genes. Thus, each of the late families consists of a nested set of 3′ coterminal

RNAs that differ in the position of the splice acceptor at the 5′end of the coding exon. The three segments of the tripartite leader are encoded at mp 17 (immediately adjacent to the MLTU cap site), 20, and 27. The late genes themselves are distributed over most of the right-hand 70% of the genome, occupying positions between about map unit 30 and 90. During the production of a typical adenoviral late mRNA, 3 introns with lengths ranging up to 20 kb are removed from the primary MLTU transcript.

In addition to the three exons of the canonical tripartite leader, some adenovirus late mRNAs contain additional 'optional' leader exons (i, x, y, and z). The largest of these is the 439 nucleotide i leader, which when present is located between segments 2 and 3 of the tripartite leader (mp 22-23). The i leader is unique in containing an open reading frame (ORF) that, in combination with a portion of the third late leader, encodes a 13.6 kDa protein. The i ORF functions in *cis* to modulate late mRNA half life;[3] the function of the protein produced is not known. Messages containing the i leader are most abundant in messages derived from the MLTU during the early phase of infection and early in the late phase, and decrease in proportion as the late phase progresses. This change in frequency may be regulated by products of early region 4 (see below). The x, y, and z leaders are encoded in the region of the genome between L4 and L5, and therefore appear only in some L5 (fiber) mRNAs. Their function is not known.

Transcriptional Activation

Transcription from the major late promoter is upregulated twice during the viral life cycle: at the beginning of the delayed early phase and again at the beginning of the late phase of gene expression. Activation of the MLP during the early phase requires the same product of early region 1A (E1A) as does activation of the traditional early regions. Early in infection, the rate of MLP-driven transcription remains low compared to that seen late. Coincident with the onset of viral DNA replication, transcriptional activity of the MLP is again increased dramatically, several hundred-fold on a per genome basis.[4] DNA replication itself is apparently required for late transcriptional enhancement to occur; both inhibitors of DNA synthesis and mutations that block replication also block stimulation of transcription. Further, in cells where late gene expression is fully underway from viruses that have been allowed to replicate their DNA, superinfecting viral genomes whose replication is prevented with inhibitors do not produce late gene products.[5] Therefore, even in cells where all of the components required for late gene expression must be present, replication per se is necessary for efficient function of the MLP on newly-introduced DNA molecules. The mechanism by which viral DNA replication induces MLTU transcription is unknown. Viral DNA replication may displace the proteins associated with viral DNA in the virion, allowing transcription factors more complete access to their binding sites.[6] Alternatively, replication might participate in the colocalization of viral DNA and transcription factors in infected cell nuclei. Replication-dependent assembly of specialized centers of viral gene expression might account not only for changes in transcription patterns, but also for alterations in the processing and export of mRNAs that accompany the early-late shift.

Transcription factors and their cognate binding sites both upstream and downstream of the MLP have been implicated in the stimulation of MLP activity at the early-late transition. The host cell transcription factor MLTF/USF binds at a site (UPE) that lies upstream of the MLP.[7] USF and UPE are required for maximal MLP activity in vitro and in transfection assays, and can confer ability to respond to the early-late transition on an ectopically located MLP. It has been reported that USF/MLTF binds to UPE only after DNA replication has begun, consistent with the observed dependence of activation upon replication. However, apparently conflicting data that UPS/MLTF binding to UPE is unaffected by replication has been published also, and viruses with mutations in UPE that abolish

USF/MLTF binding in vitro remain responsive to the early-late transition. The role of USF/MLTF in transcriptional activation at the early-late transition therefore remains unclear. Disruption of a second, nearby upstream site (a CAAT box) in mutants with an inactive UPE is apparently lethal. It is possible, therefore, that these two sites act redundantly to stimulate transcription from the MLP, the loss of both sites preventing sufficient late gene expression to support viral growth.

Downstream of the MLP, a cluster of three binding sites interact with two multimeric DNA binding proteins, each of which contains the viral IVa2 gene product.[8] IVa2 is an intermediate protein whose synthesis begins just before the MLP is activated. Temporally, occupancy of the downstream site precedes stimulation of transcription from the MLP, and IVa2 binding may thus contribute to enhancement. As with UPE, mutation of the downstream site does not prevent transcriptional upregulation at the early-late switch, but the mutations tested may not have completely abolished binding to all three sites in vivo. Mutants in the viral gene encoding IVa2 have not been tested for late gene expression.

Non-MLTU Late Proteins

In addition to the products of the MLTU, a few proteins derived from other regions of the genome are synthesized in substantial amounts late in infection. Two of these (IVa2 and IX) are present in the capsid. The IVa2 gene is transcribed from a promoter near the MLP but in the opposite orientation on the genome; the IX gene lies just beyond the end of E1B and is transcribed rightward. Both of these proteins appear before the onset of viral DNA replication and are therefore termed 'intermediate' proteins. Both have transcription factor activity and, as noted above, IVa2 may participate in stimulation of MLTU expression late in infection. A third protein abundantly expressed during the late phase is the viral DNA-binding protein (DBP). DBP is encoded by E2A, is present early in infection, and is required for viral DNA replication. Its action in replication is stoichiometric, and increasing amounts are required as DNA synthesis proceeds during the late phase. Presumably, its continued synthesis in the late phase reflects this requirement. The transcription of E2 during the late phase is driven by the 'late' E2 promoter, which is not active at earlier times; transcription from the 'early' E2 promoter declines as the late phase proceeds. Like the MLP, the E2 late promoter is activated by viral DNA replication. Notably, the E3 11.6 kDa protein is also produced in large amounts late in infection. This protein arises from a message containing an E3 exon spliced to the tripartite late leader. E3 11.6K is required for the efficient lysis of infected cells.

Regulation of Polyadenylation

Polyadenylation site usage in the MLTU is a temporally controlled process. Early in infection, transcripts originating from the major late promoter terminate just downstream of the L3 poly(A) site, but processing at the promoter proximal L1 site occurs nearly exclusively. In contrast, processing at all five sites occurs at roughly the same frequency following the onset of the late phase of infection. This switch in poly(A) site usage can be duplicated using recombinant adenovirus constructs encoding an MLTU containing only the L1 and L3 sites, and studies of these recombinant viruses have indicated that the basic requirements for the transition are viral DNA replication and connection of the poly(A) sites in cis.[9,10] Characterization of poly(A) site usage by many different transcription units indicates that, when two competing sites are present on the same RNA, selection is generally mediated by the affinity of the sites for 3′ end processing factors and by the location of the sites relative to the promoter and to each other. Characterization of the L1 site both in vivo and in vitro indicates that it is a much weaker substrate for 3′ end processing than the L3 site, since competition between L1 and L3 in trans or in cis on a pre-synthesized RNA

results in preferential L3 use. However, when transcription and processing are linked, the promoter proximal L1 site is preferred, with the temporal advantage overcoming the inherent weakness of the site.[11] Upstream and downstream sequence elements that enhance binding of processing factors (CPSF) to the core site are required for preferential L1 processing at early times and for the change in relative use.[10] Recent studies on the mechanism of early L1 dominance demonstrate that the presence of an unprocessed L1 poly(A) site inhibits the accumulation of stable mRNA processed at the downstream L3 poly(A) site. Processing at the L3 site occurs efficiently, but this mRNA never reaches the cytoplasmic pool of steady-state mRNA.[12]

Regulation of Splicing

The several distinct mRNAs expressed from the MLTU arise from alternative splicing of identical precursors. Like polyadenylation, alternative splicing in the MLTU is regulated over the course of infection, most dramatically in L1.[13] Two major mRNAs, encoding the 52/55K and IIIa proteins, are produced from L1 as a result of alternative splicing of the 5′ leader to either of two splice acceptors. Usage of these sites is subject to temporal control; the 52/55K site is used exclusively at early times of infection and with decreasing frequency thereafter, while IIIa splicing is detected only during the late phase. When splicing to these two acceptors is examined in extracts from uninfected cells, splicing to the promoter-proximal 52/55K site occurs about 10-fold more frequently than to the IIIa site. This may reflect a weaker affinity of the IIIa site for processing factors, but the spatial orientation of these two sites is also important; if the IIIa site is placed first on a tandem construct, it is able to compete effectively with 52/55K for processing. The alteration of the balance between 52/55K and IIIa splicing is due to an increase in commitment complex formation at the IIIa site in combination with repressed 52/55K splicing activity. Early repression of IIIa splicing involves an intronic repressor element located just upstream of the IIIa branchpoint sequence.[14] In contrast to their essential role in stimulation of splicing activity, binding to this repressor element by proteins from the SR family of splicing factors mediates repression. The steady state levels of SR proteins remain constant throughout infection, suggesting that they are somehow modified to alleviate IIIa repression at late times. A requirement for viral late protein synthesis for efficient IIIa splicing may also indicate involvement of a viral protein in this process.

Alternative splicing of the tripartite leader also occurs. Messages expressed at early times contain the i leader, while the majority of those expressed late do not. Two proteins expressed from early region four (E4), the products of ORFs 3 and 6, have opposing effects on tripartite leader assembly, one enhancing and one reducing the proportion of i leader-containing mRNAs.

Nuclear Organization

Viral DNA replication and late gene expression occur at discrete locations within the nucleus of an infected cell.[15] Although much is known about the biochemistry of replication and gene expression, the spatial organization of these processes and the relationship between them is just beginning to be understood. Uninfected cells contain discrete subnuclear structures which may be involved in RNA maturation. For example, interchromatin granules (IG) are not transcriptionally active, but contain splicing factors and poly(A) RNA. Viral infection induces formation of new subnuclear domains and alterations of pre-existing structures. At early times of infection, small electron-dense nuclear inclusions correspond to sites of viral replication and transcription. These sites contain viral DNA and spliced and unspliced viral RNA, but splicing factors retain their normal distribution in IGs separate from these locations. The onset of the late phase is accompanied by the formation of

structures containing large amounts of single stranded DNA (ssDNA) and the E2A-encoded DBP. Foci of active viral replication and transcription occur in partial coincidence at the periphery of these ssDNA accumulation sites, with transcription sites extending to more distant locations. The colocalization of splicing factors with sites of active transcription demonstrates that these factors are redistributed from interchromatin granules to centers of viral replication at this time, and that transcription and processing are linked. At times of maximal late transcription, splicing snRNPs and splicing factors are found in large clusters that correspond to the enlarged interchromatin granules (IGS) observed during the late onset of the phase. Viral transcription occurs in areas separate from the snRNP clusters, but snRNP clusters accumulate viral RNA posttranscriptionally, indicating that their formation may be an important step in viral late mRNA production. Recent studies have shown that the snRNP clusters contain polyadenylated RNA and spliced tripartite leader exons, suggesting that they may serve as an intranuclear accumulation point for processed RNA before transport.

Adenovirus also alters the organization of other nuclear substructures during the course of infection. Nuclear domain 10 (ND10) are dense spherical particles containing at least four different proteins, including the proto-oncoprotein PML. The pathogenesis of acute promyelocytic leukemia is correlated with a redistribution of the PML protein, and PML is also redistributed in adenovirus infection from ND10 to a nuclear fibrous meshwork. The E4 ORF3 protein colocalizes with PML in these fibers, and is both necessary and sufficient for this effect.[16] The function of these organelles and the significance of PML redistribution are not yet known; ND10 do not colocalize with snRNP containing organelles, and the ORF3-induced PML containing fibers do not colocalize with sites of RNA splicing.

mRNA Export

After maturation in the nucleus, mRNA must migrate from the final point of processing to a nuclear pore for export. These processes require at least two viral proteins, the early region 1B 55 kDa protein (E1B 55K) and the E4 ORF6 protein.[13] Viral mutants with disruptions of E1B 55K exhibit reductions in viral late protein synthesis that parallel reductions in cytoplasmic mRNA accumulation. Since reduced nuclear accumulation and cytoplasmic stability of the relevant transcripts are unable to account for the observed decrease in cytoplasmic mRNA, this phenotype probably reflects a decrease in mRNA transport. Dependence upon E1B 55K for transport extends to all mRNAs expressed during the late phase, including transcripts derived from the IX, IVa2, and E2 late promoters as well as the MLTU. Transport of early-expressed mRNAs does not require E1B 55K, including the MLTU-derived 52/55K mRNA which becomes strongly dependent upon E1B 55K at late times. The longest mRNAs in the late families show the greatest dependence upon 55K for transport. This may reflect the presence of unused splice sites in these messages that would normally be recognized as unprocessed and result in nuclear retention.

E4 ORF6 mutants also display defects in viral late protein synthesis and reductions in cytoplasmic mRNA accumulation. Identification of a physical interaction between E4 ORF6 and E1B 55K in infected cells and the phenotypes of double mutants indicate that the two proteins function as a complex. The complex of E4 ORF6 and E1B 55K has recently been demonstrated to shuttle between the nucleus and cytoplasm under the direction of the ORF6 protein, and this protein complex is therefore an attractive candidate for a direct role in nuclear export of viral late mRNA.[17]

In the late phase infected cell, adenovirus mRNA constitutes only around 20 percent of total RNA transcribed, although 90-95 percent of the mRNA reaching the cytoplasm is viral in origin. Like efficient export of viral mRNA, inhibition of host mRNA transport requires both E1B 55K and E4 ORF6, and it seems likely that these two phenomena reflect a single

underlying mechanism. It has been speculated that viral mRNAs are transcribed and processed in areas of the nucleus which are not well connected to the transport apparatus and that re-localization of a cellular protein to areas of viral transcription is required for efficient transport. Consistent with that proposal, E1B 55K localizes to the periphery of viral replication centers in an E4 ORF6-dependent manner.[18] The further possibility that the complex of E4 ORF6 and E1B 55K interacts with a cellular protein required for transport and sequesters it at sites of viral replication would contribute both to inhibition and selective export.

Inhibition of Translation of Host mRNA

During the late phase of adenovirus infection, infected cells produce viral proteins almost exclusively. This is despite the persistence in the cytoplasm of host mRNA that can be translated in vitro if extracted. Selective inhibition of host mRNA translation occurs by two distinct mechanisms. First, the activity of the cellular translation initiation factor eIF-4F is reduced late in infection.[19] eIF-4F is a multimeric cap-binding protein complex that stimulates translation of most mRNAs, probably by facilitating the melting of secondary structure found at their 5' ends. Because of the presence of the tripartite leader, which is relatively free of secondary structure, adenovirus late mRNAs are less dependent on eIF-4F than most host messages. This permits continued translation of viral late mRNAs as eIF-4F activity, and translation of host mRNA, declines. Reduced activity of eIF-4F is the result of under-phosphorylation of one of its components, eIF-4E, induced by expression of an as yet unidentified viral late protein(s).

Adenovirus infection also inhibits host cell translation by a second mechanism that exploits a central element of the interferon-induced antiviral state.[20] Double-stranded RNA produced by adenovirus infection activates the cellular protein kinase R (PKR), also induced by interferon. When activated, PKR is capable of phosphorylating the translational initiation factor eIF-2a, inhibiting its function and thus nonspecifically preventing translation. To subvert PKR-dependent inhibition of translation, adenoviruses encode one or two small VA (for virus associated) RNAs that bind to and antagonize activation of the kinase. Production of these RNAs specifically rescues viral protein synthesis despite activation of PKR. The basis of the specificity of the activity of the VA RNAs is at the moment uncertain, although it is likely to be the result of cocompartmentalization of VA RNA and viral mRNAs, perhaps in the centers of viral gene expression that develop late in infection.

Finally, it should be noted that the viral late protein 100K specifically stimulates translation of viral mRNAs with and without the tripartite leader, although it is not required for inhibition of host cell RNA synthesis. The mechanism of this effect is not known, although 100K binds RNA and is a component of adenoviral hnRNPs.

Conclusion

Adenovirus late gene expression has served with distinction as a paradigm for the study of eukaryotic gene expression. It is likely that its usefulness for this purpose is just beginning. Regulation of posttranscriptional events in mRNA production and the functional organization of gene expression in subnuclear domains, for example, are only two universal features of host cell gene expression that seem ideal targets for study in the adenovirus system.

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CHAPTER 8

Role of Endoprotease in Adenovirus Infection

Joseph Weber

With the possible exception of very simple viruses, most viruses appear to encode at least one virus-specific endopeptidase. In addition to facilitating the orchestrated fragmentation of polyproteins of RNA viruses, these proteolytic enzymes may also be involved with the suppression of host protein synthesis, the regulation of virus assembly, the egress and subsequent uncoating in another cycle of infection, of both RNA and DNA viruses.The endopeptidase encoded by adenoviruses (AVP) appears to be involved in several of these functions. Most of the literature concerns the protease of human adenovirus type 2, but there are good reasons to believe that the proteases of other adenovirus serotypes will be very similar. (For a review, see ref. 1.)

The gene for the protease is located near the middle of the genome, just downstream of the gene for the major capsid protein hexon. The enzyme is translated from a small, tripartite leader bearing mRNA expressed in the late phase of infection from the major late promoter. At 36 and 52 h p.i., approximately equal levels of enzyme were detected in the cytoplasmic and nuclear fractions.[2] No recognizable nuclear localization motif has been identified and it is not known by what means the enzyme is transported to the nucleus.

The enzyme is packaged into virus particles (estimated at 10-40 molecules per virion) as the integral protein, possibly in association with the viral DNA via four large clusters of positive charge on the protease.[3] The ts1 mutant (P137L) of Ad2 prevents encapsidation of the otherwise active AVP, resulting in unprocessed virions at the nonpermissive temperature which fail to uncoat in a subsequent infection. Unlike many other proteases, there is no evidence of proteolytic maturation in AVP. The basal activity of the enzyme is significantly boosted by an 11 residue cleavage product (pVIc) from the C-terminus of capsid protein pVI which forms a thiol bond with Cys-104.[4,5] The sequence of this peptide in Ad2 is GVQSLKRRRCF, with residues 1, 7, 8, 10 conserved in other virus serotypes. All of the seven viral proteins, accounting for approximately 3326 cleavage sites, digested by the enzyme are either internal or in part disposed internally in the virion: L1-52K scaffolding protein, pIIIa, pVI, pVIII hexon-associated capsid proteins, pVII and pX core proteins and pTP linked to the viral DNA. The order of cleavages and their relationship to virus assembly and maturation is not known. Mutants, such as in capsid proteins, which prevent virus assembly fail to execute these cleavages, yet assembly occurs in the absence of active enzyme, as in the case of ts1, suggesting that virus assembly triggers the activation of the enzyme. It has been suggested that the 50 copies of protease per virion might slide along the viral DNA to the 3326 cleavage sites disposed in the semi-crystalline interior of the virion.[6]

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TABLE 8.1. Adenovirus endoprotease sequences

Virus	Source	EMBL/GEN BANK accession No.	Number[4] of amino acids
H2	HUMAN	J01917[3]	204
H3	HUMAN	X13271	209
H4	HUMAN	M16692	201
H12	HUMAN	X73487[3]	206
H40	HUMAN	L19443[3]	205
H41	HUMAN	M21163	214
CAV1	DOG	Y07760[3]	206
CAV2	DOG	U77082[3]	206
MAV1	MOUSE	M33995	204
OAV	SHEEP	U40837[3]	201
PAV3	PIG	U33016	203
BAV2	COW	U44124	204
BAV3	COW	X53990	204
BAV7	COW	X53989	202
FAV1[1]	CHICKEN	U46933[3]	206
DAV1[2]	DUCK	Y09598[3]	202
EAV2	HORSE	L80007	200

[1]CELO, chicken lethal orphan virus; [2]egg drop syndrome virus; [3]complete genome; [4]includes initiator menthionine

In addition to viral proteins, cellular proteins may also be cleaved and indeed cytokeratin K18 and possibly K7 have been shown to be digested.[7] Cleavage results in the depolymerization of the cytoskeletal network and may accelerate cell lysis, thereby promoting viral spread. Other proteins which contain cleavage sites have also been digested in vitro, but only after denaturation to expose the site.

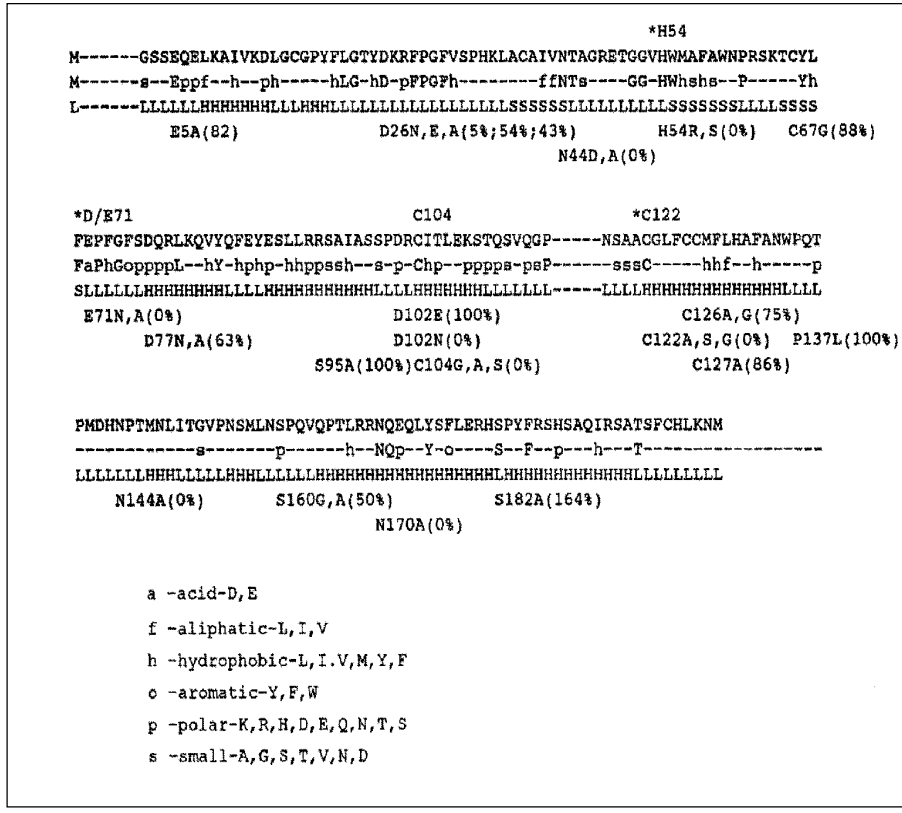

Fig. 8.1. Sequence features of the adenovirus protease. This abbreviated figure, displaying only the protease of human Ad2, was derived from a multiple sequence alignment of 17 protease sequences (listed in Table 8.1) using PILEUP (Wisconsin Genetics Computer Group). The active site triad of H54, C122 and D71(or E71 in all other serotypes) is indicated with stars. C104 forms the thiol bond with C10′ of the activating peptide. The third line displays residue conservation among the seventeen sequences as follows: Upper case letters indicate identity; lower case letters represent a selection of amino acids with similar functional or physical properties as defined before[16] and shown below the figure. The fourth line shows the secondary structure (L, loop; H, helix; S, strand) of Ad2 taken from the PDB 1AVP file using RasMol. Subsequent lines indicate mutations (P137L is the ts1 mutation and the only mutation available in the virus) and in vitro enzyme activity in brackets. Residue numbering is according to the Ad2 sequence.

Several types of indirect experimental evidence suggest that the protease is also required early in infection to mediate the release of virus particles from endosomes.[8,9] The failure of ts1 particles, which are devoid of protease, to leave the endosome, is the most compelling evidence for a role of the protease in endosomal lysis.

Currently, 17 distinct protease genes have been sequenced from adenoviruses infecting a variety of species (Table 8.1). The translated amino acid sequences range from 201 to 214. Thirty residues are identical (15%) and 63 residues (46%) are conserved in identical

positions among these sequences, all mostly in the N-terminal half of the molecule (Fig. 8.1). The atomic structure of the Ad2 enzyme complexed with its activating peptide has been solved.[3] Both the sequence and the structure are unique and unrelated to existing databank entries. Surprisingly, the disposition of active site residues (H54, E71, C122) is identical to that of papain (H159, N175, C25) and so is the location of Q115 of AVP and Q19 of papain, which is presumed to participate in the formation of the oxyanion hole of the latter. The identity of the active site triad is confirmed by mutational analysis.[10,11] Thus, AVP is a cysteine protease in a different class from both the papain superfamily and the other viral cysteine proteases.

The Ad2 enzyme is a 204 amino acid monomer of 24,838 Daltons. AVP complexed with the pVIc peptide has a K_m of 5 mM and its activity is optimal at pH 8, and 45°C in the presence of 1 mM thiol compounds and positively charged polymers. The enzyme is specific for two consensus sites (M,I,L)XGG-X or (M,I,L)XGX-G, where X is apparently any amino acid.[1] The rate of hydrolysis is influenced by the nature of the variable residues at the X sites. Furthermore, as might be predicted by the consensus sites on capsid precursor protein pVI, GX-G sites are cleaved 3-4 times faster than GG-X sites.[12] The GX-G site which gives rise to the pVIc activating peptide is conserved on all pVI sequences known so far. A survey of the sequences of all known precursor proteins reveals a remarkable conservation of the position of cleavage and the consensus sites. Human Ad4 contains one glaring exception; the iTP site (the first of two sites digested) is QRGF-G, suggesting that either this extends the consensus sequence to include Q in the P4 position (in addition to M,I,L), or more likely, that the specificity of the Ad4 enzyme is changed.[13]

Because of the absence of currently identifiable protein motifs on AVP, functions other than proteolysis are unlikely. Major unanswered questions include the following: What prevents the protease from digesting its substrates prior to virus assembly? How is the protease transported and encapsidated? Do the cleavage fragments have any function? Are any other cellular proteins cleaved? Are there any endogenous inhibitors of the protease? The unique nature of AVP and the prospect that all adenovirus proteases function similarly offers an ideal target for the development of specific inhibitors effective for the control of all adenovirus infections.[14,15]

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CHAPTER 9

Adenovirus Assembly

Susanne I. Schmid and Patrick Hearing

Assembly Intermediates

The assembly of adenovirus particles proceeds through an ordered series of assembly events (reviewed in ref. 1). The assembly of the virus particles has been probed using viral temperature-conditional mutants blocked at different stages of assembly at the restrictive temperature and by pulse-chase kinetic analyses. The first recognizable viral assembly intermediate is a light intermediate particle (buoyant density of 1.315 g/cc in a CsCl equilibrium gradient). These particles contain the capsid structural components and no or very little viral DNA and associated core proteins. Additionally, light intermediate particles contain several proteins that exit the particle during maturation (50 kDa and 39 kDa polypeptides) and may represent the adenoviral equivalent of phage scaffolding proteins. The light intermediate particles mature into heavy intermediate particles (1.37 g/cc buoyant density) with the insertion of viral DNA. The 50 kDa and 39 kDa polypeptides are released from the particle during this maturation step. The heavy intermediate particles appear to lack core proteins, which enter the particle during the next maturation step with the formation of young virus particles (1.34 g/cc buoyant density). The issue of separate or combined entry of DNA and core proteins, however, remains controversial. As the final step in maturation, the virus-encoded and encapsidated proteinase performs numerous cleavages of multiple viral proteins to generate the mature, infectious virion (see chapter 8). Four minor virus-encoded proteins (IIIa, VI, VIII and IX) appear to either enhance the assembly of subviral components and/or stabilize viral protein-protein interactions, and hence particle integrity, once formed (see chapter 2). The salient conclusion from these analyses is that adenovirus virion assembly likely follows an ordered series of maturation events, with a capsid prohead assembled as the initial target for the DNA encapsidation process. In this way, the assembly of infectious adenovirus particles may follow the paradigm of prokaryotic phage assembly.

Incomplete Particles of Adenovirus

Infection with human adenoviruses, including types 2, 3, 12, and 16, in tissue culture systems has shown that a given serotype yields several classes of viral particles (reviewed in ref. 1). These different classes can be distinguished from each other and individually purified on cesium chloride equilibrium gradients due to their distinct buoyant densities. Only one of these different types of particles constitutes complete infectious virus. The particles isolated from the remaining bands, when analyzed by electron microscopy, have a morphology that resembles that of complete adenovirus, but are only weakly or non-infectious. Therefore, they have been classified as incomplete virions. The number of bands representing discrete

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incomplete virion particles, as well as their predominance in an infected cell lysate relative to complete virions, is characteristic for each adenovirus serotype, but independent of the cell line or culture conditions. Pulse-chase experiments suggested a precursor-product relationship between incomplete and complete particles—following a pulse, radioactivity was first detected in incomplete virions and then decreased continuously with a coincident linear rise of radioactivity in complete virions. Analysis of the labeling kinetics of individual proteins further corroborated the precursor-product hypothesis—labeled hexon polypeptides appeared in incomplete particles immediately after the pulse, but with a lag phase of at least 60 minutes into mature virions. In contrast, labeled core polypeptides, which are found in association with the viral genome in intact virions, were incorporated into complete virions without a lag phase, suggesting packaging into a preformed empty particle. Additionally, the formation of incomplete particles is more sensitive to inhibition of protein synthesis than the formation of mature virions. These results strongly suggest that viral DNA and core proteins are inserted into preformed, empty capsids to yield infectious virions.

Polar Encapsidation of Adenovirus DNA

Incomplete particles of lower density than mature virions were isolated from Ad2, Ad3 and Ad7 infected cells. The lightest incomplete particles seem to be completely devoid of viral DNA. Among the other defective virions, a linear relationship between the length of the encapsidated viral DNA and the density of the corresponding group of incomplete particles was established. The size of the genome ranged from 15% of the length of the complete genome to full size. Restriction endonuclease analysis of the packaged subgenomes revealed that sequences derived from the left end are strikingly over-represented, which initially suggested that DNA packaging occurs in a polar fashion from left to right.[2,3] In support of this model, both ends of the genome were equally represented in the pool of subgenomic adenovirus DNA in infected cells.

The first indication for the presence of *cis*-acting sequences in the adenovirus genome that direct selective DNA encapsidation from the left end came from studies with naturally occurring evolutionary variants of Ad16.[4] A duplication of the left end 390, but not 290 base pairs (bp), at the right end of the viral genome allowed DNA packaging to initiate from both ends of the genome. It was suggested that the region between nucleotides (nt) 290 and 390 harbors an essential signal for polar viral DNA packaging. In separate studies, a number of Ad3 variant viruses were selected after repeated high multiplicity passage in HeLa cells.[5] Those variants carry various mutations in their left end 750 bp, but retain full capacity for growth and polar packaging of their genomes. Mutations of sequences within nt 136 to 318 did not affect viral growth, whereas the maintenance of nt 319 to 390 in all the mutants suggested that this region is indispensable. This interval coincides with the above mentioned region between nt 290 and 390 in Ad16 which appeared to direct DNA encapsidation. Sequence comparisons of Ad3 with Ad5 and Ad12, as representatives of adenovirus subgroups A, B and C, reveals that the interval between nt 237 and nt 491 is highly conserved.

Cis-acting Sequences Involved in Packaging Specificity

The corresponding *cis*-acting packaging domain of Ad5 was localized to the left end of the genome during deletion analysis of the E1A transcriptional control region (reviewed in ref. 6). The packaging domain overlaps with two distinct enhancer elements (Fig. 9.1A). Enhancer element I is repeated and specifically stimulates transcription of E1A. Enhancer element II augments transcription in cis from all the early transcription units by an unknown mechanism. An Ad5 mutant virus lacking the interval between nt 194 to 358 at the left end was nonviable. Substitution of the left end 353 bp to the right end of the genome restored

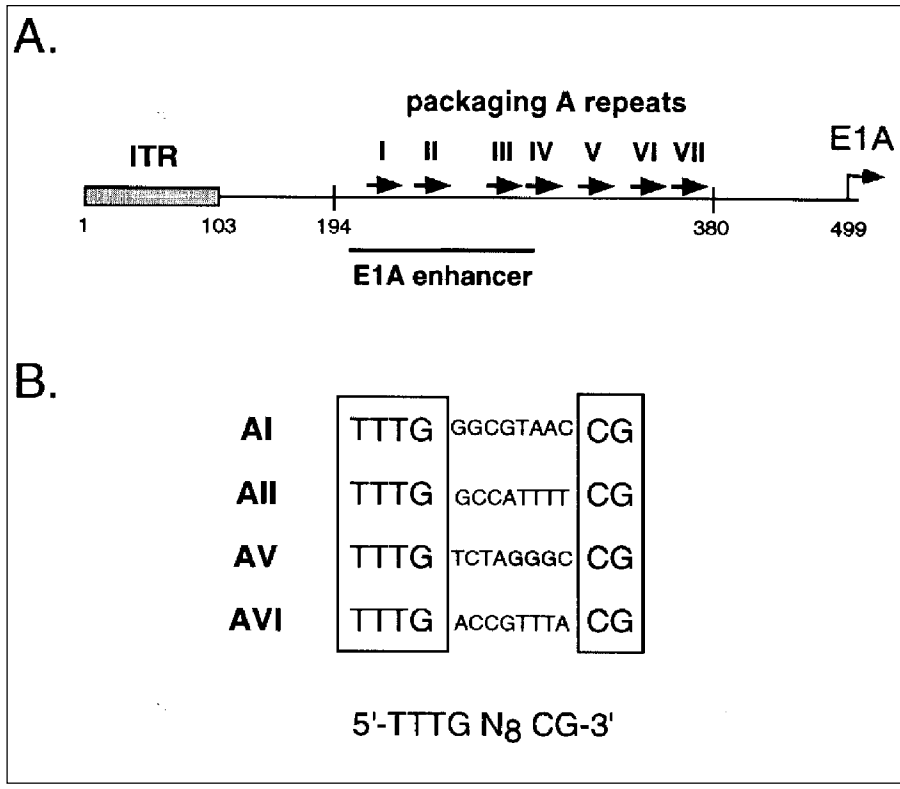

Fig. 9.1. The adenovirus type 5 packaging domain. (A) Schematic representation of the left end of the adenovirus type 5 genome. Nucleotide positions, relative to the left terminus, are indicated by numbers. The inverted terminal repeat is represented by a grey box. Viral packaging repeats are termed A repeats I to VII (arrows) and are located between nt 194 and 380. The E1A transcriptional start site is indicated by an arrow at nt 499. Viral enhancer elements I and II are designated as E1A enhancer. (B) The packaging repeat consensus motif. Shown is an alignment of A repeats I, II, V and VI. Nucleotides comprising the bipartite consensus motif for A repeats I, II, V and VI are boxed and enlarged. The consensus motif is shown at the bottom.

virus viability, as previously described with Ad16 variants. The fact that the Ad5 packaging domain indeed represents an independent, functional unit was demonstrated by the construction of a series of mutant viruses that contained the packaging domain deleted and reinserted at different locations.[7] The Ad5 packaging domain can be inverted or moved more than 100 base pairs toward or away from the left terminus without a reduction in virus growth. However, the Ad5 encapsidation signal must be positioned relatively near either terminus of the viral chromosome for activity. Due to an absolute requirement of sequences in the ITR for initiation of DNA replication, it has not been possible to determine yet if this region also functions in cis to support viral DNA packaging.

By analyses of a series of deletion, insertion and linker scanning mutations within the packaging domain, seven AT-rich repeats were identified as functional packaging elements.[8-10]

They share a loosely defined consensus motif 5′-TTTG(N_8)CG-3′ (Fig. 9.1B). Sequential deletion of these repeats from either side of the packaging domain revealed that they are functionally redundant. In spite of their functional redundancy, the different packaging elements are not functionally equivalent to each other. Two pairs of A repeats, repeats I and II as well as repeats V and VI, represent functionally more significant members of the A repeat family, and are separated from each other within each pair by exactly 21 base pairs or two helical turns of the DNA. Putative packaging factors bound to these repeats would be positioned on the same face of the DNA helix, possibly interacting with each other and/or with additional factors bound outside the packaging domain.

Trans-acting Components May Be Involved in Packaging

That a limiting trans-acting component interacts with the *cis*-acting packaging domain was strongly indicated in cotransfection experiments.[9] Cotransfection of cells with Ad5 DNA and a plasmid carrying an excess of the packaging domain resulted in a substantial decrease in virus yield as compared to cotransfection of wild type genomes with nonspecific plasmid sequences. Presumably, the presence of an excess of packaging elements competed for a *trans*-acting factor preventing the formation of a functional packaging complex on the Ad5 genome. This notion was supported by the fact that total levels of viral DNA as well as late mRNAs were not affected, which indicates that, consistent with a packaging defect, the observed defect must have occurred very late in infection. Analyses of mutant viruses that carry alterations in the spacing between the packaging domain and the left terminus also support the notion that *trans*-acting components interact with the packaging elements, since the precise spacing between the packaging domain and the genomic end is important for efficient packaging, in certain virus contexts.[8,9] Finally, in coinfection experiments, it was observed that viruses carrying a greater number of A repeats packaged viral DNA more efficiently that viruses carrying fewer A repeats, even though the levels of total nuclear virus DNA available for packaging was equivalent.[10] These results suggest a competition within infected cells between the different viral genomes for a limiting *trans*-acting packaging component(s).

The search for packaging proteins has been difficult. Considering adenovirus-encoded proteins, the IVa2 protein, which appears to be present in light intermediate particles but not in heavy intermediates or mature particles, represents a possible candidate for a scaffolding or packaging substrate recognition product.[11] Other viral proteins that may have a direct involvement in the packaging process are suggested through the analysis of viral mutants and include the single-stranded DNA binding protein (DBP), the covalently-linked terminal protein (TP), and the L1 52/55 kDa proteins. A temperature-sensitive DBP mutant (ts19) accumulates light intermediate particles at the restrictive temperature.[12] Similarly, an L1 52/55 kDa protein mutant (ts369) accumulates incomplete particles at the restrictive temperature that carry only small segments of the left end of the viral genome.[13] What role these proteins play in the packaging process is unknown. Lastly, a possible role of TP in viral packaging was suggested by a codon insertion mutant of TP which maintains full replicative properties in vitro yet is nonviable in vivo.[14,15] This phenotype is consistent with a defect in DNA packaging, as are the positive or negative effects on packaging efficiency described above of different spacing mutations between the packaging domain and the viral genomic terminus.[8,9]

Virus Release from Infected Cells

The final event in the virus life cycle is the release of newly formed virus from the infected cell. Wold and colleagues have shown that the E3 11.6K protein (termed ADP, adenovirus death protein) induces the lysis of cells at late times after virus infection. This

facilitates virus release and subsequent virus spread. A description of the function of the E3 gene products may be found in chapter 26.

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CHAPTER 10

Development of Adenoviral Vectors for Gene Therapy

Dai Katayose and Prem Seth

In recent years adenoviruses have been extensively used as vectors to deliver foreign genome into mammalian cells (reviewed in refs. 1-5). Adenoviruses have certain features, which make them attractive vectors for gene transfer to target cells. Some of these properties include their ability to infect a broad range of cell types, including dividing as well as nondividing cells, the ease with which adenovirus genome can be manipulated, and the ability to obtain high titers. There are essentially two ways by which adenoviruses can be used to deliver foreign DNA into cells.[1] One approach utilizes introduction of the foreign cDNAs into the adenovirus genome, resulting in recombinant adenoviruses containing the gene of choice. In the second approach, the ability of adenovirus to enter the cytosol by disrupting the endosome membrane is used to enhance the delivery of the foreign DNA into the cells. In this chapter we describe these two methods of adenovirally-mediated DNA delivery.

Recombinant Adenoviral Vectors

In this approach, the cDNA of choice is inserted into the adenoviral genome resulting in the generation of a recombinant adenoviral vector. Most of the work has been conducted using human adenoviruses type 2 and 5 belonging to group C. This is mainly because of our greater understanding of their genome, and also because this group of adenoviruses has never been shown to induce tumors in any animal model.[6,7] Following sections describe the principles and methods currently being applied to construct recombinant adenoviruses. A discussion of the key features of recombinant adenoviruses and how the various problems associated with their usage are currently being solved will be also presented.

Replication-Deficient E1⁻ Adenoviral Vectors

For most gene transfer purposes, it is desirable to use replication-deficient recombinant adenoviral vectors. The principle behind construction of such vectors is to delete the DNA sequences essential for viral replication and replace them with the foreign cDNA. To construct replication-deficient adenoviral vectors, the most commonly used deletions are E1A and E1B sequences which can be easily substituted for the foreign cDNA. Since upto 105% of the viral genome size can be packaged into the intact virion, the size of the foreign insert can be about 1.8 kb longer than the viral sequence deleted to generate replication-deficient adenoviral vector.[8] However, such recombinant viral genomes will not replicate in cells unless E1 proteins are supplied in trans. Therefore, to grow E1-deleted viruses, one uses cell line such as 293, which is a human embryonic kidney cell line transformed with the left-end of

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adenoviral genomic DNA (about 4 kb). 293 cells provide adenoviral E1 sequences, left ITR, and cis-acting packaging sequences, and protein IX sequences[9] and have been extensively used to generate E1-deleted recombinant adenoviral vectors.

There are several approaches which can be used to isolate $E1^-$ recombinant adenoviral vectors. In one approach an adenoviral genomic DNA is cut at convenient restriction sites, such as Cla1 and Xba1 sites, present at the left end of the genome of dl309 mutant virus.[10] The right end of the genomic piece of DNA (the larger fragment of about 33 kb) is isolated and used as the source of viral backbone. cDNA of choice is cloned into a shuttle vector which provides the E1A enhancer sequences, packaging sequences, protein IX sequences, a heterologous promoter, foreign cDNA, poly(A) signal, and an adenovirus homologous sequence. The large genomic piece of viral DNA and the shuttle vector containing the cDNA can be either ligated in vitro and transfected in 293 cells or both the DNAs can be costransfected in 293 cells. Homologous recombination between the two DNAs in 293 cells generates a recombinant adenoviral DNA in which the E1 sequence has been replaced by the cDNA.[2,3] This recombinant DNA will package to form the recombinant adenoviral particles in 293 cells. Viral plaques can be isolated and screened for the absence of E1 sequences and for the presence of the foreign cDNA by PCR (polymerase chain reaction). The recombinant adenoviral particles devoid of E1 sequences and containing the foreign cDNA can be grown to high titers in 293 cells.

Another approach utilizes a plasmid DNA in which a circular form of adenoviral DNA, including the viral ITRs, has been cloned into the bacterial plasmid, and can be used as the source of viral backbone. The bacterial plasmid also provides the bacterial origin of replication and the antibiotic resistance genes and hence can be grown in bacterial cultures. One such plasmid is pJM17[11] (Fig. 10.1). Recombinant adenoviral vectors can be constructed by homologous recombination between pJM17 and the shuttle vector containing the cDNA and other sequences as described above. As pJM17 DNA is too large to be packaged into adenovirus particles, this minimizes the formation of any background viral plaques.[11]

One can also rescue $E1^-$ deleted adenoviruses by introducing selectable markers such as herpes simplex virus thymidine kinase cloned in the E1 region.[2] The recombinant viral particles can be rescued in the presence of ganciclovir. Other marker genes, which have been used to assist the screening of the recombinants, are the genes for green fluorescent protein, or β-galactosidase proteins in the virus backbone.[2] Another approach to rescue recombinant adenoviral vectors utilizes adenovirus genome cloned into the yeast artificial chromosome (YAC), which can be manipulated in yeast cells by homologous recombination.[12] The recombinant DNA formed can be used in 293 cells to produce recombinant adenoviral vectors.

Using the above described methods, one can introduce about a 4 kb foreign expression cassette, into the adenoviral genome. In order to insert longer cDNAs one can use viral backbones in which the E3 region of the adenoviral genome has also been deleted. One adenoviral mutant commonly used is dl327, which lacks most of the E3 sequences, and also provides a convenient Cla1 site at the left end of the genome to delete the E1 region. Thus, in this approach dl327 is cut with Cla1, the large genomic DNA piece is isolated and subjected to recombination with the shuttle vector containing the cDNA.[13] Similarly, bacterial plasmid such as pBGH10 and pBGH11 have been cloned in which E3 region is deleted.[14] Since E3 proteins are not required for viral replication, one can still use 293 cells to rescue the recombinant adenoviral particles deleted of E1 and E3 sequences. Using E3-deleted viral backbones, one can clone up to 7.5 kb of foreign cDNA into the adenovirus genome.

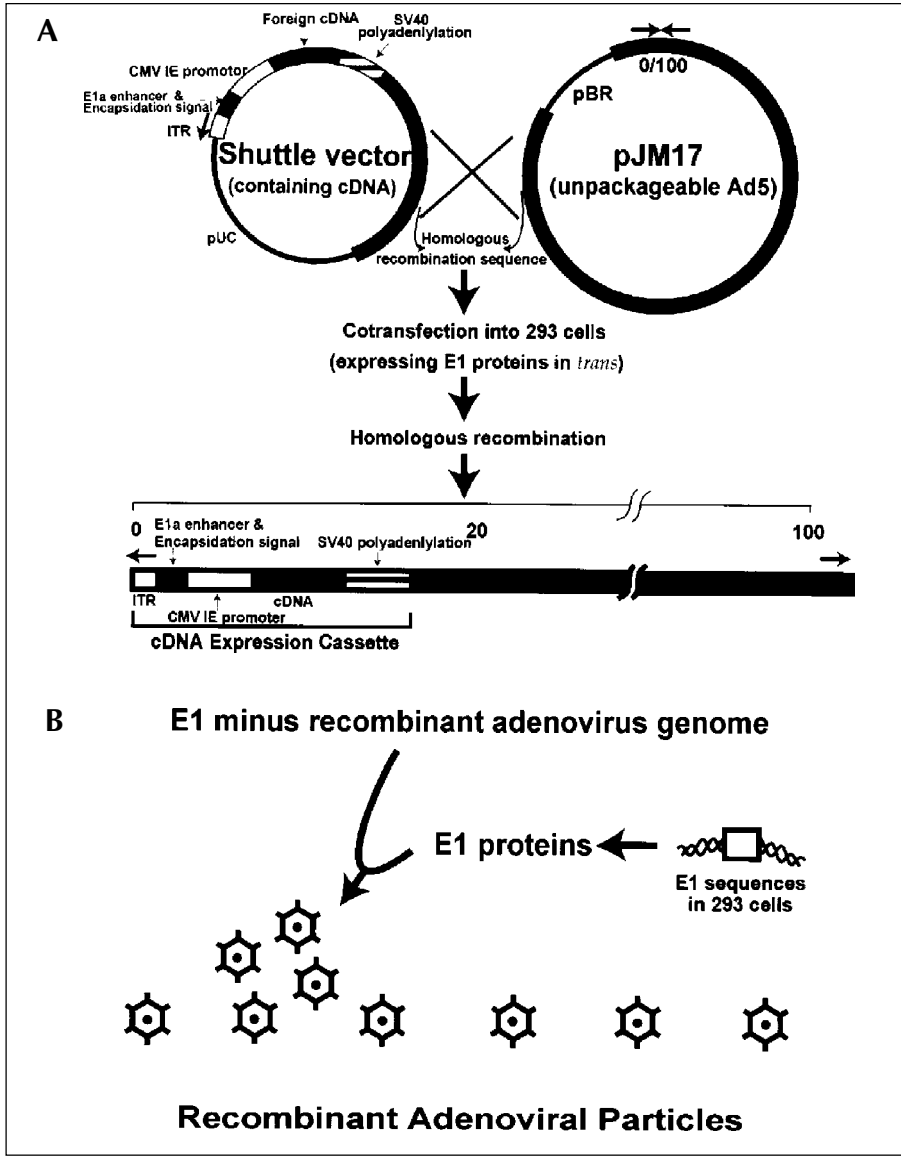

Fig. 10.1. Construction of recombinant adenoviral vectors. (A) cDNA of interest is cloned into a shuttle vector which provides a cDNA expression cassette (adenovirus ITR, E1 enhancer, adenovirus encapsidation signal, CMV promoter, and SV40 polyadenylation signal). Homologous recombination sequences are also cloned in this vector. (B) Adenovirus genome (e.g., pJM17 shown in the figure) and the shuttle vector containing the cDNA are cotransfected in 293 cells. Intracellular homologous recombination between the two DNAs results in an E1-recombinant genome; the numbers 0, 20, 100 represent the approximate map units. This recombinant genome is replication defective. However, in the presence of E1 proteins (provided in trans by 293 cells), the recombinant genome will replicate and form adenoviral particles. Reproduced from ref. 1 with permission.

Key Features of E1⁻ Adenoviral Vectors and Recent Improvements

It has been shown that most cell types are easily infectable by recombinant adenoviruses. Moreover, recombinant adenoviruses have also been shown to mediate high level transgene expression in both dividing and non-dividing cells.[1] Thus, first generation recombinant vectors (E1-deleted) overexpressing a variety of transgenes have been extremely valuable to address many basic research questions such as understanding the mechanisms of cell cycle progression and apoptosis in a variety of cell types in vitro.[1-4,15-17] Moreover, the ease with which adenoviruses can be administered directly into various organs and tumor sites, including intraperitoneum and intravenous routes has helped us to evaluate their efficacy and safety in numerous animal models for in vivo pre-clinical research (see chapters 11-17). They have also been tested for possible clinical applications for cystic fibrosis and cancer gene therapy (see chapters 29-33).

Adenoviral vectors will target essentially any cell type that provides adenovirus receptors, so these viruses lack cell and tissue specificity. Therefore, many attempts are being made to introduce tissue specificity by modifying the viral capsid proteins in such a way that adenoviruses can be made to enter cells through other receptors. Furthermore, tissue specific promoters, enhancers and other transcriptional elements are being employed in an attempt to generate cell and tissue specific adenoviral vectors (also see chapter 20).

One of the limitations of the use of recombinant adenoviruses is that adenoviral-mediated gene expression is transient. Transgene expression generally lasts for only a few days in immunocompetent animals to a few months in immunocompromised animals. Many reasons can be attributed for this short term gene expression. First, adenoviral vectors rarely integrate into host genome; thus the transgene expression is not expected to be permanent. The lack of long term expression has also been attributed to the use of foreign viral promoters (such as RSV and CMV) generally used for driving the transgene. Use of alternative cellular promoters to prolong transgene expression has been employed in some laboratories.[2] Another reason for short term expression is due to the immunogenicity associated with adenoviral vectors. The input adenoviral particles themselves can elicit an immune response. This immunogenicity is further enhanced in the first generation of vectors, by low level viral gene expression (see below). Both cell-mediated and humoral immunity have been observed against the viral and the foreign transgene (see chapters 25-28 for details).

Although E1-deleted vectors are replication-deficient, at higher doses (greater than 100 pfu/cell) they have been shown to produce low levels of viral transcripts and proteins presumably due to low levels of viral replication. Moreover, some mammalian cells types can also provide certain proteins (such as IL6) which can substitute for the transactivating function of E1 proteins resulting in viral replication.[3] It has also been shown that continuous passaging of E1-deleted vectors into 293 cells can result in the homologous recombination between the recombinant adenoviral genome and the adenoviral sequences integrated in 293 cells, resulting in the formation of replication competent adenoviruses (RCA).[18] (also see chapter 24). The presence of RCA in the viral stocks can also function as a helper virus for the replication of the recombinant viruses, which could have adverse effects on the individual treated with such vectors. The wild type adenoviral contamination can also potentially induce transformation in the host cells, though the two commonly used adenoviruses Ad2 and Ad5 have never been shown to induce tumors even in experimental animals (see chapter 23). The appearance of RCA during viral propagation can be partly solved by using packaging cell lines such as a variant of A549 (human lung carcinoma cells), which contains left end of the virus genome (505-4034 nt).[19] Since these cells lack the extreme left sequences of adenovirus genome (left ITR, and the packaging sequences), and also do not contain any overlap sequences present in the E1⁻ adenoviral vectors, homologous recombination between the recombinant adenoviral genome and cellular DNA is not possible, thus preventing the formation of RCA.[1-3]

Replication-Defective E2a and E4 Deleted Adenoviral Vectors

In addition to generating new packaging cell lines, the viral backbones have also been modified to provide minimum viral transcriptional units. It is known that an adenovirus mutant H5ts125 which contains a temperature sensitive mutation in an E2a-encoding 72 kDa DNA binding protein, replicates less efficiently at non-permissive temperature (37˚C) than at permissive temperature (32˚C).[20] In fact, recombinant adenoviral vectors expressing β-galactosidase constructed using such a mutant in E1-deleted backbone were shown to express adenoviral proteins at a much lower level than other E1-deleted vectors.[2,3,21] This vector expresses the transgene for much longer periods (70 days) in an immunocompetent mouse model, and much diminished infiltration of $CD8^+$ cells were observed in the livers of the infected animals.[21] Recombinant adenoviral vectors in which E1 and E2 sequences have been deleted have also been constructed.[2,3,22] Since E2 encoded proteins are critical for viral DNA replication and regulation of the viral gene expression, absence of E2 in addition to E1 further diminishes the replication potential of such vectors. However, to rescue such a vector, packaging cell lines, which provide both E1 and E2 proteins in trans, are needed.[2,3,23]

Another transcriptional unit of the adenoviral genome, which can be additionally deleted in recombinant adenoviruses, is the E4 region (ORF3 and ORF 6). However, the generation and propagation of $E1^-/E4^-$ adenoviral vectors requires 293 cells, which in addition to E1 proteins, also provide E4 proteins.[24] Due to the fact that E4 protein products are required for the export of viral mRNA from the nucleus, vectors deleted for E1 and E4 region are not able to synthesize late gene products. These vectors, therefore, are incompetent for viral replication in mammalian cells.[3,25,26] Moreover, the deletion of E4 sequences also minimizes the chances of RCA contamination, since recombinant adenovirus containing E1 sequences, but devoid of the E4 region, is still replication incompetent in the target cells (which do not express E4 proteins) used for gene transfer. In fact, an interesting strategy to prevent the formation of RCA is to use recombinant adenoviruses in which E4 sequences have been cloned between the left ITR and the *cis*-packaging sequences.[2,3] Thus, homologous recombination between this adenoviral genome and the cellular sequences results in a recombinant adenovirus, which is $E1^+/E4^-$, and hence not capable of viral replication. Recombinant adenoviruses ($E1^-E4^-$) containing the foreign cDNAs have been shown to express the transgene at relatively high levels,[27] and are under extensive pre-clinical testing in animal models.

Adenoviral Vectors with Longer Adenovirus Genome Deletions

In recent years, adenoviral vectors that are essentially devoid of any viral genome have been constructed.[28-31] This generally requires the use of helper adenovirus. In one approach, adenoviral genome sequences for L1, L2, VAI, VAII, and preterminal proteins, are replaced by the foreign cDNA fused with a selectable marker gene.[28] This DNA is coinfected with a helper wild type adenovirus (or an E1-deleted adenovirus) into 293 cells in the presence of an appropriate selection pressure. Under these conditions, the recombinant adenoviral particles deleted of viral sequences but containing the foreign cDNA are generated and can be rescued. The size differences between recombinants and the helper virus allow recombinant adenoviral particles to be partially separated on CsCl2 gradients. However, such preparations of the recombinant adenoviruses are invariably contaminated with the helper wild type virus. Using such helper virus approaches, recombinant adenoviral vector containing dystrophin, β-galactosidase and CFTR, but devoid of all viral coding sequences, have been successfully produced.[28-31] With several rounds of amplification, a titer of approximately 10^7-10^8 pfu/ml of the recombinant adenoviruses has been obtained.

To avoid the problem of helper virus contamination in the final preparations, another approach to generating recombinant adenoviral vectors containing minimum adenovirus genome utilizes a Cre-*lox* recombination system described by several laboratories.[2,3,32,33]

The basic principle underlying these methods is to first generate a variant of the 293 packaging cell line expressing the P1 bacterial phage recombinase Cre, a *lox*P-specific enzyme (293Cre).[34] In parallel, *lox* sites are introduced adjacent to the packaging sequences in an $E1^-$ adenovirus genome. This viral genome serves as a helper virus. The cDNA of interest is cloned into a plasmid, which provides the *cis*-acting elements, adenoviral ITRs, and the packaging sequences. This vector also contains non-adenoviral stuffer sequences such as lambda DNA.[32] The two pieces of DNA are costransfected into the 293Cre cell line. The Cre activity will direct the recombination with the helper virus genome at the lox site. This results in the deletion of the *cis*-acting packaging sequences present in the helper virus, thus disabling the helper virus from packaging into the viral particles. However, the viral proteins are still expressed in trans through this restricted helper genome, thereby allowing the assembly of the recombinant adenovirus containing the cDNA, and the non-adenoviral stuffer sequences. Such recombinant adenoviruses are free of any viral related transcripts and can be purified on a $CsCl_2$ gradient to high titers.[32] The viral titers can be further increased by serial passages in 293Cre superinfected with the helper virus. The final viral preparations have titers of 10^{10} pfu/ml, and contain less than 0.01% of helper virus contamination. The recombinant adenoviral vectors produced by such methods should not replicate in target cells, and hence are not expected to elicit any immune responses, at least to newly synthesized viral proteins.[2,3,35] However, the extensive in vivo use of these vectors has not yet been reported.

Replication Competent Adenoviral Vectors

While for most gene transfer purposes it is desirable to utilize replication-incompetent adenoviral vectors, under certain circumstances it might be advantageous to use replication competent viruses. As discussed earlier, it is possible to package an adenoviral genome 5% larger than the wild type adenoviral genome. Thus, without deleting any viral sequences, one can introduce about 1.8 kb of foreign cDNA into the adenoviral genome. Since the E3 region of adenoviruses is not needed for viral DNA replication, the E3 region (up to 3.5 kb) can be removed from the virus backbone and replaced by the foreign cDNA, thus allowing the introduction of larger cDNA sequences into the recombinant adenovirus. Of course, to rescue such recombinants, one needs a shuttle vector which contains the homologous viral sequences from the right end of the adenoviral genome. As described earlier, the sources of genomic DNA can be either adenovirus genome restricted with an enzyme located at the 5′-end of the genome or an adenoviral genome cloned into the appropriate plasmids.[2,3] Using this cloning strategy, many replication-competent recombinant adenoviral vectors have been generated.[2,7] This approach is particularly useful for generating recombinant vectors in which high level protein expression is needed. One such application includes the production of live vaccines, as discussed in chapter 17.

Another approach utilizing adenoviral vectors which should replicate under defined cellular conditions, for example, only in the cells expressing certain oncogenes or defective tumor suppressor genes, is a subject of intense investigation and will be discussed in detail in chapter 21.

Adenovirally-Mediated Enhancement of DNA Delivery and the Concepts of Molecular Conjugates

Adenoviruses can also be used to enhance DNA delivery into the cells using methods in which the DNA molecule is not introduced into the recombinant adenovirus genome, but instead remains outside the adenoviral genome, either unlinked or linked by physicochemical means. This process of adenovirally-mediated DNA delivery exploits the ability of adenoviruses to enter cells by receptor-mediated endocytosis. As described in chapter 4, a

key step in this pathway is the escape of the adenovirus from the membrane-limited endosomes into the cytoplasm by disrupting the endosome membrane. This has led to the idea that if adenovirus were incubated with another molecule, the latter would be internalized into the common endosomes; hence, the disruption of the endosome membrane by the adenovirus would release the content of the endosomes (virus + cointernalized molecules) into the cytosol. In fact, when human adenovirus type 2 is incubated with proteins such as epidermal growth factor (EGF) linked with gold particles (EGF-gold) and the entry of EGF-gold is followed by electron microscopic examination, many more gold particles are detected in the cytosol.[1] This phenomenon has been confirmed by biochemical assays using EGF linked with *Pseudomonas exotoxin* (PE-EGF). Entry of PE-EGF into the cell can be followed by the inhibition of protein synthesis. When cells were incubated with PE-EGF alone, there was minimum cytotoxicity to the cells. However, if PE-EGF was internalized into the cells in the presence of adenovirus, the latter enhanced the release of PE-EGF into the cells, resulting in at least 3-4 log increase in the toxicity of PE-EGF.[36,37] By the 1980s it became clear that adenoviruses belonging to group C (type 2, type 5) can be used to increase the delivery of a variety of molecules, including carbohydrates and proteins, into target cells.[1]

In recent years, the concept of adenovirally-mediated enhancement of protein delivery has been extended to nucleic acids. (Fig. 10.2). It has been shown that the incubation of cells with adenoviruses and plasmid DNAs can increase the transfection efficiency of the DNA by at least 3-4 logs.[38] Some of the plasmids tested are those coding for marker genes such as luciferase, β-galacatosidase, and therapeutic genes such as *Pseudomonas exotoxin*.[38,39] This adenovirally-mediated DNA delivery is dependent upon the concentration of the adenovirus used, and can be further augmented (10-100 fold) by monocationic and polycationic liposomes such as lipofectin and lipofectamine.[39] In other studies, adenoviruses have been used to deliver plasmid DNA to the cells after directly linking the plasmid DNA to the adenovirus. In our laboratory we have shown that the foreign proteins or DNA can be directly conjugated with the hexon coat protein of adenovirus, and these conjugates can deliver large amounts of the foreign molecules into the cells.[40] However, such molecular conjugates still enter into the cells through adenovirus receptors.[40] To introduce cell and tissue specificity, many versions of these conjugates have been generated. In one strategy, adenovirus complexed with polylysine can be linked with plasmid DNA conjugated to a ligand such as transferrin. Adenovirus-DNA-transferrin conjugates have been shown to be internalized through the transferrin receptor.[41,42] In other modifications, the knob of adenoviral fiber protein can be genetically engineered in such a way that other ligands can be crosslinked to fiber. Such molecular conjugates enter the cells through the crosslinked ligand. Alternatively, antibodies to fiber can be conjugated with adenovirus, and such complexes can be further crosslinked with ligands such as fibroblast growth factor or transferrin.[43] Other investigators have modified penton base of adenovirus or used penton base to crosslink bispecific antibodies to increase the viral tropism for gene delivery.[44] Adenovirus can also be conjugated with other viruses such as AAV and retroviruses, to modify their tissue tropism. Some of these approaches will be further described in chapters 18 and 19.

These approaches of adenovirus-mediated enhancement of DNA delivery have some features which are potentially very useful. It has been shown that the infectious adenoviral particles are not needed for this process, as inactivation of the adenovirus genome by UV light or proselin does not diminish the ability of adenovirus to increase the release of DNA into the cells.[45,46] Moreover, empty adenovirus capsids and dodecons devoid of adenoviral genomic DNA also have limited capacity to lyse endosome membrane, and hence may be potentially useful for gene delivery.[38,40,47] Using these approaches, one can deliver DNA molecules, which normally can not be packaged into recombinant adenoviral particles. These

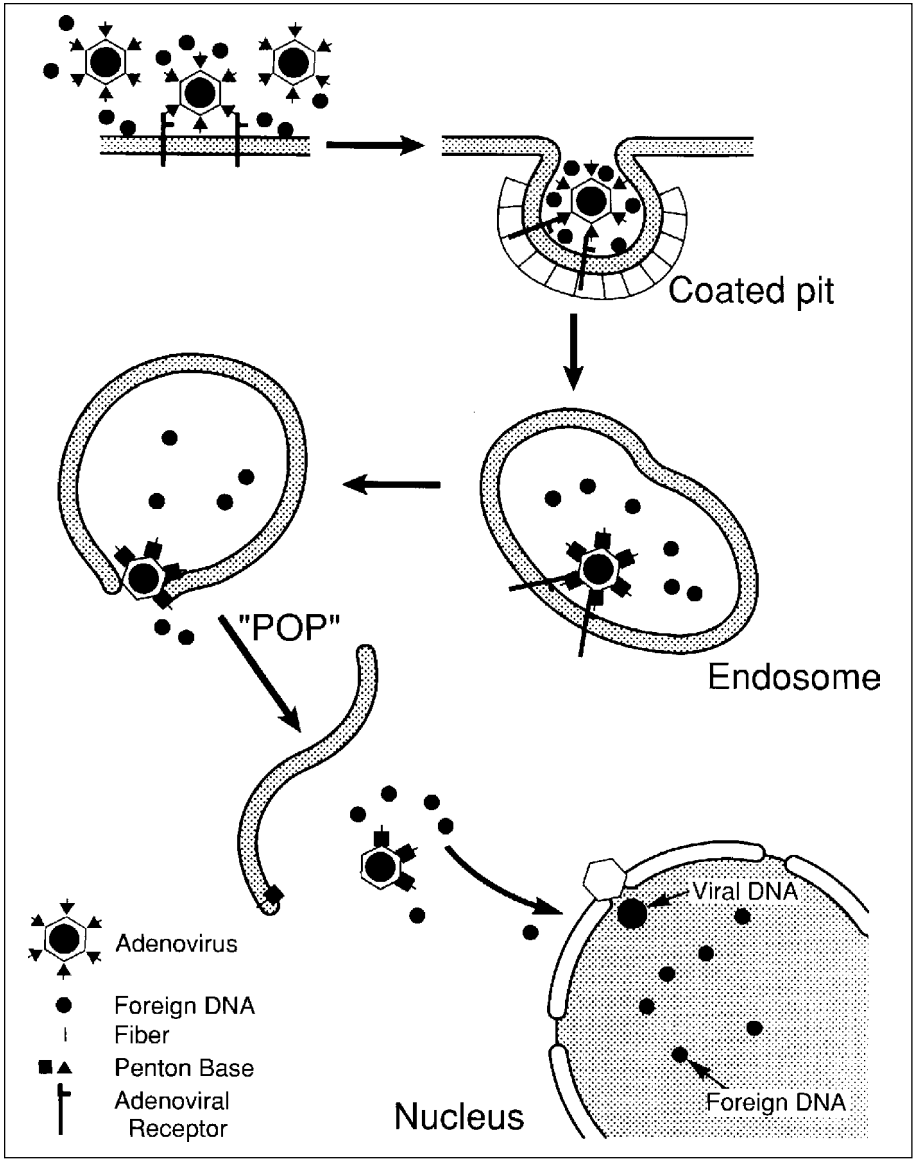

Fig. 10.2. Adenoviral-mediated enhancement of DNA delivery to cells. In this approach, adenovirus is mixed with plasmid DNA (shown as small black circles), and cointernalized into the cells. As discussed in chapter 4, during the entry of adenovirus into the cells, adenovirus escape the membrane-limited endosomes by disrupting the endosome membrane. As shown in the diagram, during this lysis event, other cointernalized molecules such as DNA can be also released into the cells. See text for details.

types of DNA include large pieces of genomic DNA (greater than 35 kb) and toxin genes (such as Diphtheria toxin, *Pseudomonas exotoxin* gene). While these approaches of adenovirally-mediated DNA delivery have found many applications in in vitro research, very little data is yet available for in vivo applications.

Conclusion

In the last few years much progress has been made in developing adenoviral vectors for gene therapy. While most of the first generation vectors constructed were $E1^-$ and/or E3-deleted, it is now possible to essentially delete all the adenoviral genome and replace it with the cDNA or even gene of interest. In recent years, significant progress has also been made in developing targetable adenoviral vectors. In preclinical settings, many applications for recombinant adenoviruses expressing a variety of genes have been reported where a high level but transient gene expression is sufficient for in vitro research and in vivo gene therapy purposes. However, as will be described in many chapters throughout this volume, applications of recombinant adenoviruses for gene therapy are likely to be hampered due to the immunological responses directed against both the adenovirus back bone, as well as the transgene protein product. Therefore, research efforts need to be continued toward circumventing these immunologic problems.

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the adenoviral vectors in vivo or by first treating the target cells ex vivo followed by transplantation of the cells into the patients' body. Anti-cancer approaches that utilize adenoviral vectors and that have been extensively subjected to pre-clinical evaluation for cancer gene therapy are outlined in Table 11.1 and discussed further in this chapter.

Direct: Toxic Transgene Products

Many recombinant adenoviral vectors expressing genes that encode proteins that will kill cancer cells or inhibit their growth have been constructed and extensively studied. Some of the commonly used approaches are discussed here.

Transfer of Tumor Suppressor and Cell Cycle Regulatory Genes

Loss of tumor suppressor function is commonly associated with many human malignancies. Several tumor suppressor genes have been isolated in recent years. One tumor suppressor gene that is frequently dysfunctional in many cancers is *p53*. It has been appreciated for some time now that overexpression of p53 through viral and non-viral vectors can inhibit cell growth either by inducing a cell cycle arrest and/or by inducing programmed cell death (apoptosis). To test the effect of wild type p53 expression on the cells expressing mutant *p53*, recombinant adenoviruses expressing p53 have been constructed in several laboratories.[4] Infection of cancer cells with adenoviral vectors carrying wild type p53 (Ad-wtp53) has been demonstrated to produce high levels of p53 protein in a number of epithelial cancer cell lines. These include breast, prostate, ovary and neuroblastoma cell lines, to mention just a few.[4] While all cell lines expressing adenovirally mediated p53 show growth inhibition, these vectors are much more cytotoxic to cancer cells expressing a mutant form of endogenous *p53*[5] (Fig. 11.1). Normal human cells are particularly resistant to the cytotoxic effects of Ad-wtp53.[5] Further study of molecular mechanisms of p53-mediated cytotoxicity have shown that all cells expressing Ad-wtp53-mediated p53 protein undergo a cell cycle arrest at G1/S. However, the more sensitive cells, in addition to cell cycle arrest, also undergo apoptosis.[5] The molecular processes involved in p53-mediated apoptosis are not clearly understood at present. The expression of several genes is upregulated during p53-mediated apoptosis.[6] These include *Bax* , *Fas*(CD95/APO-1) and *IGFBP3* (insulin-like growth factor binding protein 3).[6] Similarly, p53 can also downregulate the activity of many genes, some of which are survival factors for the cells. Examples of these genes include the FGF-1 (*fibroblast growth factor receptor*), *thyroid receptor β-1*[8] and *insulin-like growth factor-1*.[9,10] Furthermore, p53 protein can also activate pathways leading to apoptosis by interacting with other cellular proteins.[6] Thus, p53-mediated apoptosis is an active area of research in which adenoviral vectors expressing wild type p53 protein can potentially be very useful.

In recent years, many laboratories have reported the effects of recombinant adenoviral vectors expressing wild type p53 on inhibiting tumor growth in animal models.[4] Human cancer cells (lung cancer cells, head and neck cancer cells, prostate cancer cells and breast cancer cells, among others) when pre-infected with these vectors and subsequently injected into the nude mice show an inhibition of tumor formation. In fact, direct injection of these vectors into pre-established tumors in nude mice leads to a significant inhibition of tumor growth. Cells from these Ad-wtp53-injected tumors in vivo have been shown to exhibit high levels of *p53* expression and to undergo apoptosis. Notably, however, direct injection of adenoviral vectors into the tumor mass can infect only a limited number of cells (generally less than 5% of the total cell population). Hence, it has been postulated that adenovirally-mediated p53 expression and cell death may lead to a "bystander effect," i.e., inhibition of growth of cells which are not infected with the recombinant virus and hence do not express exogenous wild type p53 protein. Several mechanisms of this putative bystander effect have been proposed. Downregulation of the angiogenesis factors such as

CHAPTER 11

Adenoviral Vectors for Cancer Gene Therapy

Prem Seth, Yu Katayose, and Amol N.S. Rakkar

Cancer can be considered a "genetic" disorder, and thus a legitimate target for gene therapy, in as much as it arises as a result of a stepwise accumulation of genetic defects in a clone of cells. In the last decade or so, several epigenetic and genetic events have been characterized that contribute to tumor initiation, progression, and metastasis. It is now well understood that gain of a dominant oncogene such as *ras* mutation, or loss of a tumor suppressor gene such as *p53* can induce an uninhibited cell growth leading to tumorigenesis.[1] In addition, the failure of the immune system to recognize and eliminate the tumor cells allows the cancer cells to proliferate and accumulate further genetic defects.[2] Other contributors to tumor progression include tumor generated factors that enhance angiogenesis, such as vascular endothelial derived growth factor (VEGF), and alterations in expression of adhesion molecules in the cancer cells that allow the cancerous cells to dislodge from their primary microenvironment and metastasize to distant sites.[3]

This multitude of genetic defects makes the cancer cells a "moving target" and poses a daunting challenge to gene therapy. Traditionally, the introduction of foreign genetic material with a therapeutic intent, i.e., gene therapy, has been based on the replacement principle, and geared towards long term expression of proteins that are defective in monogenetic disorders. Gene therapy of cancer, however, necessitates more complicated and innovative approaches because of the aforementioned complex nature of the underlying process. For instance, in sharp contrast to the long term expression required for management of genetic deficiency disorders, a transient high level expression of genes aimed at killing the cancer cell or stimulating the immune system may be more desirable for gene therapy of cancer.

While an increasing number of systems designed for transferring genetic material to cancer cells are currently under development, this review will focus on adenoviral vectors. In fact, adenoviral vectors may be particularly suited to gene therapy of cancer, as they can be easily grown to high titers, infect a wide variety of cancer cells and result in a high level of expression in the infected cells. A number of strategies using adenoviral vectors for treating cancer have been proposed and are currently in various stages of pre-clinical and clinical evaluation (reviewed in ref. 4). For cancer gene therapy purposes, one can design adenoviral vectors to express therapeutic genes to correct any number of steps leading to tumor progression. The most straightforward approach is to introduce genes which will directly kill the cancer cells or inhibit cell growth. One can also kill the cancer cells by activating the immune cells responsible for the tumor destruction. In addition, one can also target the various factors responsible for angiogenesis and metastasis. Depending upon the strategy used, the target cells can be either cancer cells or other normal cells (such as T-cells, dendritic cells). For cancer therapy, one can conduct gene transfer either by direct administration of

Adenoviruses: Basic Biology to Gene Therapy, edited by Prem Seth.

Table 11.1. Approaches for adenoviral-mediated cancer gene therapy

Direct: Toxic transgene products
•transfer of tumor suppressor and cell cycle regulatory genes •suicide genes-Enzyme/prodrug approach •expression of antisense oncogenes and ribozymes to dominant oncogenes
Indirect: Immunomodulatory approaches
•expression of cytokines •expression of costimulatory molecules •expression of tumor specific antigens
Other novel strategies
•combination of direct and indirect approaches •expression of molecules that affect angiogenesis, cell adhesion and metastasis •chemosensitization and radiosensization approaches •tissue-specific promoter driven transgene expression •expression of cell surface receptors to target cancer cells •exploiting adenoviral-mediated DNA transfection for delivery of toxin genes and generation of tumor vaccines •replication competent adenoviruses

vascular-derived endothelial growth factors or upregulation of angiogenesis-inhibiting factors like thrombospondin 1 could be important components of this bystander effect.[11] It is also conceivable, though not yet proven, that the adenovirus backbone and the p53 protein can activate an immune response directed against the cancer cells.

Adenovirally-delivered human wild type *p53* has also been shown to enhance sensitivity of cancer cells to chemotherapeutic agents as well as external radiation. This observation was initially made in lung cancer cells treated, in vitro and in vivo, with a combination of cis-platin and the adenoviral vector.[12] We have observed similar results even in multi-drug resistant breast cancer cells in vitro.[13] Others have observed increased sensitivity to radiation in cells infected with adenovirus expressing p53. In cell lines derived from head and neck and ovarian cancer, external beam radiation induced increased toxicity in cells infected with an adenoviral vector expressing wild type *p53* in vitro.[14,15] Notably, this enhanced radioresponse was observed in vivo in xenograft models of both these cell lines.

Based on these in vivo results, clinical protocols using adenovirus expressing p53, alone or in combination with chemotherapeutic agents, are currently accruing patients and are discussed in chapters 30-32.

The retinoblastoma gene (*Rb*) is a prototypic tumor suppressor gene. Studies have demonstrated that tumorigenicity of the treated non-small cell lung carcinoma and bladder carcinoma cells in nude mice is suppressed following treatment by a recombinant adenovirus vector expressing the N-terminal truncated retinoblastoma (Rb) protein (pRb94).[16] Notably, gene therapy of established human Rb^- and Rb^+ bladder xenograft cancers in nude mice by this vector resulted in regression of the treated tumors. In another recent study, decreased tumor cell proliferation, inhibition of tumor growth, and

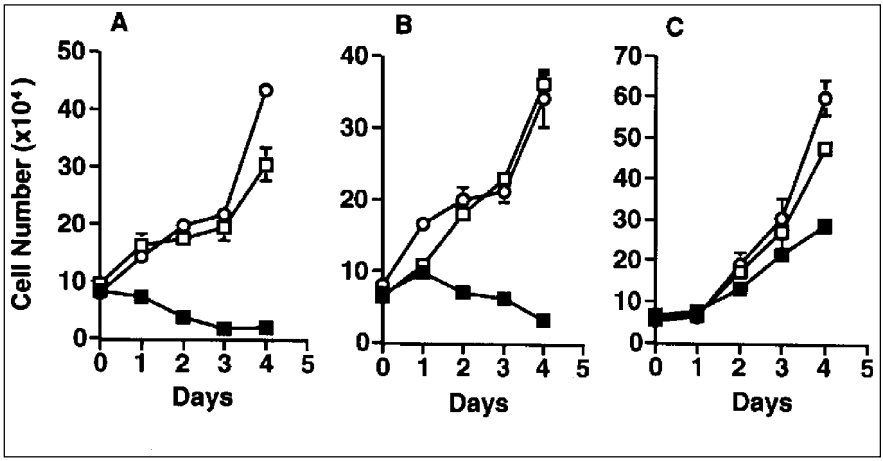

Fig. 11.1. Effect of Ad-wtp53 and AdControl on cell growth. 5 x 10^4 cells were plated in triplicate on 6 well plates, exposed to Ad-wt*p53* (10 pfu/cell) or AdControl (10 pfu/cell) and cell number counted on each day. Shown are cell number of: (A) H-358 cells: uninfected (○), exposed to Ad-wt*p53* (■), exposed to AdControl(□); (B) MDA-MB-231 cells: uninfected (○), exposed to Ad-wt*p53*(■), exposed to AdControl(□); (C) MCF-7 cells: uninfected (○), exposed to Ad-wt*p53*(■) exposed to AdControl(□). Values shown are mean ± SE. (Reproduced from ref. 5 with permission.)

prolongation of life span was observed when spontaneous pituitary melanotroph tumors arising in immunocompetent *Rb* +/- mice were treated with a recombinant adenovirus carrying a full length *Rb* cDNA.[17]

Another tumor suppressor gene frequently mutated or absent in some cancers is *p16*^{INK4A}. The protein product of this gene is a cyclin dependent kinase inhibitor. A recombinant adenovirus expressing p16^{INK4A} has been constructed and evaluated for their effects on cell growth.[18] Human cancer cells infected with Ad-p16 produce high levels of p16^{INK4A} protein, and show growth inhibition. These inhibitory effects are much more pronounced in cells devoid of endogenous p16^{INK4A}, but containing wild type retinoblastoma protein.[18] Thus, cancer cells defective in p16^{INK4A} are good targets for gene therapy by Ad-p16. In addition to p16^{INK4A}, adenoviral vectors expressing other cyclin kinase inhibitors, p21$^{WAF1/Cip1}$ and p27^{Kip1}, have also been reported.[19-21] Overexpression of both p21$^{WAF1/Cip1}$and p27^{Kip1}inhibits cell growth and induces G1/S arrest in the infected cells.[19,20] In addition, Ad-p27 infection has been shown to induce apoptosis in a variety of cancer cells[21] (Fig. 11.2). Some studies have appeared in which inhibition of tumor growth has been shown after infection with Ad-p21 or Ad-p16. In vivo studies in syngeneic mice with established s.c. prostate tumors have demonstrated that the rate of growth and final tumor volume were reduced in mice that received intratumoral injections of an adenoviral vector expressing p21$^{WAF1/Cip1}$ and that survival was extended.[22,23] Similarly, in lung cancer as well as esophageal cancer cells, infection with Ad-p16 resulted in significant growth inhibition.[24,25] So far, no Phase 1 clinical trial evaluating adenoviral vectors transducing cyclin dependent kinase inhibitors has been initiated.

Adenoviral expression of *Bcl-xs*, a dominant negative repressor of the anti-apoptosis genes *Bcl-2* and *Bcl-xL*, has been demonstrated to induce apoptosis in cancer cells arising

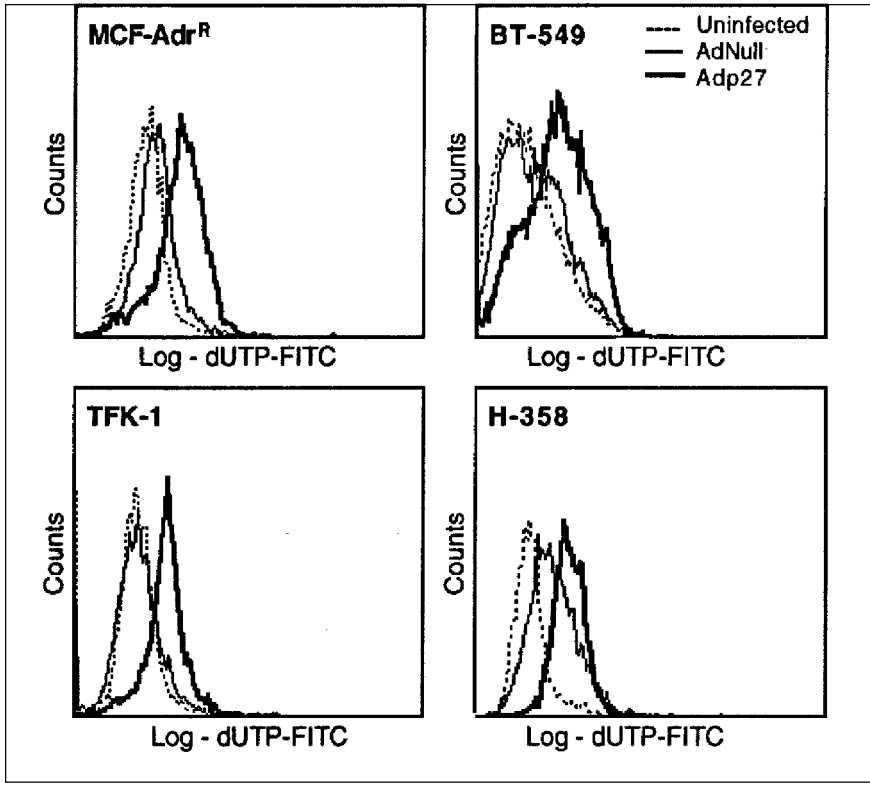

Fig. 11.2. Flowcytometric TUNEL analyses for apoptosis on cancer cell lines. Cells (2 x 10^6) were uninfected (•••••) or infected with 10 pfu/cell of Ad-p27(▬) or AdNull(▬). After 72 h, cells were harvested and analyzed by flowcytometer TUNEL assays. DNA fragments were labeled with biotin-dUTP and detected by binding to avidin-FITC. Reproduced from ref. 21 with permission.

from a number of different organs.[26,27] Furthermore, intratumoral injections of adenoviral vector expressing *Bcl-xs* into MCF-7 tumors in nude mice resulted in a 50% reduction in the tumor size.[27]

The transcription factor E2F-1 plays a crucial role in the progression of eukaryotic cells through the cell cycle. A recombinant adenovirus vector containing the transgene *E2F-1* under control of the cytomegalovirus promoter has been shown to induce high levels of the E2F-1 protein in human breast and ovarian carcinoma cell lines and also to result in the induction of apoptosis in breast and ovarian cancer cell lines.[28] Of note, this induction of apoptosis by E2F-1 seems to be independent of the *p53* status of these cells, as it could be observed in cell lines that have a mutant *p53*. Another putative tumor suppressor that has been expressed via an adenoviral vector is the promyelocytic leukemia gene (PML).[29] Infection with this vector led to significant reduction in growth rate and tumorigenicity of prostate cancer cells and, in a nude mice model of prostate cancer, intratumoral injections of this vector resulted in retardation of tumor growth in 64% of the animals.[29]

Some recent reports have focused on combinations of adenoviral vectors delivering tumor suppressor genes to cancer cells. Whereas adenoviral delivery of *p16*^{INK4A} or *p53* on their own did not lead to apoptosis, simultaneous exposure of cells to adenoviral vectors expressing *p16*^{INK4} and *p53* induced apoptosis and led to inhibition of tumor growth in human hepatocellular carcinoma xenografts in nude mice.[30]

Suicide Genes-Enzyme/Prodrug Approach

In this approach, cells are exposed to recombinant adenoviral vectors expressing suicide genes in the presence of a pro-drug. The product of the suicide gene (generally a non-mammalian enzyme) converts the pro-drug into the active toxic species, which will kill the cells. Several enzymes/pro-drug systems have been studied.

One extensively studied suicide gene is herpes simplex virus thymidine kinase (HSV*tk*) which converts ganciclovir into the phosphorylated forms of ganciclovir. The latter can inhibit DNA synthesis and hence kill the cells that express HSV*tk* . Adenoviruses expressing HSV*tk* (Ad.HSV.*tk*) have been constructed and well characterized.[31-33] Cells of various origins when exposed to HSV*tk* virus produce high levels of the enzyme activity. Moreover, when these infected cells are concomitantly exposed to ganciclovir, a 2-3 log increase in the cytotoxicity of ganciclovir has been reported. Interestingly, a "bystander effect" involving toxicity to cells not expressing the enzyme has been observed in this system. Thus, when different percentages of Ad.HSV.*tk*-infected cells were mixed with mock-infected cells, and exposed to ganciclovir, a cell population with only 5-10% infected cells resulted in cell death in 100% of the cell population. This is presumably due to the transfer of the small molecular weight active moiety of the drug from infected to non-infected cells through gap junctions between cells. The effects of Ad.HSV.*tk* have been extensively studied in animal models of various cancers. Among them are C6 glial tumors, 9L glioblastoma tumors and MATB mammary adenocarcinoma tumors in rats. Several xenografts of human cancers in nude mice, including hepatocellular carcinoma, head and neck cancer, mesothelioma and prostate cancer, have also been shown to be effective targets for Ad.HSV.*tk*.[31-34] In many of these models, it has been shown that direct intratumoral injection of Ad.HSV.*tk* along with simultaneous systemic administration of ganciclovir led to a significant inhibition of the tumor growth and an increase in the animal survival. Based on these results, clinical trials using Ad.HSV.*tk* have been initiated.[35] The details of one such trial are discussed in chapter 33.

Another enzyme-prodrug approach utilizes the bacterial cytosine deaminase (CD) enzyme, which can convert the prodrug 5-fluorocytosine (5-FC) into the toxic species 5-fluorouracil (5-FU). Recombinant adenoviruses expressing *E. coli* cytosine deaminase (AdCD) have been constructed and characterized.[36-38] Infection of breast cancer cells and colorectal carcinoma cells in vitro with AdCD resulted in high levels of CD activity. When cancer cells infected with AdCD were exposed to varying amounts of 5-FC, up to 3-4 log increase in the cytotoxicity of 5-FC was observed. Mixing AdCD-infected cells with uninfected cells, followed by exposure to 5-FC, indicated that less than 10% of the cancer cells had to be infected to kill all cells in the tissue culture dish, again suggesting that bystander effects might be playing a role in inducing cytotoxicity[36](Fig. 11.3). One advantage of this system is that the toxic drug 5-FU can readily diffuse into the medium and enter the neighboring cells, thus bypassing the need for gap junctions required for the phosphorylated ganciclovir as discussed above. Human xenografts of MDA-MB-231 breast cancer cells and HT29 colorectal cancer cells, when infected with AdCD delivered intratumorally and treated with intraperitoneal 5-FC, showed a significant inhibition of tumor growth in vivo[36,38] (see Fig. 11.4 for the results from the MDA-MB-231 model). This adenoviral vector is also being currently tested in Phase 1 clinical trial.[39]

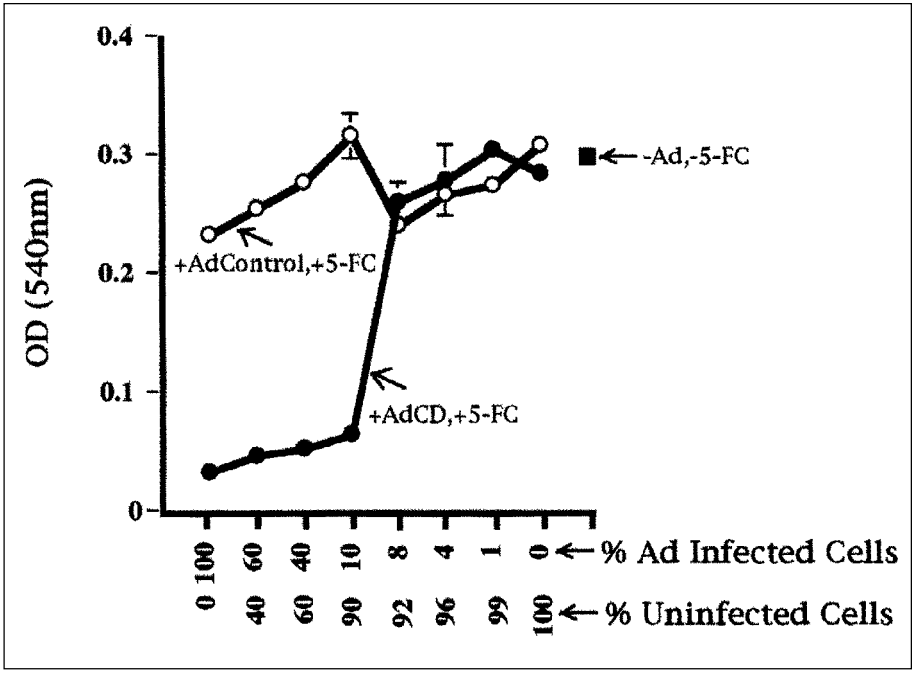

Fig. 11.3. Bystander effects of AdCD. MDA-MB-231 cells were infected with either AdCD or AdControl for 24 h. Virally infected cells were mixed with uninfected cells in different ratios shown in the Figure. After 5 days, cell viability was measured by MTT cell proliferation assays. Shown are the absorbance readings obtained when different ratios of infected and uninfected cells were mixed as shown in the Figure. Results shown are the average of the triplicate determinations ± SE. Reproduced from ref. 36 with permission.

Expression of Antisense Oncogenes and Ribozymes to Dominant Oncogenes

It is well known that development of many cancers is associated with the expression of dominant oncogenes.[1] One oncogene that has been well studied is the *ras* gene. Point mutations in the *ras* gene result in the production of a mutant Ras protein, which can constitutively activate signal transduction pathways and thus lead to uncontrolled cell growth. Therefore, abrogation of this uncontrolled activity of Ras is an interesting anti-cancer strategy. To this end, adenoviral vectors have been designed that produce antisense *ras* sequences. Infection of cancer cells expressing mutant Ras with these antisense vectors has shown a significant inhibition of cell growth in vitro.[40] Another approach targeted to cells expressing mutant Ras protein is to generate adenoviral vectors expressing ribozymes to the *ras* gene. Production of hammerhead ribozymes has been shown to cleave the mutant *ras* RNA specifically and inhibit cell growth.[41] In one study, it has also been shown that direct injection of these ribozyme-producing vectors into tumors derived from EJ human bladder cells grown in nude mice significantly inhibited the tumor growth.[42]

Besides *ras*, other oncogenes which have been targeted through adenoviral vectors are *HER-2/neu* (*erbB2*), *MYC* and *IGF-1* (insulin-like growth factor 1). For instance, infection with an adenoviral vector expressing an endoplasmic reticulum (ER)-directed monoclonal antibody, anti-erbB2 sFv (Ad21), has been shown to downregulate cell surface levels of

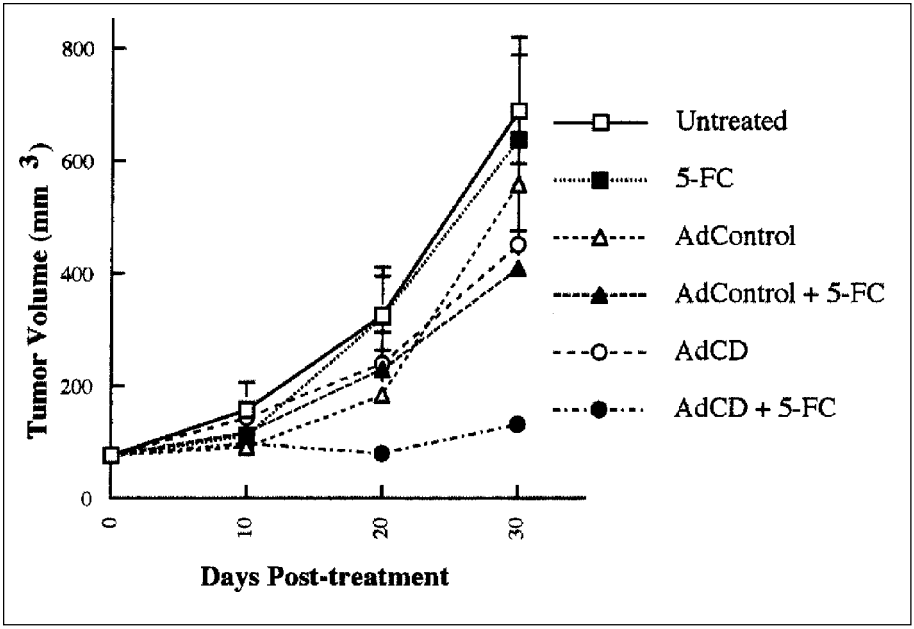

Fig. 11.4. Effects of AdCD infection on the growth of MDA-MB-231 tumors grown as xenografts in nude mice. MDA-MB-231 cells (5 x 10^6 cells/animal) were injected subcutaneously in nude mice and tumors formed. Animals were then divided into six groups (n=6 in each category). One group was mock infected, the second group treated with AdControl (10^9 pfu/0.1 ml volume), the third group with AdCD (10^9 pfu/0.1 ml volume), the fourth group with 5-FC alone, the fifth group with a combination of AdControl and 5-FC, and the sixth group with a combination of AdCD and 5-FC. 5-FC was administered intraperitoneally 12 h post viral injections, and twice daily thereafter for the next 10 days (10 mg/20 g animal weight). Tumor sizes were measured every third day. Shown are the average of the tumor volumes ± SE. Reproduced from ref. 36 with permission.

HER-2/Neu. This results in apoptosis in ovarian and breast cancer cells that overexpress HER-2/Neu, but not in cell lines with lower levels of HER-2/Neu.[43] This approach is currently being evaluated in a Phase I clinical trial.[44] Recombinant adenovirus expressing hammerhead ribozymes to *HER-2/neu* and another growth factor, pleiotropin (*PTN*), have been constructed and demonstrated to deplete the respective RNAs and proteins, thus leading to abrogation of HER-2/Neu and PTN-dependent cancer cell proliferation.[45] HER-2/Neu expression has also been shown to be reduced following adenoviral transduction of the adenoviral type 5 E1a.[46]

MAD, an antagonist of the *MYC* oncogenes, has been expressed in astrocytoma cells via an adenoviral vector, thereby leading to a decrease in cell proliferation and a diminished malignant potential.[47] Adenovirally mediated overexpression of an antisense molecule to insulin-like growth factor has been shown to reduce tumorigenicity of lung cancer cells and prolong survival of nude mice bearing lung cancer xenografts.[48] So far, adenoviral vectors expressing antisense or ribozymes have not been tested in the clinical setting. However, one can anticipate that such trials will be initiated in the near future.

Indirect: Immunomodulation Through Recombinant Adenoviral Vectors

The failure of normal immune surveillance mechanisms, leading to an inability to recognize cancer cells as "foreign" cells, is an integral component in the process of tumor development. Recent research has identified numerous mechanisms that operate to allow cancer cells to evade host immunity. These include lower expression of MHC class 1 and class II proteins, decreased growth and differentiation of effector immune cells and defects in expression of co-stimulatory molecules.[2] Thus, utilizing adenoviral vectors to activate the host immune system, or to attempt to bypass some of these defects by introducing genes that alter the local immune microenvironment, is an attractive anti-cancer strategy. Additionally, these strategies, if successful, have the potential to nullify a major drawback of approaches using adenoviral vectors, i.e., their inability to infect more than approximately 10% of cells in tumor mass, thus making these approaches truly "systemic" in scope.

Immunomodulatory approaches utilizing adenoviral vectors can be grouped into the following subcategories: expression of various cytokines, expression of co-stimulatory molecules, and expression of tumor antigens. In future it might be also feasible to generate cancer vaccine using adenoviruses.

Expressing Cytokines via Recombinant Adenoviruses

One of the earliest adenoviral vectors used in studying this approach was a replication-competent adenovirus expressing γ interferon. Administration of this vector directly into a murine tumor model prevented the growth of these tumors.[49] Moreover, a subsequent tumor challenge failed to establish tumors, thus providing evidence for a "vaccine" effect. Adenoviral vectors expressing the cytokine IL-2 have been studied in different animal models.[50] One extensively studied model is a polyoma middle T-antigen transgenic model which gives rise to spontaneous breast cancer tumors. Using this model, it has been demonstrated that direct injection of these vectors inhibits tumor growth and leads to 40-50% of the animals studied becoming tumor free. Limited analysis of the immunological parameters showed an increase in lymphocytes in the tumors. Mice with regressed tumors were protected from a second tumor challenge, demonstrating long lasting immunity against tumor cells. Some laboratories have employed other murine models and have reported significant effects of IL-2.[4,50]

Adenoviral vectors expressing IL-4 and IL-12 have also has been reported. Adenovirus-mediated in vitro transduction of murine IL-4 resulted in a 61% reduction in tumorigenicity of breast cancer cells in the polyoma middle T-antigen transgenic model, while direct intratumoral injection of this vector led to complete regressions in 50% of animals with pre-existing tumors.[51] Increased infiltration of eosinophils was observed in ex vivo as well as in vivo IL-4 treated animals. "Cured" animals were protected from a tumor challenge. Similar results have been reported after a single administration of a recombinant adenovirus expressing murine IL-12 into pre-existing breast cancers in the polyoma middle T-antigen model.[52] Overall, response rate was 92%, with 31% complete responses. Protection from a second challenge was again observed. Interestingly, this study provided evidence for alteration in the local cytokine milieu, as increased interferon production was observed within the tumor as well as in the draining lymph nodes.

Another cytokine that has been studied is GM-CSF (granulocyte-macrophage colony-stimulating factor). In the Lewis lung cancer mouse model, AdGMCSF (adenoviral vector expressing murine GM-CSF)-transduced Lewis lung cancer cells showed suppressed tumorigenicity.[53] In addition, injection of transduced cells led to slower tumor growth and increased survival even in animals with pre-existing tumors. This effect could be explained

by the observed specific anti-tumor cytotoxic T lymphocyte activity or the accumulation of dendritic cells in the tumor mass observed in this study.

The systemic use of certain cytokines is limited by severe toxicity. In this setting, recombinant adenoviruses may be particularly useful, as they can deliver the cytokine directly into the tumor microenvironment. Furthermore, these vectors can deliver engineered forms of the cytokines that retain the therapeutic effects but have limited toxicity. For instance, it has been demonstrated that intratumoral adenoviral delivery of a mutant murine TNF-α resulted in permanent tumor regressions with minimal toxicity when compared to adenoviral delivery of murine TNF-α, which was significantly more toxic and even fatal in a murine transgenic breast cancer model.[54]

Recombinant Adenoviruses Expressing Costimulatory Molecules

Adenoviral vectors expressing the costimulatory molecules B7-1 and B7-2 have been evaluated. In two tumor models that express defined protein epitopes, grow in mice and kill their hosts with no evidence of immune response, overexpression of B7-1 mediated by a recombinant adenovirus has been shown to be capable of eliciting specific cellular immunity.[55] Animals exposed to these cells after adenovirus-mediated ex vivo transduction of B7-1 were also protected from a subsequent challenge with non-transduced tumors. An adenoviral vector expressing the costimulatory molecule B7-2 has also been described to be effective in retarding the growth of established parental P815 tumors in mice.[56]

Recently, a combinatorial approach using a single adenoviral vector to express IL-12 as well as B7-1 (AdIL12-B7-1) has been reported.[57] In mice bearing tumors derived from a transgenic mouse mammary adenocarcinoma, a single intratumoral injection of AdIL12-B7-1 led to complete tumor regression in 70% of treated animals. Interestingly, coinjection of two different viruses expressing either IL-12 or B7-1 induced complete tumor regression in only 30% of animals treated at this dose.

Recombinant Adenoviruses Expressing Specific Tumor Antigens

Studies evaluating the ability of recombinant adenoviruses to directly function as an immunogen have been reported from the Surgery Branch of the National Cancer Institute, Bethesda, USA. In a novel study, Chen and coworkers showed that immunization of Balb/c mice with recombinant adenoviral vectors encoding a model antigen, the bacterial protein β-galactosidase, was able to elicit a specific immune response in their splenocytes.[58] Furthermore, adoptive transfer of these splenocytes produced dramatic regressions of pulmonary metastases from a colon adenocarcinoma cell line that expresses the same bacterial antigen. Using a higher titer virus and exogenous IL-2 administration, they could obtain regressions even in pre-established pulmonary metastases in the same animals.[58]

Similar observations have been made with recombinant adenoviruses expressing human melanoma tumor antigens MART1 and gp100 (Ad2CMVMART1 and Ad2CMVgp100).[59] Ad2CMVMART1 infection of cancer cells negative for MART1 expression resulted in recognition and specific lysis by MART1-specific CTLs. Immunization of C57BL/6 mice with Ad2CMVgp100 protected the mice from murine melanoma challenge with B16 cells. This effect was abrogated by depleting the animals of $CD8^+$ cells, but not $CD4^+$ T cells. These studies show that recombinant adenoviruses encoding tumor antigens are a feasible anti-cancer approach and, in fact, these vectors are currently being evaluated in clinical studies.

A recent innovation to this strategy of presenting tumor antigens via adenoviruses involves the use of dendritic cells to present these antigens. Dendritic cells (DC) are potent antigen-presenting cells that can activate cytotoxic T cells. Recombinant adenoviruses have proven to be the most efficient way to express foreign genes in human DC.[60] More interestingly,

dendritic cells transduced to express a model antigen, β-galactosidase, have been shown to induce protective antitumor immunity against a cancer cell line expressing the same antigen.[61] In another study, intravenous (i.v.) administration of adenovirally transduced DC presenting the MART1 antigen was superior to direct i.v. administration of the adenoviral vector in inducing MART1 specific immune response, as well as protection against a challenge of a cell line stably transfected with MART1.[62]

Other Novel Strategies

Using adenoviral vectors for anti-cancer strategies is a rapidly evolving field leading to frequent reports of new ideas and innovations of pre-existing approaches. Some of these novel concepts are described below:

Combinations of Direct and Indirect Approaches

In addition to the previously mentioned combinations of tumor suppressor genes (*p53* and $p16^{INK4A}$) and immunomodulatory approaches (co-stimulatory molecules and cytokines), combinations of directly toxic approaches and immunomodulatory approaches have also been examined. These include combining adenoviral vectors expressing the suicide gene HSV*tk* with adenoviral vectors expressing murine IL-2 or GM-CSF.[63] Animals treated with a combination of all three were shown to develop long term immunity to metastatic colon cancer and prolonged survival in contrast to animals treated only with Ad.HSV.*tk* and ganciclovir. In another report, combination treatment with adenoviral vectors expressing the cyclin dependent kinase inhibitor $p21^{WAF1/Cip1}$ and an immunomodulatory molecule, $H\text{-}2K^b$, resulted in greater reduction of tumor growth than with either vector alone.[64] Furthermore, it could be demonstrated that the immune response against the $H\text{-}2K^b$ gene product involved in tumor regression was potentatiated by expression of the $p21^{WAF1/Cip1}$ gene.[64]

Expression of Molecules That Affect Angiogenesis, Cell Adhesion and Metastasis

In recent years, remarkable progress has been made in understanding the molecular mechanism of angiogenesis and metastasis. This in turn has spawned the construction and characterization of a number of adenoviral vectors transferring genes that can modify these processes. For instance, construction of an adenoviral vector that expresses a novel, secretable form of the antiangiogenic protein platelet factor 4 (sPF4) has recently been reported.[65] Vector-mediated sPF4 results in tumors show decreased vascularity and grow slowly in vivo. Notably, transduction of established intracerebral gliomas by an sPF4-expressing adenoviral vector resulted in reduced tumor-associated angiogenesis and prolonged animal survival. These data support the feasibility of anti-angiogenic strategies utilizing adenovirally mediated gene transfer. Another adenoviral vector that has been recently described expresses C-CAM, an androgen-regulated cell adhesion molecule that acts as a tumor suppressor in prostate cancer development.[66] Interestingly, delivery of a single dose of C-CAM adenovirus was demonstrated to repress the growth of PC-3-induced tumors in nude mice for at least 3 weeks.

Another novel anti-cancer strategy targets the increased secretion of metalloproteinase enzymes (MMPs) that is implicated in cancer cell invasion and metastasis. Recombinant adenoviruses capable of expressing the MMP-9, TIMP-1 or -2 genes have been generated and shown to efficiently express these enzymes in human MCF-7 breast adenocarcinoma cells.[67] Although in vivo studies with these vectors have not been reported thus far, this area appears to be very promising.

Chemosensitization and Radiosensitization Approaches

In recent years, a number of adenovirus-based approaches have been proposed that attempt to increase the sensitivity of cancer cells to chemotherapy and radiotherapy. As mentioned previously, adenoviral delivery of wild type p53 has been demonstrated to have a chemosensitizing effect. In another approach, adenoviral delivery of the liver cytochrome P450 gene (CYP2B1) to cancer cells has been shown to lead to substantial chemosensitization to the oxazaphosphorines like cyclophosphamide and ifosfamide that require activation by the cytochrome enzymes.[68] Similarly, infection of breast cancer cells with an adenoviral vector that expresses the adenoviral type 5 E1a has been shown to downregulate expression of HER-2/Neu and lead to increased sensitivity to paclitaxel.[46]

Others have explored novel adenovirus-based strategies to increase radiosensitivity of cancer cells. For instance, the gene therapy strategy of toxin gene conversion of nontoxic prodrug to chemotherapeutic drug in combination with radiation therapy has been applied to the treatment of cholangiocarcinoma. In this study, the *E. coli* enzyme cytosine deaminase (CD) was expressed via an adenoviral vector in a human cholangiocarcinoma cell line (SK-ChA-1) and was shown to convert the non-toxic prodrug 5-fluorocytosine (5-FC) to 5-FU, a well known radiosensitizing agent. Radiobiological survival curve parameters demonstrated an interactive cytotoxic effect in vitro when viral infection and prodrug therapy were combined with external beam radiation exposure. Interestingly, combined treatment of SK-ChA-1 tumors with AdCMVCD, 5-FC, and radiation in animals resulted in significantly greater survival, time to tumor regrowth, and doubling time compared to the nonradiation treatment group. Significantly greater change in tumor size, smaller ratio of final tumor size to original tumor size, and smaller final tumor size were also observed in the radiation treatment group compared to the no radiation treatment group.[37] In vivo regulation of gene transcription, spatially as well as temporally, by ionizing radiation is another approach that has been explored recently. In this strategy, the radiation-inducible promoter region of the Egr-1 gene was linked to the gene encoding the radiosensitizing and tumoricidal cytokine, tumor necrosis factor α (TNF-α). A replication-deficient adenovirus (Ad5.Egr-TNF) was then used to deliver the Egr-TNF construct to human tumors growing in nude mice. Combined treatment with Ad5.Egr-TNF and 5,000 cGy (rad) resulted in increased intratumoral TNF-α production and increased tumor control compared with treatment with Ad5.Egr-TNF alone or with radiation alone. Furthermore, this increase in tumor control was achieved without an increase in normal tissue damage when compared to tissue injury from radiation alone.[69]

Tissue-Specific Promoter-Driven Transgene Expression

With an aim to express transfected genes specifically in tumor cells, and not in the infected normal cells, the promoter elements of genes usually expressed only in tumor cells have been used to drive expression of adenovirally transduced genes (also see chapter 20 for details). For instance, an adenoviral vector in which the expression of the HSV*tk* gene is controlled by the carcinoembryonic antigen (CEA) promoter has been shown to confer sensitivity to ganciclovir only in CEA-producing gastric cancer cell lines.[70] Similarly, a recombinant adenovirus containing the murine α-fetoprotein (AFP) promoter constructed to direct hepatocellular carcinoma (HCC)-specific expression of the human interleukin 2 (IL-2) gene has been evaluated. This recombinant adenoviral vector produced HCC-specific IL-2 gene expression three to four orders of magnitude higher in AFP-producing HCC lines compared to non-AFP-producing non-HCC lines.[71] A similar result was obtained in vivo in the treatment of established human HCC (Hep 3B/Hep G2) xenografts growing in CB-17/SCID mice. Intratumoral injection of this vector resulted in growth retardation and

regression in a majority of established hepatic tumors with a wider therapeutic index and less systemic toxicity.[71]

Expression of Cell Surface Receptors to Target Cancer Cells

In addition to the targeting approaches using tissue specific promoters and monoclonal antibodies mentioned in previous sections, another novel targeting strategy based on adenovirally transferred cell surface receptors has been proposed recently. For instance, an adenoviral vector has been used to induce the expression of the murine gastrin-releasing peptide receptor (GRPR) both in vitro and in vivo. A radiolabeled bombesin analog [125I]-mIP-bombesin) has been shown to bind with high affinity to GRPR and to localize to intraperitoneal ovarian tumors.[72] This approach may prove to be very useful in targeted delivery of radiolabeled and other cytotoxic molecules to cancer cells that are expressing the adenovirally transduced receptor.

Exploiting Adenovirally Mediated DNA Transfection: Delivery of Toxin Genes and Generation of Tumor Vaccines

Replication defective adenoviruses can enhance delivery of DNA to tumor cells by their ability to disrupt the endosomal vesicles transporting the plasmid DNA (also see chapters 4 and 10 for details). However, this is true only for cells that express the adenoviral receptors on their surface. An interesting application of this observation involves increased delivery of *Pseudomonas exotoxin* plasmid into cells, resulting in increased toxicity only to cells that express the adenoviral receptors on their surface but not to cells deficient in adenovirus receptors, such as hematopoietic $CD34^+$ cells[73] (Fig. 11.5). This differential toxicity of adenovirally dependent enhancement of DNA delivery can be exploited to purge bone marrow contaminated with cancer cells. This is further discussed in chapter 22.

Taking advantage of adenovirally mediated enhancement of DNA delivery, a tumor vaccine has been generated from a murine melanoma clone by using a technique called adenovirus-enhanced transferrinfection (AVET).[74] Two consecutive vaccinations with these transfected cells resulted in protection from development of tumors on subsequent challenge, thereby implying that this method can be used with allogeneic tumor cells or synthetic antigens.

Replication Competent Adenoviruses

All of the anti-cancer strategies discussed thus far in this chapter have utilized replication deficient vectors. However, an exciting new idea is to employ replication competent adenoviral vectors. In a pioneering work, Bischoff et al have reported that a mutant adenovirus that does not express the 55 kDa E1B protein can replicate in *p53*-deficient tumor cells but not in cells with a functional *p53*.[75] Injection of this virus into *p53*-deficient human cervical carcinoma cells grown in nude mice led to complete regressions in a remarkable 60% of animals. As discussed in chapter 21, this virus is currently being evaluated in clinical trials. In another study based on a replication competent adenovirus, the prostate-specific antigen enhancer/promoter construct was used to drive the E1A gene. This resulted in preferential growth and replication of this virus in prostate cancer cells expressing the prostate-specific antigen, compared to cells that did not express this antigen.[76]

Conclusion

The use of recombinant adenoviral vectors for gene therapy of cancer is a relatively young field. In spite of many limitations of adenoviral vectors mentioned in the previous chapter, in the last several years numerous reports have appeared in which adenoviral vectors have been successfully used for in vitro and ex vivo research. More importantly, in various

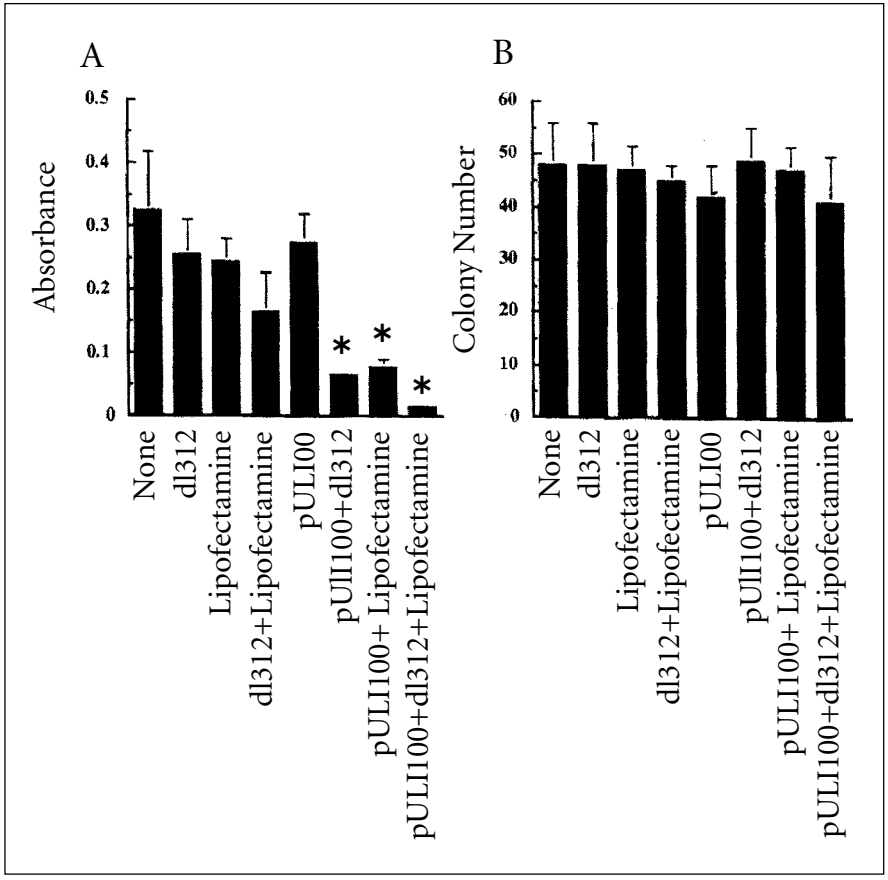

Fig. 11.5. Ad-mediated enhancement in the cytotoxicity of plasmid pULI100 in breast tumor and bone marrow cells. MDA-MB-231 (500), and $CD34^+$ bone marrow cells (1000) (purity of $CD34^+$ cells was about 15%) were transfected with pULI100 plasmid (5 μg/ml) in the presence of lipofectamine (1μg/1 μg of plasmid DNA) and of dl312 (100 pfu/cell). Cell survival was estimated for MDA-MB-231 by a colorimetric assay using sulforhodamine, and for $CD34^+$ cells by colony forming assay. Panel A shows the absorbance reading in MDA-MB-231 cells, representing the growth of these cells treated under different conditions, and Panel B shows the colony number from $CD34^+$ cells treated under the same conditions. Results shown are the average of triplicate determinations ± SD. Statistically significant cytotoxic effects (p values <0.05) are indicated by ✱ on top of the respective bars. Reproduced from ref. 73 with permission.

animal models based on a variety of anti-cancer strategies using these vectors, the initial results obtained have been encouraging enough to justify further evaluation in clinical trials. In the next few years, mature data from these early trials will guide further development of this field.

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CHAPTER 12

Adenoviral Vectors for Cardiovascular Diseases

Noel M. Caplice, Timothy O'Brien, and Robert D. Simari

Cardiovascular diseases affect over 60 million people in the United States, and resulted in over 150 billion dollars in expenses in 1996 (American Heart Association, 1997). As such, the potential for clinical benefits from cardiovascular gene transfer is great and the targets for therapy are numerous. Over the last two decades, the development of gene transfer to treat cardiovascular diseases has progressed from concept to clinical trials. This evolution has been advanced by an increased understanding of the inherent requirements for efficient gene transfer, identification of therapeutic targets and the development of improved vectors. The normal heart and vasculature consist of multiple cell types suitable as targets for gene transfer. In addition, in diseased states, the cellular composition of each tissue is altered quantitatively and qualitatively, increasing the complexity and possibly the difficulty of gene transfer. Adenoviral vectors have several advantages for gene transfer to the cardiovascular system, including the ability to transduce quiescent cells. The use of these vectors has been critical to allow the development of gene transfer in pre-clinical animal models of human disease. However, the requirement for the use of adenoviral vectors to obtain efficient gene transfer in these models of cardiovascular disease has delayed clinical application due to concerns regarding toxicity.

Vector Requirements for Cardiovascular Disease

Requirements for the ideal vector for gene transfer in cardiovascular disease would include the lack of toxicity and the ability to efficiently transduce targeted cells in a regulatable manner. Requirements for ideal vectors are determined by phenotypic and genotypic characteristics of the cells and their distribution within each tissue. Ideal gene transfer vectors for the heart and vasculature have not been developed.

Delivery to the Heart

The normal heart is composed of terminally differentiated cardiac myocytes and cells capable of replication, including fibroblasts and vascular endothelial cells (EC). The primary targets for gene transfer to the heart are cardiac myocytes. Potential disease targets include cardiac hypertrophy, heart failure, myocarditis and ischemic heart disease. Although systemic (intravenous) delivery results in minimal cardiac myocyte expression,[1] direct delivery to the heart has resulted in enhanced efficiency of gene transfer.[2,3] Delivery of adenovirus to cardiac myocytes can be performed with either direct injection or infusion into coronary arteries. The former technique results in a discrete area of infected myocytes expressing transgenes, while the latter results in a more diffuse pattern of transgene expression.[4]

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Delivery to the Vasculature

Normal arteries are composed of a trilaminar structure consisting of an inner intima containing endothelial cells and, in humans, vascular smooth muscle cells. The middle layer, or media, is separated from the intima by the internal elastic lamina and is composed of vascular smooth muscle cells (VSMC). The outer layer, or adventitia, is separated from the media by the external elastic lamina and is composed of fibroblasts as well as EC and VSMC from the vasa vasorum. Low rates of cell proliferation are seen in normal arteries. However, cellular proliferation occurs in response to injury. In atherosclerosis, there is the intimal expansion consisting of cellular infiltration and extracellular matrix, including cholesterol and calcium deposition. Vector delivery to the arterial wall can be performed via lumenal or adventitial approaches. An ideal vector would be capable of transfecting target cells without local or systemic toxicity.

Comparisons with Other Vectors

Adenoviral vectors have been used extensively for cardiovascular gene transfer due to their ability to infect large numbers of nondividing and dividing cells in vitro and in vivo, their capacity to accept relatively large DNA inserts and the ease of production of high titer stocks. Transfection efficiencies using conventional methodologies for in vitro gene transfer to neonatal and cardiac myocytes with DEAE-dextran, calcium phosphate, lipofection and retroviruses were disappointing. Comparison with nonviral vectors demonstrated the superiority of adenoviral vectors in transfecting cardiac myocytes in vivo.[3,5] Direct injection of first generation (E1, E3 deleted) recombinant adenoviral vectors into adult immunocompetent rats resulted in expression of transgene from myocytes at the injection site for up to 60 days. In comparison to optimized doses of injections of plasmid DNA, adenoviral delivery resulted in 10-5000 fold increase in transgene expression. This susceptibility to adenoviral infection might be explained by the known susceptibility of myocytes to coxsackie B viruses and the recent isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. In neonatal rats, expression has been demonstrated for over 225 days.[5] This enhanced expression in neonatal animals supports the role of an immune response in limiting the duration of expression from adenoviral vectors. Adenoviral gene transfer to cardiac myocytes adjacent to areas of myocardial infarction has been documented in rat hearts, suggesting applicability of this technique in diseased states.

Early studies of vascular gene transfer were performed with DNA liposomes or with retroviral vectors.[6] DNA liposomes were limited in their ability to transduce large numbers of vascular cells and by the brief duration of expression from the plasmids. Studies with vascular plasmid delivery have demonstrated predominantly endothelial cell transfection with occasional intimal and medial VSMC transfection. Retroviruses were limited by their inability to infect nondividing cells, which represent the majority of vascular cells even in injured arteries. Adenoviral vectors provided an opportunity to infect larger numbers of vascular cells in vivo, including medial VSMC, albeit in a transient fashion (Fig. 12.1).[7]

Potential for Toxicity

Concerns regarding the toxicity of myocardial adenoviral-mediated gene transfer have been raised. Wild type adenovirus is known to be a cause of myocarditis and adenoviral vectors have potential cytopathic effects. In early animal studies with direct injection of adenoviral vectors, a marked leukocytic infiltrate was seen at the site of injection and transgene expression.[3] A subsequent study failed to document any adverse local cardiac or systemic physiologic effects of this inflammatory response in minipigs.[4] Phase 1 studies in humans will help define the safety profile of these first generation adenoviral vectors.

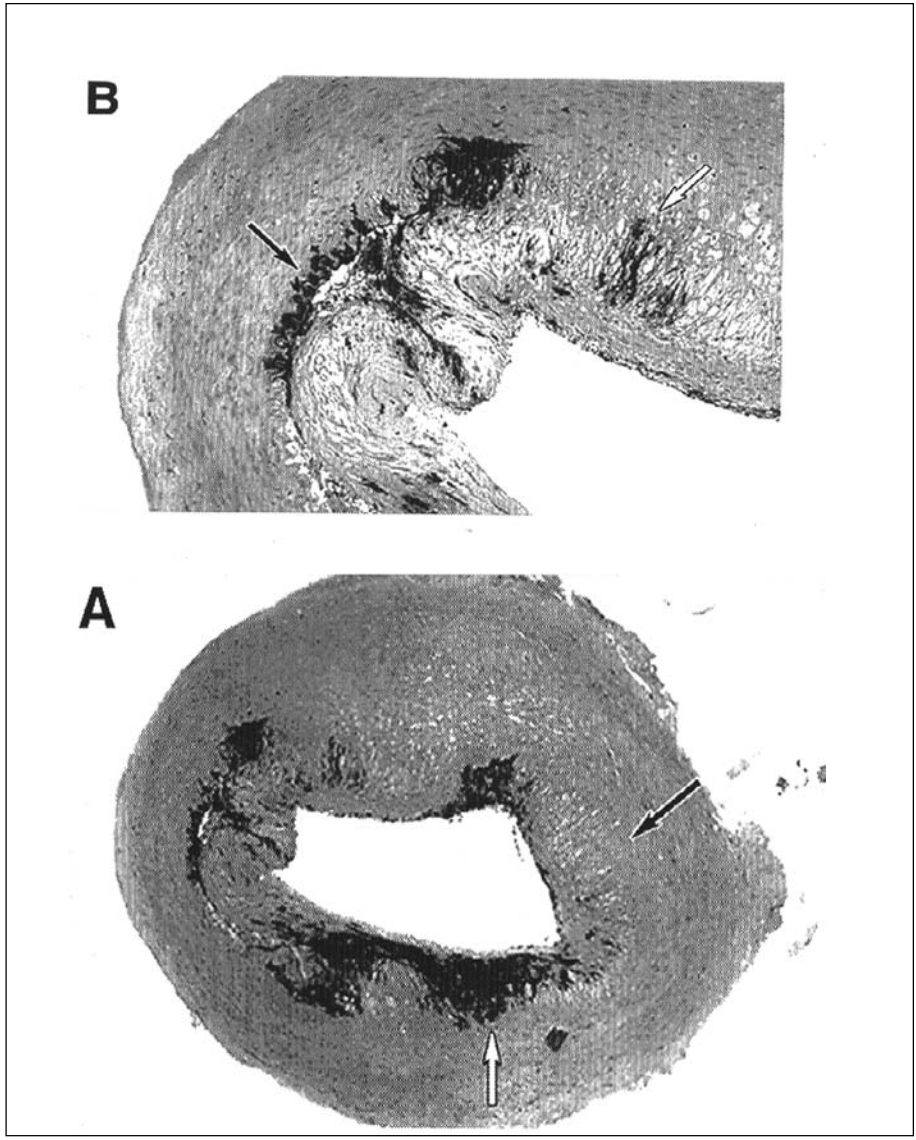

Fig. 12.1. Transgene expression in atherosclerotic rabbit arteries 2 days following balloon injury and infection with an adenoviral vector expressing human placental alkaline phosphatase (AP). (A) AP expression was observed in the intima and media of balloon injured atherosclerotic arteries. Black arrow denotes internal elastic lamina. White arrow denotes dark AP histochemical staining. x30. (B) Gene expression in the media was prominent along a dissection plane where the internal elastic lamina was fractured, as designated by the black arrow. The white arrow denotes the internal elastic lamina. x77. (Permission requested from Rockefeller University Press.)

Limitations of the vascular applications of adenoviral delivery have been highlighted in the work of Dichek and colleagues. They demonstrated that adenoviral delivery to normal rabbit arteries resulted in vascular cell activation, inflammation and intimal formation.[8] In contrast, rats do not seem to develop this response to vascular adenoviral delivery, suggesting important species differences. In addition, they showed that established immunity in rats precludes efficient gene transfer from these first generation vectors.[9] These studies have generated considerable concerns regarding the safety and efficacy of the use of these vectors for clinical studies. However, the human immune response may have important quantitative and qualitative differences from these experimental models and transfer would not likely be performed in normal vessels but in atherosclerotic arteries, which are inherently inflammatory. Prior studies failed to detect additional vascular inflammation following local adenoviral delivery in atherosclerotic models, albeit in naive animals.[7]

Specific Enhancements of Adenoviral Vectors for Cardiovascular Targets

Enhancement of myocardial gene transfer has been achieved using physical, biochemical and genetic means. Donahue and colleagues have described an in vitro technique to almost universally infect myocytes with adenoviral vectors using intracoronary perfusion in a Langendorff preparation of rabbit hearts.[10] This study demonstrated that optimal transfer efficiency was associated with transfer at 37˚C in crystalloid solutions with high titer viral stock (1.6×10^9 pfu/ml) at exposures up to 60 minutes. This technique might be adaptable to clinical cardiac bypass surgery. Replication-deficient adenoviral vectors have been combined with plasmid DNA and polylysine to take advantage of the endolysosomal properties of adenovirus to enhance delivery of plasmid DNA to cardiac myocytes by using a replication-deficient adenovirus.[11] This system eliminates the need for the preparation of new recombinant viral vectors for testing. Cardiac myocyte-specific gene expression from an adenoviral vector has been achieved using a gene under the control of the ventricle-specific myocyte light chain 2.[12] This vector, when injected into the left ventricular cavity of rats, resulted in heart-specific transgene expression in spite of systemic distribution of adenovirus. These adaptations, in addition to further reduction and replacement of adenoviral DNA in future generations of vectors, might provide for less toxic andenhanced, specific delivery to cardiac myocytes, the goal for cardiac gene therapy.

With arterial gene transfer, targeting has been performed by the use of localized gene delivery. Recently, targeting of vascular smooth muscle cells has been performed using a transcriptional targeting approach. Kim and colleagues took advantage of the smooth muscle cell specific promoter SM22α to target VSMC.[13] They demonstrated that expression of a reporter gene driven by the SM22α promoter was limited to VSMC. Targeting adenoviral gene expression to VSMC might have important advantages when introducing a cytotoxic vector.

Preclinical and Clinical Studies of Cardiac Gene Transfer Using Adenoviral Vectors

Following the clear demonstration of the feasibility of adenoviral mediated cardiac gene transfer, several therapeutic strategies have been tested in vitro and in preclinical animal models. These strategies are aimed to genetically modify cardiac myocytes having autocrine, paracrine or endocrine effects (Fig. 12.2). In heart failure, myocyte contractility is attenuated, providing a substrate for fatal arrhythmias. In vitro, gene transfer has been

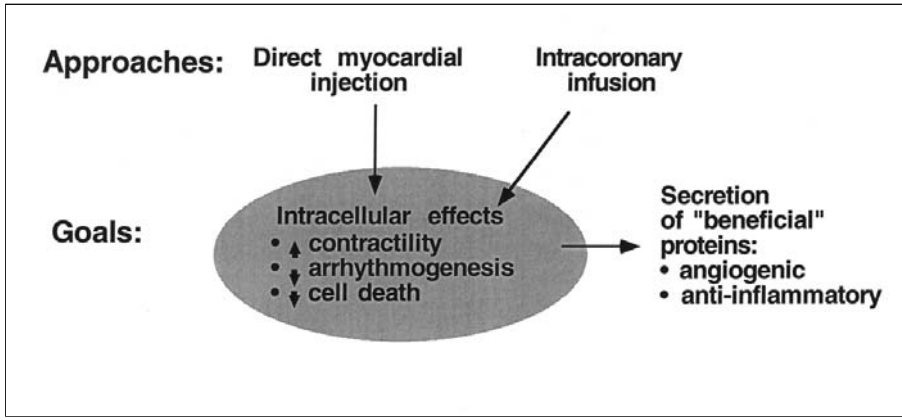

Fig. 12.2. Strategies for "therapeutic" cardiac myocyte gene transfer.

used to enhance myocyte contractility in normal or failing myocytes using adenoviruses expressing slow skeletal troponin I[14] or components of the β_2-adrenergic receptor system,[15] and excitability has been altered by overexpressing potassium channels.[16] Attempts to provoke cell cycle reentry of terminally differentiated cardiac myocytes have included in vivo adenoviral delivery of E2F-1.[17] However, this strategy results in apoptosis of postmitotic adult ventricular myocytes. In contrast, expression of Bcl-2 with adenoviral delivery inhibits p53-induced apoptosis of cardiac myocytes.[18]

Gene transfer to cardiac myocytes to deliver proteins locally may provide opportunities for sustained and local peptide therapy not achievable by other methods. To deliver interleukin 10 and TGF-β to rabbit cardiac allografts, intracoronary infusion of recombinant adenoviruses was performed.[19] The effects on allograft rejection are yet to be determined, but the potential remains for gene delivery during the period between harvesting and transplantation. Angiogenesis, or the growth of new blood vessels, is an important clinical goal in the treatment of ischemic heart disease. Intracoronary adenoviral delivery of FGF-5, a secreted member of the angiogenic fibroblast growth factor family, has been shown to improve cardiac function in ischemic areas in a porcine model.[20] Importantly, this study determined that 98% of the virus delivered is taken up by the heart, and demonstrated the lack of systemic toxicity with this approach. These results have generated a multicenter clinical trial of a related gene.

Preclinical Studies of Vascular Gene Transfer Using Adenoviral Vectors

The use of adenoviral vectors for vascular gene transfer has permitted the testing of several important hypotheses regarding the development and treatment of vascular disease. The vectors tested have uniformly been first generation vectors, while the strategies tested have included antiproliferative, antithrombotic and vasoactive approaches. The majority of studies have involved the use of adenoviral delivery to prevent intimal expansion in models of vascular injury.

The first "therapeutic" use of vascular gene transfer was performed in the laboratory of Elizabeth Nabel at University of Michigan.[21] This cytotoxic strategy used an adenovirus expressing herpesvirus thymidine kinase (tk) and exposure to the prodrug ganciclovir to

kill proliferating cells (and neighboring cells via the bystander effect) and to inhibit intimal expansion following balloon injury in a porcine model. Subsequently, this strategy has been tested in normal rat carotid arteries and in atherosclerotic rabbit iliac arteries.[7] A similar approach using cytosine deaminase sensitization to 5-FC has reinforced the conclusion that killing proliferating cells could limit intimal formation following vascular injury.[22]

Cytostatic approaches to inhibit intimal formation have been successful using vectors expressing p21, a cyclin-dependent kinase inhibitor[23] and a constitutively active form of Rb.[24] These approaches do not require the administration of a prodrug to activate the transgene product. The induction of apoptosis in the vessel wall has been shown using an adenovirus expressing Fas ligand.[25] Interestingly, this approach limited intimal formation and attenuated the immune response to viral delivery. Thus, coexpression of Fas ligand may provide an important tool to modulate the immune response to adenoviral gene transfer.

Modulation of vascular thrombosis using adenoviral delivery has been shown using transfer of genes expressing anticoagulant proteins. These approaches demonstrate the ability to manipulate the hemostatic system using gene transfer and might have applications to prevent the progression of atherosclerosis, a process dependent in part on thrombosis. However, antithrombotic gene transfer strategies suffer from the inherent delay in transgene expression following delivery.

Regulation of vasoactivity using adenoviral delivery of endothelial nitric oxide (NO) synthase has been shown to have antiproliferative effects[26] as well as vasoactive effects following luminal or adventitial delivery.[27] Adventitial adenoviral delivery of NOS has been shown to create a "neo-endothelial" source of NO for the vessel. Like NO, the natriuretic peptides act through a cGMP-dependent pathway and may have multiple vascular effects. Adenoviral-mediated gene transfer of C type natriuretic peptide (CNP) decreased intimal formation in the rat model of vascular injury.[28] Taken together, these studies suggest the importance of modulation of cGMP in the vascular response to injury using adenoviral gene transfer.

Conclusion

Cardiovascular diseases are attractive targets for developing gene therapies. The cell types to be transduced and the nature of the tissues in which they reside currently require adenoviral delivery for the application of effective "therapies" in preclinical models. These preclinical studies have demonstrated the ability to modulate gene expression in cardiac and vascular tissues to attenuate and treat cardiovascular disorders. Early clinical studies with first generation vectors are under way. Extrapolation of the preclinical and early clinical studies to widespread clinical use will require further modification of the adenoviral vector to improve its safety profile and to increase the duration of expression from cardiovascular tissues.

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CHAPTER 13

IAP-Based Gene Therapy for Neurodegenerative Disorders

Stephen J. Crocker, Daigen Xu, Charlie S.Thompson, Peter Liston, and George S. Robertson

Neurodegenerative diseases are steadily becoming more prominent as human life expectancy increases. Gene therapy holds tremendous promise for the long term treatment of neurodegenerative disorders. The potential successes of gene therapy as a clinically useful therapeutic will be determined by two key components: the vector(s) used and the payloads they carry. The major constraints at the present time for the use of adenoviral delivery systems include the duration of vector-derived gene expression, targeting of vectors to specific cell or tissue types, and toxicity. At the same time, however, the number of candidate genes for potential delivery is immense and growing daily. Ultimately, the clinical potential provided by a candidate gene for the treatment of a neurodegenerative disorder requires a detailed understanding of the molecular pathway(s) in which the virally-delivered protein product will function.

Programmed cell death, or apoptosis, is a process of cell suicide brought about by the activation of an internal mechanism of gene expression which serves to dismantle the cell. While programmed cell death is a fundamental process required for the proper development and homeostasis of an organism, dysregulation of apoptosis is believed to be the cause of many pathologies. For instance, cancer and several autoimmune diseases can be conceptualized as failure to execute programmed cell death, while the development of many neurodegenerative disorders may be the consequence of inappropriate apoptosis. Consequently, to lessen the severity or halt the progression of a neurodegenerative condition, a gene therapy designed to control apoptosis may offer a wide range of potential clinical applications.

It is becoming increasingly clear that a family of enzymes, called caspases, play a quintessential role in the initiation and execution of neuronal cell death. Specifically, recombinant mouse technology has shown that caspase-3 is a key component in developmental neuron death.[1] Recent studies have also described caspase-3 activation in several models of neurodegeneration and demonstrated that inhibition of this caspase can enhance neuron survival.[2] Hence, a factor capable of blocking caspase-3 activation may have significant utility for the treatment of neurodegeneration disorders. A family of mammalian proteins which function as potent inhibitors of caspases 3 and 7 have recently been cloned[3,4] and characterized.[5,6] These inhibitor of apoptosis (IAPs) proteins provide one of the most promising payloads for neurodegenerative gene therapy. This chapter will discuss the

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potential uses of adenovirus mediated IAP-based gene therapy for the treatment of three neurodegenerative disorders: stroke, optic neurodegeneration and Parkinson's disease.

The IAP Gene Family

Baculovirus IAPs were identified as proteins encoded by insect viruses which enabled their propagation by blocking defensive apoptosis of the infected cell.[7] Subsequent work has revealed that these genes represent a highly conserved anti-apoptotic strategy found in organisms ranging from *C. elegans* and *Drosophila melanogaster* to birds and mammals.[4]

The first mammalian IAP to be isolated was neuronal apoptosis inhibitor protein (NAIP), a candidate gene for the degenerative motor neuron disease spinal muscular atrophy.[8] This gene is homologous to two baculovirus inhibitor of apoptosis proteins (Cp-IAP and Op-IAP) and is partly deleted in individuals with type I spinal muscular atrophy. The isolation of NAIP prompted the search for other human genes that might encode IAP-like proteins. First to be identified was a gene located on the X chromosome at q24-25 which encodes a protein termed XIAP (for X-linked IAP) that contains three BIR motifs and a RING zinc finger.[4] BIR, for baculoviral inhibitor of apoptosis protein repeat, a motif originally observed at the amino-terminus of viral IAP proteins, are approximately 65 amino acids in length and appear to be required for the ability of IAPs to prevent apoptosis. Next to be identified were two new human cDNAs, *hiap-1* and *hiap-2*.[4] Both of these genes encode proteins that contain three BIR motifs and a RING zinc finger and map to chromosome 11.[4] The high degree of conservation (72% for the overall amino acid sequence) between HIAP-1 and HIAP-2, as well as the location on chromosome 11, is suggestive of a duplication event. In contrast, XIAP exhibits only 44% and 42% conservation with HIAP-1 and HIAP-2, respectively. NAIP is much more distantly related to the other IAPs, with only 25-30% conservation observed. Moreover, NAIP lacks the RING zinc finger present in baculovirus and the other human IAPs. Nevertheless, there is a high degree of homology through the BIR domains, especially with HIAP-2, with which NAIP exhibits 58% identity. In addition, at least two of the IAPs, HIAP-1 and HIAP-2, have a caspase recruitment domain (CARD) motif.[9] Although CARD motifs have been identified in several caspase pro-domains and adapter proteins where they mediate interaction with other CARD containing proteins, a role for this domain in the IAPs has not been established. The structural organization of these proteins is summarized in Figure 13.1.

Function of IAP Proteins

Overexpression of IAP proteins using recombinant adenovirus vectors suppresses apoptosis triggered by a variety of stimuli including growth factor withdrawal, oxidative stress, transfection of pro-caspases, TNFα and Fas ligand binding.[4,10] The breakthrough in understanding IAP function came with the demonstration that IAP proteins can bind and inhibit certain members of the caspase family.[5,6,11] The activation of caspase-3-like enzymes constitute the terminal 'executioners' of apoptosis[12] since they inactivate DNA repair enzymes,[13] degrade cytoskeletal and nuclear scaffolding proteins and proteolytically activate an endonuclease which generates DNA laddering characteristic of apoptosis.[14] Caspases cleave many additional cellular targets, only some of whose effects are understood in the context of apoptosis (reviewed in refs. 12,15,16). The IAP proteins function at a point further along the cell death pathway than other anti-apoptotic proteins. XIAP, HIAP-1 and HIAP-2 have been demonstrated to inhibit the proteolytic activity of caspases 3 and 7, both at the level of the proenzyme and the fully activated tetramer conformations. This activity has been refined to the second BIR domain of XIAP, which is both necessary and sufficient for caspase inhibition.[17] No function has yet been attributed to the RING zinc finger of the IAPs, nor to the unique carboxy-terminus of NAIP.

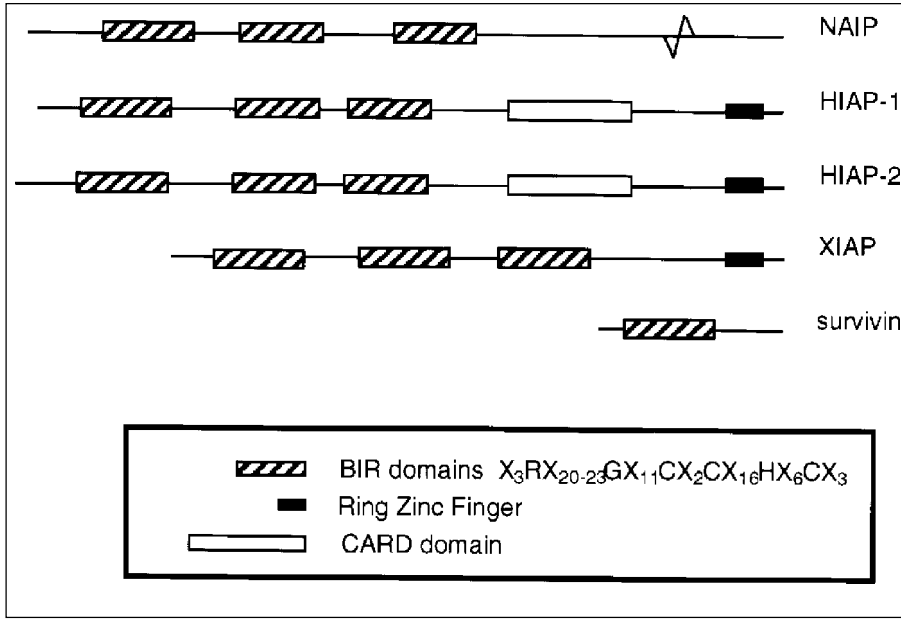

Fig. 13.1. Schematic representation of the IAP family of proteins. BIR domains and RING zinc fingers are indicated by the hatched and black boxes respectively. The carboxy-terminus of NAIP has been truncated in the figure. The CARD domains of HIAP-1 and HIAP-2 are shown as white boxes.

The number of proteins involved in signal transduction pathways that lead to apoptosis is immense, yet the number of anti-apoptotic proteins is by comparison very small. Although the Bcl-2 family of anti-apoptotic proteins offers potential for intervening in some or even many pathologies by inhibiting caspases, the IAPs are uniquely situated at the confluence of all apoptotic pathways. These genes may therefore offer a broad therapeutic scope pending the optimization of appropriate vectors.

IAP Gene Therapy for Stroke

Interruption of blood flow to the brain produces a state of cerebral ischemia which rapidly destroys central neurons, resulting in a stroke. Accumulating evidence suggests that ischemic neuronal death occurs by an apoptotic mechanism. Supporting this view are recent observations from several laboratories implicating caspase-3 activation in the apoptotic pathways responsible for neuronal death following a brief period of cerebral ischemia.[18,19] These findings have stimulated gene therapy experimentation on the neuroprotective potential of viral based vectors encoding anti-apoptotic genes in animal models of cerebral ischemia.

A brief episode of global ischemia produced by four-vessel occlusion results in the delayed death of CA1 neurons in the hippocampus. Loss of these neurons is thought to be responsible for deficits in behavioral measures of memory performance following transient global ischemia.[20] Work performed in this laboratory has demonstrated that adenovirally-mediated overexpression of two human IAP family members, NAIP and XIAP, reduces the loss of CA1 hippocampal neurons in a rat model of global ischemia.[19,21] Moreover, overexpression of XIAP was found

to prevent ischemia-induced deficits in spatial learning performance in the Morris water maze test, indicating that CA1 neurons protected in this manner operate properly.[19] Consistent with the observation that surviving CA1 neurons appeared to function normally, XIAP overexpression prevented ischemia-induced reductions in levels of the neuronal activity marker NGFI-A.[19] The neuroprotective effects of XIAP overexpression were associated with a substantial decrease in the number of CA1 neurons displaying caspase-3 activation and DNA fragmentation.[19] These observations suggest that IAP overexpression confers resistance to ischemic brain injury by blocking apoptosis.

Before gene therapy for stroke can be realized as a clinically useful therapy, several issues regarding this technique need to be addressed. First, since gene therapy would be conducted after the onset of a stroke, the question of whether post-ischemic delivery is efficacious must be addressed. Recent evidence indicates that delivery of a viral vector encoding an anti-apoptotic gene after an ischemic episode can reduce neuronal loss. For instance, intracerebral injection of a *bcl-2* HSV construct following an episode of cerebral ischemia has been shown to limit neuronal damage. This finding supports the contention that post-ischemic delivery of a viral vector may have utility in the treatment of stroke.

A second issue which merits attention is the route of administration of a viral construct. Intracerebral injection of adenoviral constructs, although effective in rescuing neurons in experimental animals, may not be a reasonable approach for use in humans due to the invasive nature of this technique. However, given that the blood-brain barrier is compromised following a stroke, it is possible that adenoviral vectors may enter the brain after systemic administration in individuals that have suffered a stroke.

A third matter relates to the non-specific nature of adenoviral infectivity. Current vector technology cannot target specific phenotypes such as neurons. This may prove to be a limiting factor for use of viral vectors to deliver non-secreted protein factors.

Lastly, although the central nervous system was once widely perceived to be an immunologically privileged site, it is clear that intracerebral administration of currently usedadenoviral vectors can trigger inflammation in the brain, including regions which are synaptically associated with the target area (reviewed in ref. 22). Attenuation of this confounding antigenic response to adenoviral gene delivery vectors has been attempted through drug-induced immunosuppression. These studies have suggested that co-administration of immunosuppressants can promote adenovirally mediated gene expression by blocking the humoral response to virus exposure. However, just as current vectors are less antigenic than previous generations, with the appropriate modifications future adenoviral constructs may not require adjunct drug therapy. Clearly, adaptation of this native adenoviral property will be required for clinically relevant adenovirus gene therapy.

In summary, recent advances in gene therapy for experimental stroke have generated promising data indicating that the introduction of anti-apoptotic genes into neurons can render vulnerable cells more resistant to ischemic injury. Functional assessment of these neurons reveals that IAP-induced neuroprotection is accompanied by a retention of normal biochemical and physiological activity. These findings support the potential utility of gene therapy for the treatment of stroke. However, more work remains to be done to resolve issues such as the delivery and safety of gene constructs before gene therapy for stroke becomes a reality.

IAP Gene Therapy for Optic Neurodegeneration

Inherited neurodegenerative disorders of the eye constitute a large and diverse group of diseases. Identification of specific genetic defects responsible for various disorders of the eye is proceeding rapidly and has revealed that many different mutations may underlie

the same clinical diagnosis. Retinitis pigmentosa (RP), for example, is a type of retinal degeneration characterized by progressive tunnel vision and night blindness, advancing to complete loss of vision. More than one hundred different mutations in over a dozen genes expressed by retinal photoreceptor cells or retinal pigment epithelium cells have been associated with RP thus far,[23,24] yet these genetic defects account for less than 50% of the cases of RP.[24] Numerous studies are in progress which are likely to identify genetic abnormalities underlying a variety of other eye diseases as well. This detailed knowledge of the genetic basis of many disorders of the eye would seem to make the eye a strong candidate for gene therapy. There are also other features of the eye which offer distinct advantages for a gene therapy approach. The eye has a well defined anatomy, is anatomically accessible and contains a comparatively smaller volume of tissue requiring treatment then many other organs. In addition, the translucent media within the eye allow for visual localization of the gene transfer process and therapeutic agents injected into the eye are less likely to spread to other parts of the body.

The feasibility of gene therapy for eye disease depends on the development of gene transfer vectors which can reliably deliver foreign DNA into target cells. In this regard, investigators have had success in introducing foreign genes and antisense oligonucleotides into cells of the eye using adenovirus-derived[25-27] and other viral and non-viral vectors.[28,29] These vectors vary with respect to degree of infectivity, induction of an inflammatory response, duration of gene expression and ability to target specific cell types. Given the rapid progress in this field, there is reason to believe that these features will be optimized in the near future.

The first step in the design of a protocol for gene therapy is to specify which genes to target. As other authors have pointed out,[30,31] a strategy of gene therapy aimed at correcting individual mutations causing eye disease may be difficult to implement due to the large number of mutations identified. In addition, many of these mutations result in autosomal dominant forms of disease, indicating that simply introducing a normal copy of the defective gene into cells would have little therapeutic value. An alternate strategy might be to target cellular mechanisms common to a variety of disorders.

A number of recent studies have indicated that in many disorders of the eye, including glaucoma,[30] retinal photoreceptor dystrophies,[23,24,32] ischemia[33] and optic nerve transection,[34] retinal cells die by apoptosis. In the case of glaucoma, the apoptosis of retinal ganglion cells is thought to be the consequence of pressure-induced ischemia or growth factor deprivation. Thus a strategy of gene therapy which targets apoptosis might prove to be a more practical approach because it would be applicable to a large number of different mutations. Given the recent success in reducing apoptosis in central neurons following ischemia by IAP overexpression[21] and the demonstration that the application of caspase inhibitors reduce apoptotic cell death in retinal ganglion cells following axotomy,[35] IAP-based gene therapy would seem to hold promise for the treatment of many forms of eye disease.

IAP Gene Therapy for Parkinson's Disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder which affects approximately 1 in 250 people over the age of 55. This condition is characterized by the selective loss of melanized dopamine neurons which comprise the substantia nigra pars compacta located in the ventral midbrain. Nigral neurons project to an area in the forebrain called the striatum, where dopamine plays an important role in regulating movement. Consequently, when levels of dopamine are depleted by more than 80% the clinical features of PD present as a resting tremor, bradykinesia, postural rigidity or instability and a shuffling gait.

Even though these features of PD were first described as a single syndrome by James Parkinson in 1817, an effective drug treatment for PD wasn't realized until 1967. It was discovered that administration of the L-isomer of DOPA, the precursor in the synthesis of dopamine, was able to replenish striatal dopamine levels and provide a clinically relevant therapy for the symptoms of PD.[36] In spite of this advance, L-DOPA therapy has been found to have limited long term utility, since L-DOPA does not abate the disease process, but rather temporarily ameliorates the symptoms of PD. Hence, the efficacy of L-DOPA therapy is reduced over time as the disease advances and there are fewer surviving nigral neurons to supply the necessary dopamine to the striatum. More recently, functional recovery through embryonic neural tissue grafts into the area of the substantia nigra or the denervated striatum have shown encouraging potential for a long term treatment for PD.[37] While this surgical approach has shown promise, complications such as host-graft rejection and profound graft cell death require further study in order to maximize the clinical efficacy of this treatment. Whereas current treatment regimes suffer limitations in technique or length of treatment efficacy, adenovirus-based gene therapy offers tremendous potential as a possible adjunct to present drug or transplant treatments and may even provide a new and independent, long term treatment for PD.

IAP-based adenoviral gene therapy for PD may be implemented in one of two ways, ex vivo or in vivo. For the ex vivo approach, fetal neurons which are to be implanted into the denervated striatum would be cultured and transfected with an IAP vector. Using this ex vivo gene therapy technique, reports employing glial cell line-derived neurotrophic factor (GDNF) have demonstrated alleviation of many of the symptoms by modest enhancement of neuronal graft survival in animal models of PD. While these studies have yielded encouraging results, poor graft survivability remains a limitation for this treatment approach. Incorporation of IAP proteins may remedy this obstacle by enhancing the tolerance of transplanted neurons to the rigors of the preparation and transplantation procedures. Perhaps the use of IAP gene therapy, in conjunction with a dopamine trophic factor like GDNF, may increase the potency of primary fetal neuron grafts, thereby improving the efficacy of this surgical treatment for PD.

There are several forms of Parkinsonism which likely have distinct etiologies. While several groups have recently identified candidate genes for the subtypes of juvenile, familial and typical (sporadic) PD, a gene therapy which targets the mechanism of dopamine neuron death in PD would negate the requirement for 'individual gene' therapies for each subtype of this neurodegenerative disorder. We have recently reported preliminary results[38,39] which demonstrate that direct intracerebral administration of IAP adenoviral vectors into rats can attenuate the loss of dopamine neurons following intrastriatal injection of 6-hydroxydopamine, a dopamine-specific neurotoxin. Similar results have been reported by several groups using GDNF adenoviral constructs when either pre-administered into the region of the substantia nigra[40] or striatum,[41] or administered into the nigra several weeks following a lesion.[42] While these results are encouraging, results from clinical trials using trophic factors suggest that caution should be exercised when interpreting the potential use of neurotrophic factors for the treatment of human disease conditions.[43] Therefore, since neither NAIP nor XIAP proteins have been associated with proliferative diseases, an IAP protein-based gene therapy for PD may offer a safe approach for the long term treatment of this disease while providing the prospect of treating a wide variety of Parkinson's disease etiologies.

In summary, any prospective gene therapy for PD should:

1. Attenuate the neuronal death in the substantia nigra;
2. Improve behavioral deficits through supplementing the denervated striatum with required factors (e.g., dopamine);

3. Increase the specificity and duration of vector derived gene expression;
4. Minimize the risk of iatrogenic complications; thereby
5. Reducing patient risk and the number of expensive medical interventions.

The potential successes for IAP-based gene therapy for Parkinson's disease, providing these guidelines could be achieved, would suggest that IAPs may serve as a therapeutic intervention on their own or in combination with other gene products, or perhaps as an ex vivo gene therapy adjunct to enhance present transplant techniques.

Prospects for IAP-Based Gene Therapy

Neurodegenerative diseases are a collection of heterogeneous pathologies with assorted etiologies. From this diversity has emerged the serendipitous realization that the mechanism of programmed cell death underlying many neurodegenerative conditions engages a final common pathway mediated by caspase activation. This point of convergence offers the possibility of novel treatments for a broad spectrum of neurodegenerative disorders based on the use of IAP proteins. Current efforts to optimize adenoviral vector technology are therefore likely to facilitate the clinical application of IAP-based gene therapy.

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Chapter 14

Adenovirus Vectors for Therapeutic Gene Transfer to Skeletal Muscles

Josephine Nalbantoglu, Basil J. Petrof, Rénald Gilbert, and George Karpati

Duchenne's muscular dystrophy (DMD) and the less severe Becker muscular dystrophy (BMD) result from mutations in the dystrophin gene on chromosome Xp21.[1] DMD, which afflicts approximately 1 in 3,500 male newborns, is fatal by the third decade of life in most patients, usually due to severe weakness of the diaphragm and other respiratory muscles. The subsarcolemmal protein dystrophin plays an important role in structural reinforcement of the muscle cell surface membrane by providing a structural linkage between cytoplasmic actin and the extracellular matrix, via binding of the dystrophin-associated protein complex (DPC) which spans the membrane of muscle fibers. Although precise function of dystrophin in muscle remains somewhat controversial, it is generally believed that skeletal muscles lacking dystrophin are abnormally susceptible to contraction-induced damage of the sarcolemma, which secondarily leads to muscle fiber dysfunction, necrosis and eventual replacement of the lost fibers by connective tissue.

So far, effective treatment is not available for DMD. A most promising approach is that of replacing dystrophin through gene transfer. Therapeutic strategies aimed at DMD have been tested mainly in the murine model of DMD, the mdx mouse. A chain-termination mutation in the dystrophin gene results in a truncated protein product which is rapidly degraded and thus is never localized to the subsarcolemmal region of the muscle fiber.[2] As in the human disease, the absence of dystrophin leads to the lack of the sarcolemmal dystrophin-associated protein complex composed of the dystroglycans, which no longer assemble at the surface. Although the mdx mouse remains a very useful model, mdx limb muscles do not reproduce all pathological aspects of DMD. Small caliber fibers in mdx muscles are relatively resistant to necrosis.[3] On the other hand, the mdx mouse diaphragm demonstrates major weakness and fibrosis from an early point in the animal's lifetime. In contrast to the mdx mouse, the dystrophin-deficient golden retriever dog model has a pathological profile similar to that of DMD.[4] In this model, the canine dystrophin gene has a point mutation in the consensus splice acceptor site of intron 6 which results in skipping of exon 7, reduced levels of dystrophin gene transcript and lack of dystrophin protein. These dogs have elevated serum creatine kinase activity, gross muscle atrophy, histologically detectable muscle fiber necrosis and concurrent regeneration, and cardiomyopathy.

Because of their ability to infect post-mitotic tissue and the relatively large insert capacity of vectors containing deletions in the E3 region, replication-defective adenoviruses have been the vectors of choice for transferring the dystrophin gene to muscle. The first generation Ad vectors, with an ~8 kb insert capacity, have been used to transfer a functional shortened version of the dystrophin gene, the so-called Becker dystrophin minigene, to the

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mdx mouse[5-7] and the dystrophin-deficient golden retriever dog.[8] In all studies, the protein was properly localized to the plasma membrane where it is normally located. Overexpression of the transgene and presence of cytoplasmic dystrophin were not toxic to the muscle fiber. Ad-mediated dystrophin gene transfer to neonate mdx mice protected muscle fibers, as evidenced histologically by a decrease in the number of centralized nuclei which are characteristic of the cycles of necrosis/regeneration occurring in dystrophic muscle.[6,7] Ad-mediated gene transfer also restored dystrophin-associated proteins to the sarcolemma. Furthermore, in the transduced fibers, there was a reversal of the usual loss of maximum force generation observed in mdx muscle.[9]

Determinations of muscle force production provide a sensitive and clinically important measure of potential adverse effects of Ad-mediated gene transfer on muscle cell function. Not only in neonate but also in adult mdx soleus muscles, Ad-mediated dystrophin minigene transfer is capable of alleviating the loss of force-generating capacity as well as the histopathological evidence of disease progression normally seen.[10] However this can only be achieved provided that optimal Ad particle titer is first established to prevent early myofiber toxicity,[11] and effective immunosuppression is also given to abrogate the later CTL response.[10] These observations point to potential problems, both non-immune and immune-related, which have to be overcome for successful use of adenoviral vectors in delivering therapeutic genes to muscle.

A major limitation of the usefulness of first generation Ad has been the transient nature of transgene expression: Although expression can be maintained after injection of Ad into skeletal muscles of immunologically immature neonates, in immunocompetent adult animals, transgene expression is eliminated 2-4 weeks after Ad delivery.[7,12] Long term persistence of transgene expression in adult animals has been attained only after Ad injection into muscles of immunodeficient nude[12] and severe combined immunodeficiency (SCID) mice.[7,13] In adult animals, there is evidence of a cellular and humoral immune attack directed against Ad-transduced muscle fibers and the vector, respectively. The problem of loss of transduced cells has generally been attributed to a CTL response directed against adenoviral antigens presented to the surface of transduced cells by MHC class I molecules.[14] Low level ("leaky") de novo expression of adenoviral genes has been demonstrated in Ad-infected cells including muscle, despite deletion of the E1A region from the vector.[14] Although substantial progress has been made in developing less immunogenic vectors through the inactivation (e.g., ref. 15) or deletion of all viral genome elements (e.g., refs. 16-18), this approach has at least two inherent limitations with respect to the treatment of monogenic recessive disorders such as DMD. First, the therapeutic transgene protein product would itself represent a neoantigen that could, depending upon its own intrinsic immunogenicity, stimulate host cellular immunity with attendant elimination of Ad-infected cells. Second, the capsid proteins still evoke the generation of virus neutralizing antibodies that preclude effective readminstration; it is doubtful that this problem can be overcome with further modification of the vector genome. In this regard, the immunosupressant FK506, which blocks T cell signaling by calcineurin, significantly increased the level and persistence of dystrophin gene expression after a single delivery of Ad to muscles of adult mdx mice.[19] However, FK506 was only partially effective in blocking the generation of antibodies against adenoviral capsid proteins and permitting further dystrophin gene expression after a second Ad injection.[19] In contrast, the combination of FK506 plus CTLA4Ig abrogated the immune response against adenovirus proteins and dystrophin to a degree not achievable with the use of either agent alone.[20] At 30 days after Ad injection, >90% of myofibers could be found to express dystrophin with little or no evidence of a cellular immune response against transduced fibers (Fig. 14.1). In addition, the humoral immune response was

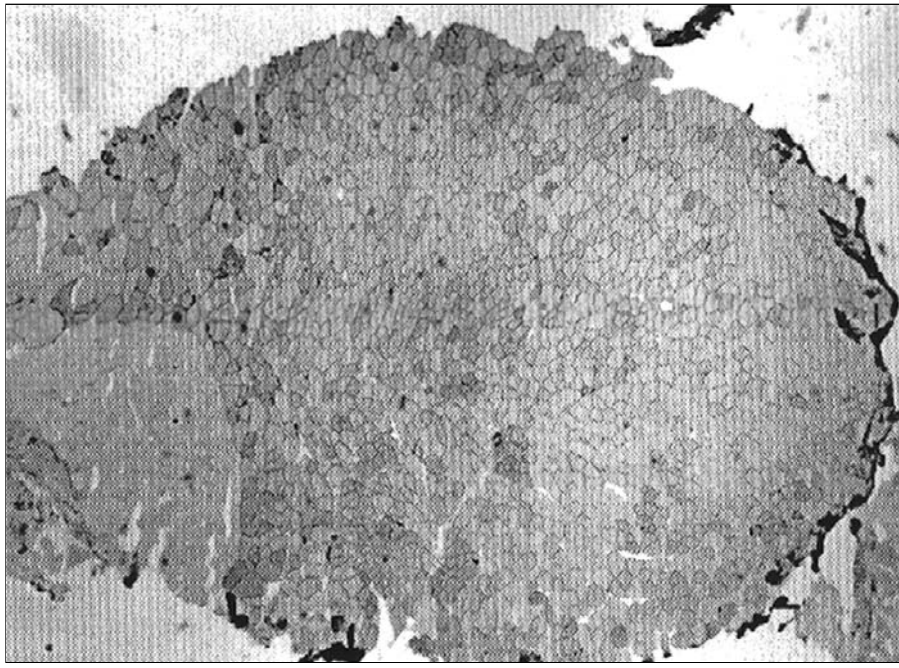

Fig. 14.1. Representative micrograph of adult mdx muscles 30 days after Ad-mediated dystrophin gene transfer in mice treated with FK506 and CTLA4Ig. Dystrophin immunocytochemistry shows the typical subsarcolemmal localization of the dystrophin minigene. Note that overexpression results in cytoplasmic staining in some fibers. The majority of the muscle fibers express recombinant dystrophin and there is minimal inflammatory cell infiltration.

markedly suppressed, and, importantly, this was associated with increased transduction efficiency following vector administration.[20]

In skeletal muscle, in particular, it has been suggested that adenoviral antigens are of little importance in generating a cellular immune response against transduced fibers.[21,22] This conclusion was based upon the observation that animals showing natural immunological tolerance to transgene-encoded proteins did not demonstrate destructive immune responses against Ad-infected myofibers. Furthermore, complete persistence of transgene expression could not be achieved even with adenoviral vectors that are deleted of all viral genes.[18,23] In these cases also, best long term expression was obtained in animals tolerized to the transgene.[24] In this regard, a particularly noteworthy finding is the immunogenic nature of dystrophin protein when expressed in adult mdx mice using Ad.[19,20] Therefore, in the case of DMD, replacing dystrophin would necessarily involve introducing a neoantigen to the host immune system irrespective of the vector used. Although this problem could be dealt with through host immunosuppression, a more attractive option would be to employ an alternative non-immunogenic therapeutic transgene. Utrophin, the autosomally-encoded homolog of dystrophin,[25] is a promising candidate for such an approach: It is expressed in the muscle of DMD patients and is therefore not a foreign protein in the setting of dystrophin deficiency. Functionally, dystrophin and utrophin have in common four structural domains and, impotantly, utrophin appears to be capable of providing structural linkage between

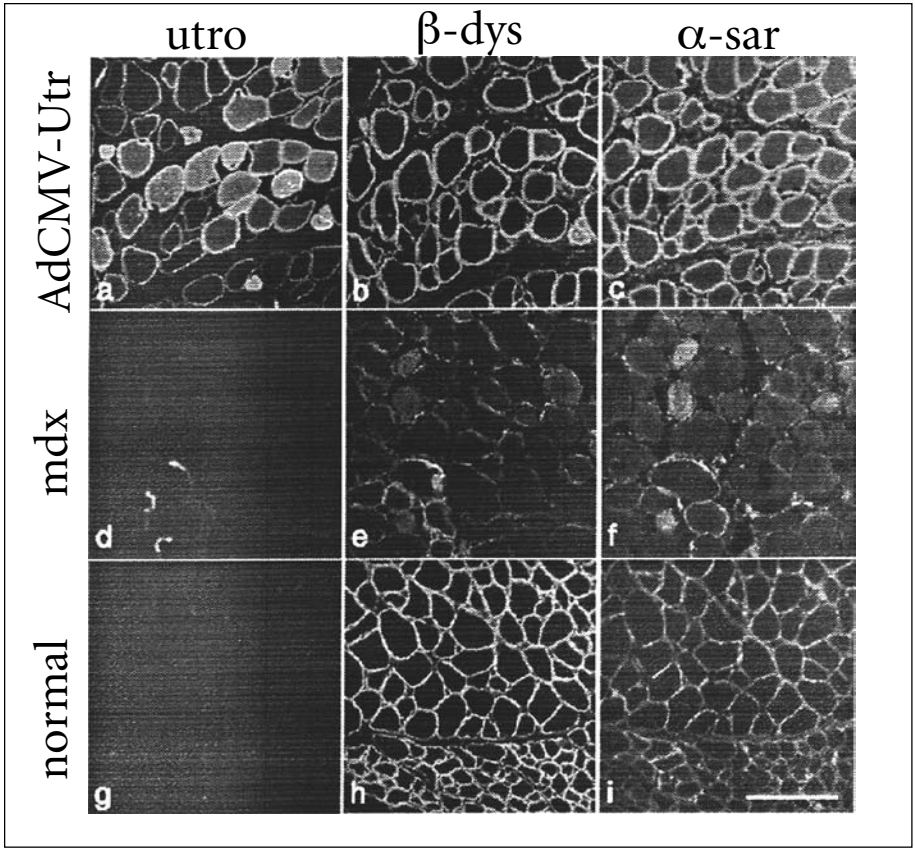

Fig. 14.2. Overexpression of utrophin restores the distribution of the DPC in the plasma membrane of dystrophic muscle. Consecutive serial sections of muscles of mdx mouse injected with Ad-utrophin (a-c); uninjected mdx (d-f); and uninjected normal mice (g-i) were processed simultaneously under the same conditions for immunofluorescence using monoclonal antibodies specific for utrophin (a, d, g), β-dystroglycan (b, e, h), and α-sarcoglycan (c, f, i). In an uninjected mdx muscle, only the post-junctional regions of muscle fibers show utrophin immunoreactivity (d). Bar = 75 μ.

cytoplasmic actin and the extracellular matrix. In mdx mice injected with a utrophin-expressing Ad,[26] the dystrophin-associated protein complex is restored to the sarcolemma (Fig. 14.2).

A major issue in gene therapy approaches to disease is efficient and widespread delivery of the therapeutic gene to all affected tissues. To date, there are no identified receptor molecules that are unique to the surface of the muscle fiber. Therefore, modification of the adenovirus fiber protein for specific targeting to muscle is not possible. However, transgene expression can be restricted to muscle by the use of muscle-specific promoters such as that of the muscle creatine kinase (MCK). Upstream regulatory sequences consisting of 1350 base pairs of the MCK gene can function in a muscle-specific manner in vivo in the context of an adenoviral vector.[27]

Significant levels of adenovirus-mediated gene transfer occur only in immature muscle[28-30] or in regenerating muscle,[7] indicating that a developmentally regulated event plays a major role in limiting transgene expression in mature skeletal muscle. A high affinity receptor for adenovirus type 5 (Ad5) has recently been identified, cloned, and shown to be a receptor shared by coxsackie B virus (coxsackie and adenovirus receptor, CAR).[31,32] A second receptor has also been postulated to play a role in adenovirus binding: The α_2 domain of MHC class I was identified as a high affinity receptor for Ad5 fiber knob using reverse antibody biopanning of a phage-displayed hexapeptide library with two virus neutralizing antibodies.[33] In developing mouse muscle, although MHC levels are not altered, CAR expression is severely downregulated during muscle maturation.[34] These results suggest that lack of expression of the primary Ad receptor CAR may limit efficacy of Ad-mediated gene transfer to matureskeletal muscle. In support of this, when CAR levels are increased in muscle cells, their susceptibility to Ad-mediated gene transfer is markedly enhanced. It is possible that inintact, mature skeletal muscle, several factors, including the extensive basal lamina surrounding mature myofibers, may limit the access of exogenously introduced virus to the muscle fiber plasma membrane. However, prior modulation of the level of CAR expression will be required to have efficient transducibility by Ad. Moreover, modulation of CARexpression levels in mature muscle may also counter the detrimental effect of directinjection of high titers of recombinant Ad into muscle.[11]

To date, the vast majority of investigations involving Ad-mediated gene transfer to skeletal muscle have employed direct intramuscular injection of Ad. A major limitation of this approach is the restricted diffusion of vector particles within Ad-injected muscles such that therapeutic gene expression remains confined to a small area surrounding the injection site (1-3 mm). In addition, direct injection of relatively inaccessible muscles such as the diaphragm with Ad would be technically difficult and clinically cumbersome in DMD patients. Although Ad injected into the systemic venous circulation tends to be mostly expressed in liver, local intra-arterial administration coupled with vasodilation holds promise for enabling regional delivery (Petrof BJ, unpublished results).

The ultimate goal of gene therapy for DMD is prevention of the progressive deterioration in muscle contractile function that generally leads to premature death as a result of respiratory muscle failure. It is clear from the above that Ad-mediated dystrophin or utrophin gene transfer remains a promising therapeutic approach for DMD.

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CHAPTER 15

Adenovirus-Mediated Gene Transfer: Applications in Lipoprotein Research

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In the last decade adenovirus-mediated in vivo gene transfer has been effectively utilized to enhance our understanding of the role that different apolipoproteins, enzymes, receptors and transfer proteins play in lipoprotein metabolism, as well as to identify potential candidate genes that modify the atherogenic lipid profile and modulate the development of atherosclerosis. The feasibility of using recombinant adenovirus for the transient correction of metabolic defects in different animal models of hyperlipidemia and atherosclerosis has been demonstrated. The field awaits the development of new adenovirus vectors that will permit safe, long term expression of a transgene at physiologic levels for human application.

Analysis of Gene Function in Lipoprotein Metabolism

Somatic gene transfer using recombinant adenovirus has been succesfully utilized to investigate and/or confirm the function of different proteins in specific pathways of lipoprotein metabolism. Thus, the important role of apolipoprotein (apo) A-I , the major structural protein of the antiatherogenic high density lipoproteins (HDL), in HDL metabolism was confirmed by expression of human apolipoprotein (apo) A-I in BALB/c mice using first generation recombinant adenovirus.[1] ApoA-I levels (168 ± 60 mg/dl) similar to normal human plasma levels were achieved in BALB/c mice. Like transgenic animal studies, apoA-I expression led to significant increases in the baseline plasma concentrations of total and HDL cholesterol, suggesting a potential strategy for the modification of the atherogenic lipoprotein profile to reduce the risk of atherosclerosis in man. Similarly, in the absence of human patients or animal models with deficiency of phospholipid transfer protein (PLTP), its role in high density lipoprotein (HDL) metabolism was investigated by expressing PLTP in C57BL/6 mice using recombinant adenovirus.[2] PLTP expression enhanced the catabolism of HDL, reducing baseline plasma cholesterol, phospholipids and HDL cholesterol as well as apolipoprotein (apo) A-I levels and led to the accumulation of lipid-poor apoA-I. These findings provided new in vivo evidence supporting the proposed role of PLTP in mediating the transfer of phospholipids between HDL and very low density lipoproteins (VLDL), accelerating the hepatic uptake of HDL surface and core lipids and ultimately, modulating the process of reverse cholesterol transport.

The physiological relevance of two major lipoprotein receptors described in the past several years, the low density lipoprotein receptor-related protein (LRP) and scavenger receptor class B, type I (SR-BI), was first investigated using recombinant adenovirus. Hepatic overexpression of SR-BI, a novel receptor proposed to mediate the selective uptake of cholesterol from HDL, led to enhanced HDL catabolism and a virtual disappearance of

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plasma HDL and apoA-I, as well as an increase in biliary cholesterol in C57BL/6 mice,[3] establishing SR-BI as an important new receptor that modulates plasma HDL concentrations. In similar studies, expression of receptor associated protein (RAP), a dominant negative regulator of LRP function, transiently inactivated LRP in control and LDL receptor-deficient mice.[4] Embryonic lethality in mice lacking LRP had previously prevented evaluation of the proposed role of this receptor in chylomicron remnant removal. The inactivation of LRP by RAP was associated with accumulation of chylomicron remnants in both animal models, establishing the importance of both LRP as well as other potential RAP-sensitive receptor pathways in remnant lipoprotein clearance. Adenovirus-mediated gene transfer of RAP has also demonstrated that RAP-sensitive receptor pathways are involved in the clearance of apoE2 and apoE3-Leiden VLDL in apoE2 and apoE3-Leiden transgenic mice[5] as well as the inhibition of VLDL clearance by apoC-I in apoC-I/LDL receptor-deficient mice.[6]

Thus, the application of recombinant adenovirus methodology to the study of protein function in vivo has markedly enhanced our understanding of the role that different apolipoproteins and enzymes, as well as receptors, play in lipoprotein metabolism.

Gene Replacement Therapy in Animal Models of Hyperlipidemia and Atherosclerosis

The potential use of recombinant adenovirus for the treatment of human monogenic disorders of lipoprotein metabolism has been evaluated by adenovirus-mediated transient gene replacement in different animal models of hyperlipidemia and atherosclerosis. ApoE-deficient mice are characterized by severe hypercholesterolemia, moderate hypertriglyceridemia and the spontaneous development of aortic atherosclerosis.[7,8] Replacement of the human apoE gene, a ligand for both the LDL receptor and the LDL-related receptor (LRP), has been performed in apoE-deficient mice by utilizing both first generation[9,10] and second generation temperature-sensitive adenovirus.[11] Expression of the major apoE3 isoform, with peak levels ranging from 1.5-600 mg/dl was detected one and three months after infusion of first and second generation adenovirus, respectively. ApoE expression led to an 80% reduction in the plasma cholesterol concentrations in apoE-deficient mice, with baseline values of 800 mg/dl on a regular diet and of 1401 mg/dl on a Western diet. A shift in the plasma lipoprotein distribution from primarily VLDL and LDL to a less atherogenic profile consisting predominantly of HDL was observed. Similar results were obtained with expression of apoE4 but not apoE2, indicating that the abnormal apoE2 isoform had less effect on lipoprotein levels.[11] Transient expression of human apoE3 for a period of one month was sufficient to markedly reduce the mean aortic lesion size in apoE-deficient mice, by approximately 62%.[10]

Gene replacement therapy using recombinant adenovirus to reconstitute LDL receptor expression in the liver has been evaluated in mouse and rabbit models of familial hypercholesterolemia (FH), a genetic disorder characterized by marked elevations in the plasma concentrations of total and LDL-cholesterol and the development of premature atherosclerosis in man. Replacement of the LDL receptor in normal BALB/c and C57BL/6 mice[12] as well as LDL receptor-deficient mice[13] led to significant reductions in plasma total and LDL cholesterol levels due to enhanced catabolism of apoB-containing lipoproteins. Similarly, gene transfer of the human[14,15] or rabbit LDL[16] receptor cDNA into the LDL receptor-deficient rabbits resulted in a ten-fold increase in hepatic LDL receptor expression and a 50-70% reduction in the baseline (500-800 mg/dl) plasma cholesterol levels. Interestingly, these animals also demonstrated a 300-400% increase in the plasma concentrations of HDL cholesterol and apoA-I, indicating an inverse relationship between plasma LDL and HDL cholesterol levels.[16] In all three studies, expression of the recombinant LDL receptor gene and its associated effect on serum lipids was transient, lasting less than

three weeks. Thus, adenovirus-mediated transient expression of the human LDL receptor reversed the hypercholesterolemic effects of LDL receptor deficiency in both mouse and rabbit models.

Recombinant adenovirus vectors have also been utilized to express intracellular as well as circulating enzymes that modulate the transport and metabolism of cholesterol and triglycerides. Unlike the plasma apolipoproteins, the required concentrations for enzyme function are in the ng-μg/ml range. However, because their site of synthesis can be different from their site of function, the replacement of these genes may be more complex. Thus, expression of either hepatic lipase (HL) or lipoprotein lipase (LPL), the two major lipolytic enzymes, require transport from their site of synthesis, either in the liver (HL) or in adipocytes and smooth muscle (LPL), to the vascular endothelium for hydrolysis of triglycerides and phospholipids present in circulating plasma lipoproteins. Adenovirus-mediated expression of human HL resulted in complete correction of the abnormal lipoprotein profile in HL-deficient mice, with a 50-80% reduction in total and HDL cholesterol.[17] Most importantly, 97% of the newly synthesized HL was heparin releasable, indicating that the human enzyme, although synthesized primarily by the liver, was virtually totally bound to the mouse vascular endothelium, the site of normal enzyme function. Similarly, Excoffon et al[18] demonstrated that ectopic liver expression of LPL transiently corrected the hypertriglyceridemia and impaired fat tolerance in heterozygous LPL deficient mice.

These combined studies demonstrate the feasibility of using adenovirus to transiently replace circulating plasma apolipoproteins, receptors and intracellular as well as endothelial-bound lipolytic enzymes in a wide range of physiological concentrations to normalize the lipid profile, as well as to modulate the development of atherosclerosis in different animal models for the human genetic dyslipoproteinemias.

Expression of Genes that Modulate Lipid Metabolism by Enhancing Alternative Lipoprotein Pathways

Partial or complete correction of the hyperlipidemia in different animal models of atherosclerosis can be also achieved by expression of genes that enhance alternative metabolic pathways which may compensate for the primary gene defect. In one study[19] a temperature-sensitive second generation recombinant adenovirus was utilized to transfer the human apoA-I gene in apoE-deficient and LDL receptor-deficient mice. Prolonged expression of human apoA-I (6 weeks to 8 months) with mean peak plasma levels of 235 and 324 mg/dl, respectively, increased plasma HDL cholesterol in both animal models, as well as reduced plasma LDL cholesterol in LDL receptor-deficient mice. Expression of apoA-I improved the atherogenic lipoprotein profile present in mice with apoE and LDL receptor deficiency by reducing the LDL to HDL ratio. Interestingly, the apoA-I concentrations, as well as the duration of apoA-I expression, were highly dependent on the type of hyperlipidemia present in the animal model. In separate studies, Amar et al[20] demonstrated significant reduction of aortic atherosclerosis, with a decrease in the mean aortic lesion area size by 43% after only transient, one month expression of human apoA-I in apoE-deficient mice. Similarly, transfer of LPL, the rate-limiting enzyme for the hydrolysis of triglyceride-rich lipoproteins, decreased plasma total cholesterol and triglycerides, as well as VLDL/chylomicron remnant cholesterol and triglycerides in both apoE and LDL receptor-deficient mice.[21] Thus, partial correction of not only the abnormal lipid profile but also atherosclerosis was achieved by enhancing either the HDL or lipolytic pathways in animal models whose primary defect involves the clearance of LDL or remnant lipoproteins.

The VLDL receptor is homologous to the LDL receptor, but has a different expression pattern, as well as a different ligand specificity. The VLDL receptor, normally expressed in nonhepatic tissues, appears to mediate the uptake of VLDL, the precursor to LDL, but it is

not believed to substantially regulate plasma lipoprotein levels in vivo. Kozarsky et al[22] and Kobayashi et al[23] evaluated the transfer of the VLDL receptor gene to the liver as a possible therapeutic intervention in LDL receptor-deficient mice. Greater than 50% reduction in the baseline plasma levels of total, VLDL, IDL and LDL cholesterol and apoB were achieved, reflecting the enhanced clearance of radiolabeled VLDL and LDL. Similar findings were observed by adenovirus-mediated expression of the human VLDL receptor in apoE2 and apoE-Leiden transgenic mice, two other mouse models of severe hypercholsterolemia.[24] Thus, both studies demonstrated reversal of the hypercholesterolemia in apoE or LDL receptor-deficient mice by hepatic VLDL receptor gene transfer. Interestingly, although the decrease in plasma cholesterol levels were similar in magnitude to the declines seen in LDL receptor adenovirus-infused mice, plasma cholesterol levels in VLDL receptor-expressing mice did not return to baseline by 3 weeks, but remained low through 9 weeks, according to Kozarsky et al,[22] leading to prolonged improvement of the hypercholesterolemia. One potential explanation for this finding is that animals infused with the VLDL receptor adenovirus failed to produce transgene-specific CTLs or antibodies and by inference did not activate T helper cells. Thus, transfer of a gene already expressed in the recipient animal model may circumvent immune response to the therapeutic gene product seen in replacement therapy of deficiency states, but still permit correction of the metabolic defect by enhancing alternative metabolic pathways.

In other studies, transient upregulation of bile acid synthesis by direct transfer of 7-α-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis, restored hepatic LDL receptor expression in Syrian hamsters fed a chow or Western diet,[25] leading to a 60-75% decrease in the baseline LDL cholesterol concentrations and an improvement of the atherogenic plasma lipoprotein profile. Adenovirus-mediated expression of another intracellular enzyme, apoBEC-1, involved in the editing of the mRNA for apoB100, the major ligand for the LDL receptor, to apoB48 mRNA, in C57BL/6 mice,[26] human apoB/apo(a) transgenic mice[27] and New Zealand White[27] or LDL receptor-deficient rabbits[28] led to significant editing activity in the liver and reduction of LDL cholesterol[26-28] and of Lp(a).[24] Finally, adenovirus-mediated gene transfer has been utilized to investigate the proposed coordinate role of hepatic lipase (HL) , a major lipolytic enzyme that hydrolyzes triglycerides and phospholipids in HDL, and lecithin cholesteryl-acyltransferase (LCAT), the key enzyme that esterifies free cholesterol present in HDL, in HDL metabolism in vivo.[29] Expression of HL in LCAT transgenic mice led to significant reductions in baseline plasma cholesterol, phospholipids and HDL cholesterol (>60%; all) as well as the formation of more homogeneous HDL, reversing the heterogeneous HDL profile in LCAT transgenic mice. These findings support a role for both HL and LCAT in modulating HDL levels, heterogeneity and function, which may ultimately affect the ability of transgenic mouse HDL to function in reverse cholesterol transport.

Structure-Function Analysis of Proteins Modulating Lipoprotein Metabolism

Our understanding of the functional and structural properties of different enzymes, receptors and transfer proteins involved in lipid metabolism have been to a large degree based on studies performed in different in vitro expression systems. However, our ability to extrapolate in vitro structure-function studies to in vivo physiological situations may be limited. Recent studies have demonstrated the feasibility of using recombinant adenovirus to express native and mutant proteins in different animal models of hyperlipidemias, permitting direct, structure-function analysis in vivo.

Kobayashi et al[30] first utilized this approach to identify potential structural domains in HL and LPL that could confer the different phospholipase functions of the two enzymes.

Thus, HL-deficient mice were injected with adenovirus expressing either native HL, native LPL or lipase mutants in which the lid covering the catalytic site of the either enzyme was exchanged. Preferential in vivo hydrolysis phospholipids were demonstrated in animals expressing either native or mutant lipases in which the lid of HL but not of LPL was present. Through these in vivo studies, the lipase lid was identified as a major structural motif responsible for conferring the different phospholipase activities between LPL and HL.

Using a similar approach, Amar et al[31] investigated the potential non-lipolytic role of HL in the metabolism of remnant lipoproteins. In addition to its classic function as a lipolytic enzyme, recent in vitro data had suggested a role of HL as a direct ligand that enhances the interaction and uptake of different lipoproteins by cell surface receptors and proteoglycans. Expression of both native HL and a mutant, catalytically inactive HL145G in apoE-deficient mice led to similar reductions in plasma total and remnant lipoprotein cholesterol, providing definitive in vivo data supporting a role for HL in lipoprotein metabolism independent of its lipolytic function. In separate studies, expression of native as well as apoBEC-1 mutants in C57BL/6 mice[32] provided additional in vivo evidence that apoBEC-1 functions as a dimer in RNA editing.

These combined studies have demonstrated the usefulness of adenovirus-mediated gene transfer for the study of the structure and function of different proteins that may modulate lipoprotein metabolism, as well as the development of atherosclerosis in different animal models (Table 15.1). Potential cautionary notes in interpreting this data include the transfer of human rather than animal transgenes that may encode proteins with different specifities and function from that of the recipient animals, the high levels of expression achieved in adenovirus studies which often exceed the physiological range, the hepatotrophic properties of the virus which may result in ectopic transgene expression in the liver, and the short duration of gene expression that may preclude achievement of steady state during metabolic studies. Despite these limitations, the phenotypic changes induced by transient gene expression in different animal models have been, for the most part, consistent with subsequent findings in transgenic animal models achieving long term expression. Thus, adenovirus-mediated transient gene expression appears to be a valuable tool for the rapid evaluation of the structure and function of different transgenes in lipoprotein metabolism, the identification of potential candidate genes for the treatment of atherosclerosis and the feasibility of gene replacement in different animal models for the human genetic dyslipoproteinemias.

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Table 15.1.

Transgene	Animal Model*	Findings	References
Apolipoproteins			
ApoA-I	BALB/c	↑ TC, ↑ HDL-C	**(1)**
	ApoE k/o, LDL-R k/o C57BL/6	↑ TC, ↑ HDL-C; improved atherogenic lipid profile	(19)
	ApoE k/o	↑ TC, ↑ HDL-C, ↓ atherosclerosis	(20)
ApoE	ApoE k/o	Transient correction of atherogenic lipid profile	(9)
	ApoE k/o	Transient correction of atherogenic lipid profile; ↓ atherosclerosis	(10)
Enzymes and Transfer Proteins			
HL	HL k/o	Transient correction of the abnormal lipid profile	(17)
	LCAT Tg	Analysis of the coordinate role of HL and LCAT in modulating the heterogeneity, function and levels of HDL	(29)
HL lid mutant	HL k/o	Identification of lipase lid as a major structural motif confering phospholipase function	(30)
HL 145G	ApoE k/o	Analysis of the role of HL in Lp metabolism independent of lipolysis	(31)

Table 15.1. con't.

Transgene	Animal Model	Findings	References
Enzymes and Transfer Proteins, cont.			
LPL	LPL k/o	Transient correction of the lipid profile and impaired fat tolerance	(18)
	ApoE k/o, LDL-R k/o	↓ TC, ↓ TG, ↓ VLDL/chylo remnants; improved atherogenic lipid profile	(21)
LPL lid mutant	HL k/o	Identification of lipase lid as a major structural motif confering phospholipase function	(30)
7-a-hydroxylase	Syrian hamster	↓ TC, ↓ HDL, ↓ LDL-C; improved atherogenic lipid profile	(25)
ApoBec-1	C57BL/6, NZW rabbit LDL-R k/o rabbit	↓ LDL-C; ↑ editing activity	(26-28)
	ApoB/apo(a) Tg	↓ TC, ↓ TG, ↓ VLDL, ↓ HDL, ↓ Lp(a), ↓ LDL-C; ↑ editing activity	(27)
ApoBEC-1 mutants	C57BL/6	↓ TC, ↓ TG, ↓ LDL; analysis of apoBEC-1 function as a dimer	(32)
PLTP	C57BL/6	↓ TC, ↓ PL, ↓ HDL-C, ↓ apoA-I, ↓ApoA-II, ↑ catabolism of HDL; PLTP mediates transfer of PL between HDL and VLDL	(2)

Table 15.1. con't.

Transgene	Animal Model	Findings	References
Receptors			
LDL-R	BALB/c, C57BL/6	↓ TC, ↓ LDL-C, ↑ catabolism of ApoB-containing Lp	(12)
	LDL-R k/o	↓ TC, ↓ LDL-C and ↑ catabolism of ApoB-containing Lp	(13)
	LDL-R k/o rabbit	↓ TC, ↑ HDL-C, ↑ ApoA-I	(14,15)
LDL-R (rabbit)	LDL-R k/o rabbit	↓ TC, ↑ HDL-C, ↑ ApoA-I	(16)
VLDL-R	LDL-R k/o	↓ TC, ↓ VLDL-C,↓ IDL-C, ↓ LDL-C, and apoB; ↑ clearance of VLDL	(22,30)
VLDL-R	ApoE2 and ApoE3 Leiden Tg	↓ TC, ↓ VLDL-C, ↓ IDL-C, ↓ LDL-C, and apoB; ↑ clearance of VLDL	(24)
SR-BI	C57BL/6	↓ TC, ↓ HDL-C, ↓ apoA-I; ↑ HDL-C catabolism establishing a role for SR-BI as an HDL receptor	(3)
Others			
RAP	LDL-R k/o, C57BL/6	Analysis of LRP function in chylo metabolism by RAP-mediated transient inactivation of LRP	(4)
	APoE2 and ApoE3 Leiden Tg	Analysis of the role of RAP sensitive pathways in ApoE2/E3 Leiden-mediated VLDL metabolism	(5)
	ApoC-I Tg/ LDL-R k/o	Analysis of the role of RAP in apoC-I mediated inhibition of VLDL clearance	(6)

*Unless otherwise specified, the animal model indicated refers to mice. Tg = transgenic; k/o = knockout; Lp = lipoprotein; chylo = chylomicron, -R = receptor

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CHAPTER 16

Correction of Serum Protein Deficiencies with Recombinant Adenoviral Vectors

James N. Higginbotham and Prem Seth

There are numerous inherited and acquired serum protein deficiencies that cause moderate to severe and even life threatening diseases in people. Some of the diseases include α_1-antitrypsin deficiency, hemophilias A and B, diabetes mellitus, and anemia. A number of these diseases have been treated by repeated subcutaneous injections of purified recombinant proteins; however, such therapies are generally expensive and inconvenient to the patient or in some cases not enough of the recombinant protein is available for prophylactic treatment. It is important that any serum protein deficiency to be studied have an accurate and reproducible assay by which the patient or physician can monitor serum levels for effective dosing. Because of problems of recombinant protein replacement, much attention has focused on the development of gene-based therapies. The use of adenoviral vectors to deliver genes encoding serum proteins has been extensively reported. Perhaps the most important advantage of adenoviral vectors is the extremely wide range of host cells they can efficiently transduce. The wide tropism of adenoviral vectors allows the use of numerous target tissues or organs like liver, lung, and muscle. Equally important is their independence of cell cycle for efficient gene transfer and expression.

α_1-antitrypsin Deficiency

α_1-antitrypsin is the major serine proteinase inhibitor (serpin) in the blood, whose major function is to inhibit neutrophil elastase. α_1-antitrypsin is a 52 kDa glycoprotein produced by hepatocytes and mononuclear phagocytes and whose sugar moieties are essential for stability in serum. The most aggressive form of α_1-antitrypsin deficiency results from inheritance of two null alleles that represents a functional "knockout" of α_1-antitrypsin. Patients with the "knockout" usually present with emphysema, which is panacinar and predominantly lower lobar, by their 30s. The most common mutant allele is PiZ, with a frequency of 0.02 in Caucasians. α_1-antitrypsin deficiency ZZ mutants in the United States are estimated at 1 in 800 for Caucasians, with significantly reduced chance of being alive by age 50, that is, 52% compared with 93% for the general public. Smokers with the ZZ mutation have an additional 10 year reduction in life expectancy. It has been estimated that less than 20% of the normal serum α_1-antitrypsin level of 2 mg/ml is necessary for adequate protection of the respiratory epithelium.[4]

One of the first reports of recombinant adenoviruses encoding a human serum protein was that of Gilardi[1] et al who constructed a first generation recombinant adenoviral vector

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having E_1 and E_3 deleted and the α_1-antitrypsin transcription unit inserted into E_1 region. The transcription unit consisted of the major late promoter and the tripartite leader followed by the human α_1-antitrypsin cDNA and SV40 polyadenylation signal. They demonstrated production of α_1-antitrypsin in vitro in HeLa and 293 cells infected with the recombinant adenoviral vector and indicated peak production of 60 μg/ml in 1 x 10^7 HeLa cells infected with an moi of 100, six days post-infection. Direct administration of this vector into the lungs and liver (through the portal vein) of cotton rats produced low levels of α_1-antitrypsin protein, which was secreted into the blood.[2,3] The low level of expression indicated a low transduction efficiency of the vector or inability of the adenovirus type 2 major late promoter to drive the α_1-antitrypsin cDNA. A more recent report of Kay and coworkers shows therapeutic levels of α_1-antitrypsin at about 700 μg/ml of mouse serum using an adenoviral vector that was E_1^-/E_3^+.[4] The potential importance of promoter choice is noteworthy in efficient transgene expression within a given species and target tissue. These investigators[4] demonstrated about a 200-fold difference in α_1-antitrypsin expression when comparing in vitro hepatoma cells and in vivo hepatocytes transduced with two adenoviral vectors differing only in their promoters. The transcription unit that consisted of the phosphoglycerol kinase (PGK) promoter, human α_1-antitrypsin cDNA, and bovine growth hormone polyadenylation signal was 20-fold more active in vitro than a similar transcription unit driven by the RSV LTR. Interestingly the RSVLTR-driven transcription unit was 10-fold more active in vivo. The next important steps will be to demonstrate phenotypic correction of a null α_1-antitrypsin mouse model and correction in a large animal model.

Factor VIII and Factor IX Deficiency

Hemophilias A and B are attractive diseases to pursue for gene therapy. Hemophilia A, also known as Christmas disease, is an X-linked bleeding disorder that results in a deficiency in clotting factor VIII (FVIII), with an incidence of about 1 in 5,000 males. The disease is characterized by frequent spontaneous and prolonged bleeding events. Of particular concern are the numerous joint bleeds that regularly develop into crippling arthropathies. Current treatment relies on injection of recombinant FVIII during bleeding episodes rather than prophylactic treatment because of high cost, limited supply, and short in vivo half life of FVIII. It is important to note that FVIII requires complexation with von Willebrand factor to remain stable in serum.

Hemophilia A has been thought to be an attractive target for gene therapy because FVIII expression need not be tightly regulated and does not require tissue-specific expression as long as the protein has access to the blood. It is also noteworthy that as little as 4-5% of normal levels (100-200 ng/ml) of FVIII may be therapeutic. However, a number of details make FVIII a difficult candidate for successful gene therapy. FVIII is a large gene in excess of 186 kb with a cDNA of over 9 kb. This large cDNA is not accommodated by most viral vectors and therefore requires deletion of the nonessential B domain to generate a smaller cDNA. This picture is further complicated by the lack of stability of FVIII in serum, and that 1 out of 5 hemophiliacs develops inhibitors to FVIII.

Despite the difficulties of FVIII gene therapy, a number of recent reports demonstrate therapeutic levels of human FVIII in canine and murine models by adenoviruses (see Table 16.1).[5,6] A recent report showed a ten-fold increase in expression of FVIII by including an untranslated exon and intron upstream of the FVIII cDNA.[7] In the same report, the authors confirmed tissue-specific expression by using the liver-specific mouse albumin promoter. In another report, sustained expression was shown using 8-fold lower doses of adenoviral vector that were "less hepatotoxic."[8] While it seems clear that high doses of adenoviral vector result in premature clearance of the vector, it is less clear that their observed persistent expression is not a strain-specific occurrence (see Table 16.1). Barr and coworkers have

Table 16.1. Adenovirally-delivered serum levels of human FVIII in animal models

Animal Model	Transgene	Peak Expression*	Length of Expression#	Reference
Dog	[illegible]	8000 µU/ml	[illegible]	5
Mouse	[illegible]	307[illegible]g/ml	[illegible]	6
Mouse	[illegible]	1046[illegible]g/ml	[illegible]	7
Mouse	[illegible]	2063[illegible]g/ml	[illegible]	8
Mouse	[illegible]	408[illegible]g/ml	[illegible]	10
Dog	[illegible]	300[illegible]% normal	[illegible]	11
[illegible]	[illegible]	1-5 µg/ml	[illegible]	12
Dog	[illegible]	300[illegible]% normal	[illegible]	13
[illegible]	[illegible]	≈ 35[illegible]g/ml	[illegible]	2
[illegible]	[illegible]	380[illegible]g/ml	[illegible]	3
Mouse	[illegible]	700 µg/ml	[illegible]	4
Mouse	[illegible]	630 mU/ml	[illegible]	14
Mouse	[illegible]	≈ 250 mU/ml	[illegible]	15
Mouse	[illegible]	91 mU/ml	[illegible]	16
[illegible]	[illegible]	52.7 mU/ml	[illegible]	16

* Peak expression is the maximum level of protein detected.
\# Length of expression is the last timepoint giving detectable protein.

clearly shown that persistence of transgene expression is highly strain-dependent and route of vector administration also has profound impact on transgene expression. [9] Though much progress has transpired, a number of limitations impede progression of adenovirally mediated gene therapy of hemophilia A. First, since adenoviruses do not efficiently integrate into the host's genome, the transgene will be diluted as the cells cycle. Second, the strong cellular and humoral immune responses generated by first generation adenoviral vectors precludes their readministration. Third, pre-established immunity is likely in place in most humans, as type 5 adenovirus is a normal human pathogen. Fourth, and perhaps most problematic, is the possibility that adenoviral vectors may enhance generation of FVIII inhibitors and thereby confound treatment.

Hemophilia B, like hemophilia A, is a sex-linked disorder with a frequency of 1 in 30,000 males. Despite the reduction in occurrence of factor IX deficiency (hemophilia B), they still make up about 20% of all hemophiliacs. A number of attributes of factor IX make it more amiable to gene therapy. First, factor IX does not require cofactor complexation for stability in serum, and factor IX is in general much more stable than FVIII. Factor IX has a smaller cDNA that does not require manipulation to be accommodated into the current viral vectors. The incidence of factor IX patients with inhibitors is significantly lower, at roughly 5% compared to 20% for FVIII patients. Adenovirus-mediated gene therapy of factor IX has seen recent successes in both mouse and dog models, as in FVIII. The first study to report therapeutic levels of factor IX in serum mediated via adenovirus showed no difference in expression levels of mice directly injected in liver parenchyma or into tail veins.[10] It also indicated that re-administration was prevented by adenoviral antibodies. Another report showed therapeutic levels in hemophilic dogs were achieved by 18 h after administration of adenoviral vector and peaked at 2.5 to 3-fold over normal levels by 48 hours[11] (see table 16.1). However, these levels of factor IX rapidly declined within three weeks to 1% and by two months to only about 0.1% of normal. Dai[12] et al were the first to indicate a solution to the transient expression of adenoviral vectors. They demonstrated expression of factor IX for longer than five months in miceimmunosuppressed with cyclophosphamide. A similar study that extended adenovirus-mediated expression of factor IX in dogs for 6 months by using the immunosuppressant cyclosporine A was reported.[13] While these reports of prolonged expression after partial immunosuppression are encouraging, similar encouraging results on re-administration are still lacking.

Erythropoietin Deficiency

Erythropoietin is a 30 kDa glycoprotein produced predominantly by the peritubular epithelium of the kidneys, and functions as a key regulator of erythropoiesis. Erythropoietin deficiency is commonly caused by some cancers, HIV infection, and chronic or acute renal failure. Erythropoietin expression is normally controlled transcriptionally by tissue hypoxia or anemia. One attractive feature of erythropoietin deficiency is the fact that the disease is an acquired model of serum protein deficiency. A recent study indicated the use of a first generation E_1^-/E_3^- adenoviral vector expressing erythropoietin.[14] They reported expression of erythropoietin in neonatal CD-1 mice injected intramuscularly (i.m.) that persisted for 120 days without immunosuppression. More recently, it was shown that the transgene encoded in the adenoviral vector is important in determining the duration of transgene expression. Tripathy[15]et al demonstrated persistent expression in four different adult mouse strains injected i.m. with an adenoviral vector encoding murine erythropoietin. In contrast, mice receiving i.m. an adenoviral vector encoding human erythropoietin broke tolerance to murine erythropoietin, resulting in anemia. This provocative report has dramatic impact on the design of future in vivo studies of adenovirally mediated transgene persistence. One important point that must be considered is that adenoviral vectors do not replicate in rodents, and the mice used in these studies are naive to adenovirus. One added problem with erythropoietin deficiency is that overexpression will result in hematocrit levels that, while tolerated by rodents, may be toxic to humans. A recent report demonstrated that first generation E_1^-/E_3^- adenoviral vector injected intramuscularly into non-human primates resulted in significant increase in hematocrit levels that persisted for 84 days.[16]

Other Potential Uses of Adenovirally-Delivered Serum Protein

Adenoviral vectors have recently been used to deliver serum proteins in a context-sensitive manner for such gene products as cytokines to increase effectiveness of tumor antigen vaccines as well as traditional vaccines. Of particular interest is the development

of adenoviral vectors to deliver the leptin hormone. Leptin is an adipocyte-derived hormone that regulates food intake, thermogenesis, body weight, and insulin sensitivity. In the last two mentioned, the utility of leptin delivery via recombinant adenovirus in rodent models of obesity and non-insulin-dependent diabetes mellitus has been demonstrated.[17-19] It remains unclear if these results can be duplicated in large animal models or humans, but the early reports are encouraging.

Conclusion

Over the last several years, increasing effort has been dedicated to further deletions in the adenoviral backbone in order to reduce leaky late region gene expression. Currently there is controversy over the ability of these added deletions to improve persistence of transgene expression. Englehardt [20,21] demonstrated improved persistence in cotton rat lung and mouse liver respectively. However, these findings are in disagreement with those published in the hemophilic dog model.[13] More recently, a number of labaratories have developed adenovirus amplicons that have only the cis-acting elements required for vector packaging and replication. While on the surface these amplicons seem very interesting, they suffer from at least two critical flaws. In order to propagate these adenovirus-based amplicons, many essential functions are provided in trans in the form of either plasmid cotransfections or helper virus. The former suffer from low yields and both almost always have contaminating helper virus. More importantly, these amplicons do not seem to have the same biological activities as first generation adenoviral vectors. A recent study has demonstrated over 100-fold reduction in transgene expression that is even less persistent than first generation vectors.[22]

Given the high efficiency of adenovirus-mediated in vivo gene delivery, their potential utility as vectors are clearly evident. Since most serum protein deficiencies are genetic disorders, it is desirable that the transgene be expressed for relatively long periods of time, or perhaps the entire life of individual. Unfortunately, the current adenovirus-based vectors do not offer such long term expression. Clearly, any usage of these vectors in vivo will require further improvements toward long term expression. Improvement to transgene persistance is a multifaceted problem that will require study of promoter shutoff, episomal maintenance, the specifics of the particular transgenes, and the humoral and cellular immune responses toward viral and transgene proteins. Pointedly, adenovirus-based vectors have been the most efficient in vivo gene delivery vectors studied to date, and further study is essential for their full development.

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Chapter 17

Adenoviral Vectors for Vaccines

Bernard Klonjkowski, Caroline Denesvre, and Marc Eloit

Several viruses can be used for the development of vectors for vaccination purposes. The human adenovirus type 2 (Ad2) and 5 (Ad5) have been used most extensively because of the greater understanding of their genome and interaction with the host cells. Nevertheless, for vaccination purposes other human adenoviruses, Ad3, Ad4 and Ad7 have also been developed, mainly because they are either not neutralized or neutralized weakly by convalescent sera of recipients of Ad2/Ad5 inoculation, a feature which makes them good tools for boost injections. The use of human adenoviruses must also not overshadow the opportunity to use animal adenoviruses, which could show similar or complementary properties. Moreover, this approach can also be dedicated to the development of veterinary vaccines. Among animal adenoviruses, until now, bovine (BAV3),[1] ovine[2] and canine (CAV2)[3] adenoviruses have been used as gene transfer vectors.

Several Deletion Mutants with Different Properties Can Be Used

Several construction strategies, summarized in Figure 17.1, can be followed depending whether the objective is to obtain a replication-competent or -defective virus; the latter being then propagated in cell lines complementing the essential deleted genes.

Replication-Competent Viruses

For the construction of a replication-competent virus, one needs not to delete genes that are essential for virus multiplication. Most often, the gene of interest is inserted in the E3 region. Because of the genome size limitation for efficient encapsidation, the E3 region is deleted to manage room for larger insertion. This region is dispensable for in vitro growth, but is implicated in the escape of host immune response (see chapter 26). Then, maintaining or even overexpressing some E3 genes seems pertinent for therapeutic gene transfer to avoid the recognition of transduced cells by CTLs specific for the transgene or viral proteins, which are expressed at low levels. However, for vaccination purposes, the deletion of E3 genes implicated in evading the immune response seems desirable.[4]

It is also possible to insert foreign genes in either the left or right part of the virus genome. However, care must be taken to keep intact the expression of, respectively, the E1 or E4 regions which are necessary for viral multiplication.[5,6]

Thus, the simplest approach to generate replication-competent adenoviral vectors is to substitute the E3 region with the foreign gene. Nevertheless, the transcription of these foreign sequences is then often initiated from viral upstream promoters (see page 167).

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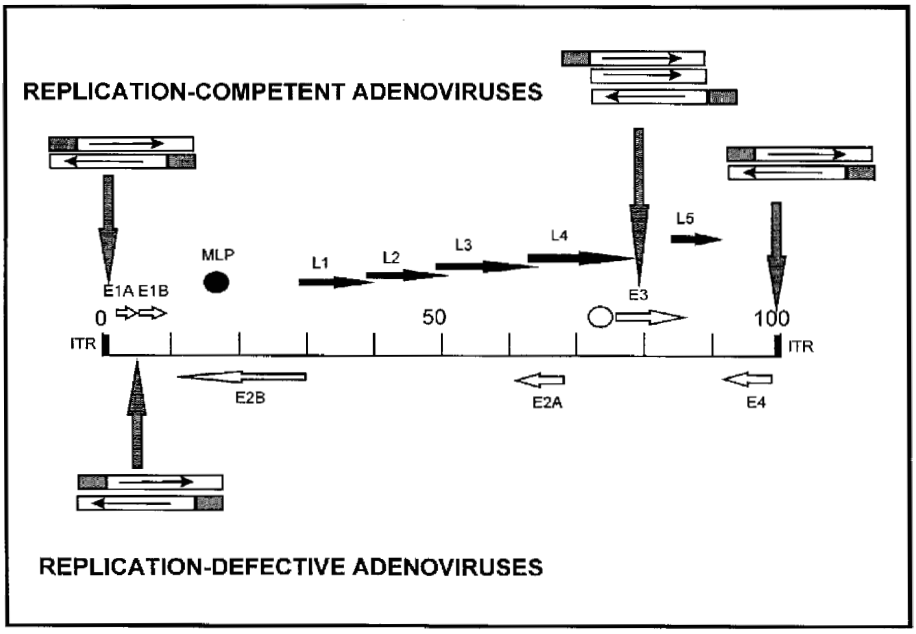

Fig. 17.1. Design of adenovirus-vectored vaccines. Virus genome: Empty arrows indicate main coding early regions; black arrows indicate main coding late regions. Regulatory sequences and introns are not depicted, except for the major late promoter (MLP, black circle) and the E3 promoter (empty circle). Inserted genes: The promoter (in gray) and the coding region (in white) are depicted. In all cases, the arrows show the direction of transcription.

Replication-Incompetent Viruses

The deletion of at least the *E1A* together with, most often, the E1B genes in an E3-deleted genetic background, leads to the generation of viruses, which are unable to replicate productively except in complementing cells like the well known 293 cell line. The deletion of E1 genes does not totally circumvent a low level of transcription from the E2 and the MLP promoters: the transduced cells then express, in addition to the transgene product, a low level of viral proteins, leading to the clearance of these cells by the host immune response. While this is clearly a limitation for the use of this kind of vector in gene therapy, this does not seem to pose a serious problem for vaccines. In fact, vectored vaccines are designed to express a highly immunogenic transgene, so the immune response against the vector itself seems marginal in this context. Nevertheless, the expression of these heterologous proteins amplifies the antibody response against the structural proteins of the injected capsids, which represents a limit for the efficacy of the subsequent boosts.

Improvements in the design of Ad vectors have been recently made with the engineering of additional deletions (particularly E4) and the development of cell lines complementing both the E1 and E4 genes. Moreover, gutless vectors devoid of any viral gene have been constructed. These approaches considerably or totally diminish the expression of viral proteins in transduced cells, increase cloning capacity and decrease the risks of generating replication-competent Ad (RCA) in virus stocks.[7-10] The deletion of these viral sequences seems to increase the persistence of the vector DNA in animal models, and sometimes the

duration of the transgene expression with a promoter-dependent mechanism.[7,8] Until now the potency of these vectors has not been tested in vaccination trials, but they might be useful, particularly to increase the size of cloned sequences, to limit emergence of RCA and to decrease the antibody response against the vector.

Efficacy and Safety of Adenovirus-Vectored Vaccines

Replication-Competent Viruses

Different human replication-competent adenoviruses have shown a high level of efficacy in eliciting both humoral and CTL immune responses. An interesting feature of these viruses is their ability to induce mucosal immunity following administration by local routes. Several trials were conducted in animal species with variable permissiveness for the vector. Hamsters developed antibody responses against HBs Ag of the hepatitis B virus or the gB glycoprotein of human cytomegalovirus following nasal administration of the relevant replication-competent adenoviruses.[11,12] Cotton rats, which are fully permissive for Ad5, demonstrated mucosal immunity after inoculation by the nasal or enteric routes of replication-competent Ad5 expressing the hemagglutinin esterase of bovine coronavirus.[13] Oral immunization of foxes and skunks with a virus expressing the G glycoprotein of the rabies virus protected the animals against challenge.[14] Partial protection of pigs, a species permissive for Ad5, was shown in animals immunized by the oronasal route with an Ad5 expressing the S gene of the respiratory porcine coronavirus.[15]

Adenoviruses other than Ad2/Ad5 were also tested: replicative Ad4 and Ad7 induced detectable antibody response in chimpanzees against the products of the HIV gag and env genes.[16] The same serotypes expressing the F and G proteins of RSV, or different antigens of HBV (HBs, HBc), were also immunogenic respectively in ferrets[17] and dogs.[4,18] The oral administration in two chimpanzees of Ad4 and Ad7 expressing Hbs was followed by antibody responses, and, after virulent challenge, partial protection in one animal and total protection in the other one was observed.[19] Besides, a single administration in humans by the oral route of an Ad7 expressing Hbs did not direct an Hbs-antibody response.[20]

Until now, use of animal adenoviruses has been quite limited. BAV3 was studied in cotton rats[21] and then developed as a replicative BAV3 vector.[1] Recently, a recombinant ovine adenovirus was used to vaccinate sheep against teniasis (*Taenia ovis*).[2]

The role of regulatory sequences in vaccine efficacy has been specifically investigated. Insertion of a foreign promoter or an exogenous polyadenylation signal in the transcription units cloned in E3 was favorable to the efficiency of the constructions, both in vitro and in vivo.[22] Nevertheless, it sometimes appeared[23] that the hierarchy in efficacy of regulatory sequence, and the dependence for expression on the replication of viral DNA, was dependent upon the coding sequence of the gene of interest. These results underline the difficulty of interpretation of the level of expression of various constructions which may rely on the level of transcription, on the stability of the mRNA and on the efficacy of translation. This also emphasizes the complexity of analysis of the results obtained with replication-competent viruses in the mouse model, which is weakly permissive for Ad5. For example, when three different replication-competent adenoviruses expressing glycoprotein G from rabies virus were tested in mice, minimum differences in the efficacy were observed, despite the diversity of the constructions: parallel orientation with the transcription of E3, with or without the insertion of a foreign promoter (SV40), or antiparallel insertion under the control of a foreign promoter (MLP). On the contrary, one of these viral vectors (parallel insertion in E3 without foreign promoter) was found to be more efficient in skunks when given by the oral route.[22] Several investigators showed that genes inserted in E3 were transcribed from upstream promoters (Fig.17.1) such as the late MLP or the E3 early promoter.[24] When Ad4

and Ad7 expressing Hbs were tested in dogs,[4] a species supporting a low level of replication of these viruses, the antibody response against Hbs was much higher for $E3^-$ viruses than for $E3^+$ viruses. Nevertheless, the interpretation of these results is difficult because the $E3^-$ and $E3^+$ constructs expressed Hbs under the control of two different endogenous adenovirus promoters: $E3^-$ viruses expressed Hbs essentially in the early phase of the cycle, while $E3^+$ viruses did so at the late phase. This could explain, at least partly, the superior results obtained for $E3^-$ vectors in dogs, because of the low degree of replication of these viruses in this species. In fact, $E3^+$ vectors induced higher antibody responses in chimpanzees, which are permissive for virus replication.[25]

The use of exogenous promoters driving genes cloned in E3 could be beneficial but could also lead to aberrant splicing of RNA due to cryptic splicing acceptor signals. Antiparallel insertion seems less efficient,[26] maybe because of the high level of transcription originating from the MLP and E3 promoters.

Replication-Incompetent Viruses

The idea of using replication-incompetent viruses as vectors for vaccines is quite recent, and is an extension of the general use of these kind of vectors for gene therapy.

The efficacy of replication-incompetent adenoviral vectors has been illustrated in several animal models or in target species, in which evidence of protection against a virulent challenge were reported: tamarin and EBV herpesvirus,[27] rat and measles paramyxovirus,[28] pig and pseudorabies herpesvirus,[29] cat and feline infectious peritonitis virus.[30] Efficacy in the vaccination against parasites like *Plasmodium voleii* was also demonstrated.[31] Induction of a protective immunity following the transfer of the gene of a nonstructural protein of the tick encephalitis flavivirus, probably linked to the development of a CTL response, was also described.[32]

Efficacy of replication-deficient adenoviral vectors is dependent on the route of administration. Both subcutaneous and intramuscular injections induce similar antibody titers and infiltrating $CD8^+$ T cell levels in liver and in spleen, while intraperitoneal or intravenous injections appear to be less efficient.[31] Local administration in mice, such as oronasal inoculation, can induce a local and/or general immunity.[33,34] In mice, a single administration of 10^8 infectious particles of an E1-deleted adenovirus expressing the SIV gag p55 antigen by intrastomachal route elicited a systemic humoral response in 40% of animals. This response was detected over one year and the CTL response for 27 weeks. A similar CTL activity was observed even in animals with no humoral response. Oronasal administration was documented as an inefficient route of administration to elicit a humoral response, but a delayed CTL response was detected in the spleen.[35]

A recombinant adenovirus expressing the gB gene of HSV was shown to induce similar antibodies and CTL levels after nasal or intraperitoneal administration, with a longer persistence after inoculation by the intraperitoneal route. Only the nasal route elicited IgA synthesis.[33] Interestingly, if the short term systemic or mucosal CTL responses were similar after general or local inoculation, induction of a long term memory was dependent on the inoculation route. After local injection of the viral vector, CTL precursors lasted over one year.[36]

In the same way, a single injection of Ad5-derived recombinant expressing the gD protein of PRV in mice elicited an humoral response and protection against challenge for at least 400 days (unpublished results).

Induction of an immune response against the viral vector is a major problem occurring during gene transfer with adenoviral vectors. Neutralizing antibodies could decrease the efficiency of later injections. Nonetheless, we observed a booster effect in rabbits, pigs and poultry even after a first injection of up to $10^{9.8}$ infectious particles.[29,37,38] A booster effect

was also observed after injection of an Ad5 expressing the EBV glycoprotein gp220.[27] However, a weak transfer efficacy could be sufficient to induce a booster effect, because of the immune memory established following the first injection. In these animals, where no immune response was detected after a first injection of a vector encoding a poorly immunogenic protein or a weakly expressed protein, later injections could be inefficient.[39] On the other hand, multiple injections of plasmid DNA encoding the same protein can induce a humoral response following repeated injections (Gonin et al, unpublished data).

Comparison of Replicative and Nonreplicative Viruses

What are the respective advantages and disadvantages of replicative and nonreplicative adenoviruses? To answer this question we built two strictly isogenic Ad5s, defective or not for the *E1A* gene which expressed the gene for gD from pseudorabies virus[6]. These viruses were evaluated after intramuscular inoculation, in the cotton rat and the mouse, species respectively permissive and very slightly permissive to Ad5. Both viruses induced similar systemic antibody titers for each dose tested (from 10^9 to 10^5 $TCID_{50}$) in all the recipient species. On the other hand, in the cotton rat, the 50% protective dose (PD_{50}) was much higher for the defective virus than for the replicative virus, whereas in the mouse the PD_{50} were similar. Insofar as no relationship could be evidenced between protection and antibody titers, it is probable that the induction of cellular immunity (in particular CTL response) was induced with lesser amount of replicative virus. These results show that, at least for the model tested, both the defective and nondefective viruses are able to induce a full protection against a virulent challenge, with protective doses probably dependant on the permissivity of the animal species. This difference seemed to rely particularly on the induction of cellular immunity.

A comparable work was carried out in the cotton rat with recombinant Ad5 defective for the E3 region and expressing the gD gene of bovine herpesvirus type 1 (BHV1).[40] The results suggested that the use of replicative viruses does not systematically induce stronger immune responses by the parenteral route but was more efficient for mucosal immunization.[41] Nevertheless, these viruses differed by several characteristics in addition to their replicative or nonreplicative nature which limited the interpretation of the results.

Mechanisms of Immune Response Induction by Recombinant Adenoviruses

Although the efficiency of adenoviruses as vaccine vectors is well admitted, the mechanisms by which they stimulate the immune response are still unclear. In the case of replication-incompetent viruses, the questions to solve are similar to those raised by DNA vaccination. The intramuscular administration of a replication-incompetent adenovirus or naked DNA allows introduction of the transgene into cells in vivo and elicitation of an immune response. One major difference between these two approaches seems to lie in the efficiency and specificity of cell transduction. Concerning injection of naked DNA into muscle, the transduction mechanism is still unclear. The DNA entry into cells seems to occur through a passive mechanism, especially efficient in the muscle fibers having a T-tubular network.[42] On the contrary, the entry of adenoviruses uses an active mechanism based on the interaction of capsid proteins with cellular receptors (see chapter 4). The use of widely spread receptors must be associated with the broad host cell tropism of Ad5.

We have expressed the gD gene of the herpes pseudorabies virus (PRV) under four different promoters with either a naked DNA or an adenoviral vector in mice and/or swine after intramuscular administration (Fig. 17.2). In our hands, the efficiency in inducing an immune response against the product of the transgene was much higher with adenoviral

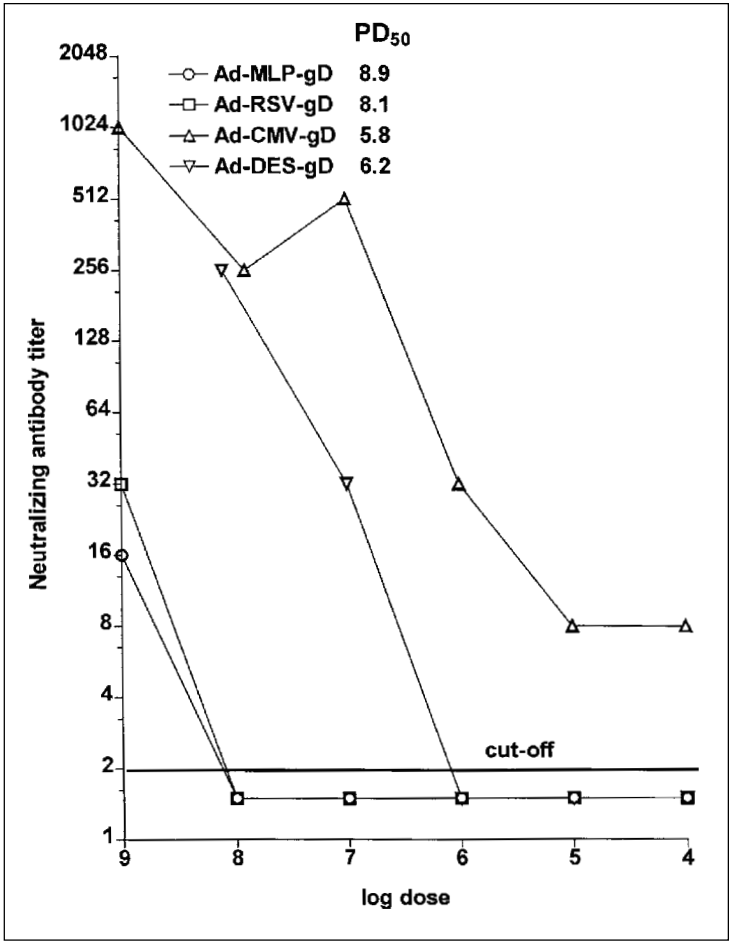

Fig. 17.2. Promoter efficiency in adenovirus-vectored vaccines used by the muscular route and comparison with DNA vaccines.[43] The gD gene of the pseudorabies herpesvirus was cloned in a replication-defective Ad5 under the control of four different promoters : major late promoter of Ad2, LTR from RSV, immediate early promoter from CMV and Desmin promoter. Mice were vaccinated once by the muscular route and challenged five weeks later by the intraperitoneal route. Neutralizing antibody responses and 50% protective doses (PD_{50}) are depicted.

vectors than with naked DNA, although the number of plasmid copies was higher than the viral particles.[43,44] Similar results were obtained with the N protein of the measles virus.[45]

How are we to explain the difference in efficiency for induction of the immune response between adenoviral vectors and naked DNA? At least three major reasons can be advanced:

1. The cell types transduced and their Ag-presenting properties. The transduced cells may not be the same with adenoviral vectors and naked DNA. We examined the expression level of the PRV gD glycoprotein from adenoviral vectors in potential

target cells of the muscle: myoblasts, myotubes, fibroblasts, macrophages and endothelial cells. According to the expression level of the transgene in vitro, the fibroblasts and the macrophages are not the major target cells of Ad5 vectored vaccines, whereas myoblasts and/or endothelial cells are. Moreover, efficiency in the induction of the antibody response and in the protection was correlated with the expression level of the transgene, at least in the myoblasts and the endothelial cells. Can these cells play a direct role in the induction of the T cytotoxic response? Cultured myoblasts express constitutively only a few MHC class I molecules, no LFA-1 and no ICAM-1 in the absence of TNF-α treatment.[46] The endothelial cells express MHC class I and certain costimulatory molecules.[47] Therefore, even if these cells are not typical antigen-presenting cells (APC), they may act as APC in some conditions such as local inflammation.[48] The major APC, the dendritic cells present in muscle,[49] were not examined in our study. Others have shown that mouse dendritic cells are poorly infectable by recombinant Ad5 virus in vitro. However, this may not be a limitation, as very few infected dendritic cells are probably sufficient to stimulate naive T cells.

2. The global efficiency of entrance into cells and the resulting level of Ag production. In this model, the targeting of cells is not a problem; rather, the level of Ag expression is the crucial point. The adenoviral vectors are more efficient in entering cells than naked DNA. Therefore, the level of Ag production is probably higher with adenoviral vectors. This hypothesis implies that the transduced Ag-producing cells are able to transfer the Ag to APCs, and that the more that Ag is produced and released, the more the Ag is recaptured and presented by APCs. Ag transfer seems conventional in the case of T helper response restricted to MHC class II molecules. Surprisingly, Ag transfers have also been proposed to explain the results of T cytotoxic activity in experiments of grafting genetically modified cells in mice[50-52] (Fig. 17.3). Ag transfer may be obtained after death of producing cells and endocytosis of the free Ag or phagocytosis of the dead producing cells. This pathway for presentation on MHC class I molecules is not the classical one, but has already been described.[53] Moreover, another mechanism of Ag transfer has been demonstrated in vitro: dendritic cells are able to present influenza Ag derived from apoptotic infected cells and induce cytotoxic activity.[54] It would be interesting to see if infection of certain cells with defective Ad recombinant vectors may lead to apoptosis.
3. The local inflammation induced by the vaccine itself. Indeed, naked DNA induced a very low inflammation process compared to recombinant adenoviruses, even replication incompetent ones.

It is possible that these three aforementioned mechanisms are not exclusive and perhaps other unidentified mechanisms would explain the difference between the efficacy of adenoviral vectors and plasmid DNA for vaccines.

Prospects for Use

To improve the prospects for the use of vaccines, we need to develop approaches for local routes of administration, for at least two reasons: facility of administration and induction of mucosal immunity. To date, only viral-vectored vaccines, whether replicative or not, have clearly shown their efficiency compared to DNA vaccines, for which lipid-DNA complexes will perhaps be necessary to obtain a satisfactory efficacy. Moreover, the use of recombinant replicative vaccines also has the advantage that lower doses of vectors can be used, but exceptions do exist.[40] There are nevertheless questions relating to the dissemination of these viruses, particularly in the case of local routes of administration. In addition, for veterinary applications, the use of replicative strains requires development and production

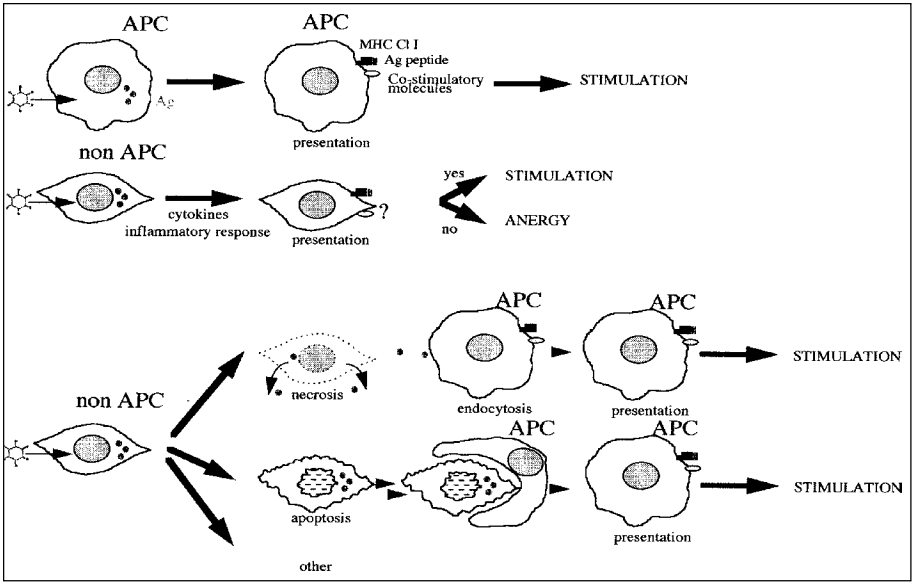

Fig. 17.3. Possible mechanisms of induction of T cytotoxic immune response by recombinant adenoviral vectors. (A) The transduced cell (APC or nonprofessional APC) presents processed Ag on MHC class I molecules. In nonprofessional APC, the presence of costimulatory molecules may be determinant for the success of the T cell stimulation. (B) The transduced cell is not able to present the processed Ag on MHC class I molecules. The Ag produced is transfered to the APC after cell lysis, apoptosis or another unknown mechanism.

of almost as many vectors as there are animal species. Lastly, replicative strains are likely to induce a strong immune response against the vector, probably limiting the efficacy of the boost injections. The nonreplicative adenoviral vectors have for their part an important potential of efficacy, while their modification by multiple deletions must lead to lower biosafety concerns.

Lastly, another field of application has just recently been opened: one of the traditional limitations of the vaccination of the younger people or animals with live or inactivated vaccines is the interference with maternal antibodies, when the mothers are immune because of previous infection or vaccination. This is particularly frequent in veterinary medicine in livestock, where the mothers are vaccinated and newborns must be vaccinated early because of the infectious risks linked to high-density breeding. We have recently shown that the transfer of the PRV gB, gC and gD genes by a replication-defective adenovirus in one day old piglets born to immune mothers overcame this inhibition (ref. 44 and unpublished results). Comparable results using the same type of defective vector were also obtained for glycoprotein G from the rabies virus in the mouse. Under the same conditions, a vaccinia replicative vector was ineffective, perhaps because of a mechanism of induction of the immune response at least partly founded on the release of the antigen from infected destroyed cells, undoubtedly more sensitive to the presence of circulating antibodies.[55]

Conclusion

Several strategies may be used to improve adenovirus-based vaccines. One would be to identify the cells which can be used as targets for the installation of an immune response, both for parenteral and local routes. Then vectors would be developed with a specific design, e.g., optimization of the regulatory sequences for better expression, targeting of the virus. Another way to improve the efficacy is to enhance immunity against the product of a transgene by the use of adjuvants[56] or cytokines.

It is nevertheless clear that the background knowledge on adenovirus-based vaccines is not yet sufficient to make a final judgment of the advantages and the limitations of these vectors compared with other viral vectors or genetic vaccination. Nevertheless, the currently available elements indicate that they seem to have certain advantages, in particular for mucosal immunization and for vaccination of neonates born from immune mothers.

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Chapter 18

Strategies to Adapt Adenoviral Vectors for Gene Therapy Applications

Joanne T. Douglas, Meizhen Feng, and David T. Curiel

The Generation of Targeted Adenoviral Vectors by Immunological Modifications of the Fiber Protein

Full utility of adenoviral vectors is potentially undermined by their broad tropism profile. In this regard, for gene therapy purposes it would be desirable to derive adenoviral vectors that will accomplish gene delivery in a "targeted", cell-specific manner.[1-3] In order to restrict gene delivery exclusively to target cells, it is necessary to prevent the interaction between the knob domain of the adenovirus fiber and its cellular receptor, which plays the major role in the determination of adenoviral tropism. Since the specific amino acid residues in the knob which recognize the cell surface receptor have not yet been identified, it is not currently possible to ablate this binding site by employing genetic techniques such as site-directed mutagenesis. However, by employing a neutralizing anti-knob monoclonal antibody chemically conjugated to a ligand recognizing a specific cell surface receptor, it should be possible to target the adenoviral vector to this novel receptor (Fig. 18.1).

To test this concept, we chose to target the high affinity folate receptor which is overexpressed on the surface of several malignant cell lines, including ovarian, lung and breast carcinomas. We then rationalized that for our purposes of developing a targeted adenoviral vector by immunological methods, it would be preferable to employ the Fab fragment of the antibody, rather than the intact immunoglobulin. In this manner, we sought to prevent the two antigen-binding arms of the parent antibody from crosslinking different viruses to form large aggregates which might prove refractory to cellular uptake. Therefore, we conjugated folate to the neutralizing Fab fragment of an anti-knob monoclonal antibody designated 1D6.14. This Fab-folate conjugate was complexed with AdCMVLuc, an adenoviral vector carrying the luciferase reporter gene, and was shown to redirect adenoviral infection of KB cells, a human nasopharyngeal carcinoma cell line, specifically via the folate receptor.[4] Furthermore, this resulted in a level of gene transfer comparable to that achieved by native adenoviral infection, which is in marked contrast to the inefficient infection exhibited by retargeted retroviral vectors.

We then sought to modify adenoviral tropism to accomplish infection of target cells via the high affinity fibroblast growth factor receptor, which is overexpressed on a variety of tumor cells including glioma cells and ovarian, pancreatic and breast cancer cells. Basic fibroblast growth factor (FGF-2) has previously been used to target cytotoxic molecules and DNA to high affinity FGF receptors. The Fab fragment of the neutralizing anti-knob monoclonal antibody was chemically conjugated to FGF-2 and the resulting conjugate was complexed with AdCMVLuc and used to retarget Ad infection of four tumor cell lines

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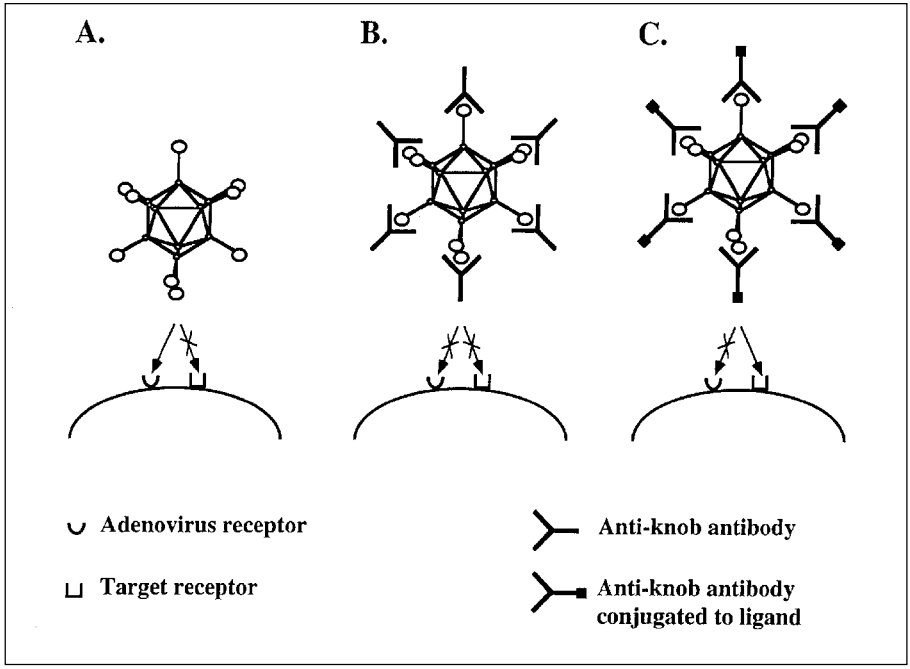

Fig. 18.1. Strategy for immunological retargeting of adenoviral vector. (A) Adenoviral attachment to cells is accomplished by the high affinity binding of the knob domain of the fiber to the primary receptor. (B) When complexed with a neutralizing antibody directed against the knob domain, the adenovirus is unable to bind to its cellular receptor. (C) Conjugation of a cell-specific ligand to the neutralizing antibody is hypothesized to permit binding to a novel target receptor on the cell surface.

which overexpress high affinity FGF receptors: Swiss 3T3, a murine fibroblast cell line; PANC-1, a human pancreatic epitheloid carcinoma cell line; SK-OV3.ip1, a human ovarian adenocarcinoma cell line; and D54 MG, a human glioma cell line. In each case, the Fab-FGF-2 conjugate was shown to redirect adenoviral infection specifically via the FGF receptor.[5] Moreover, the levels of gene transfer observed by the retargeted viruses were significantly higher than observed for the unmodified viruses after infection via the native pathway. The biological basis of this phenomenon is not yet known, but is currently the focus of investigation in our laboratory to determine whether it results from an increased efficiency of binding, internalization or gene transfer of the adenoviral vectors retargeted to the FGF receptor.

We then extended this immunological targeting strategy to achieve efficient adenovirus-mediated gene transfer to human cancer cells which are normally resistant to adenoviral infection due to a lack of primary receptors for the fiber. In this case, the target was Kaposi's sarcoma (KS), the most common neoplastic disease associated with human immunodeficiency virus type 1 (HIV-1) infection. Acquired immunodeficiency syndrome (AIDS)-related KS presents as a disseminated and aggressive tumor, leading to significant morbidity and mortality. At present, there is no effective therapeutic regimen for AIDS-KS, suggesting that gene therapy is a rational approach to the treatment of this disease.

However, AIDS-KS cell lines have shown to be refractory to transduction by a variety of viral and nonviral vectors, a problem which threatens to undermine any gene therapy approach. Since an autocrine loop involving FGF and its cognate receptor has been implicated in the development of AIDS-related KS, we hypothesized that it might be possible to transduce KS cells by exploiting the FGF receptor. Therefore, the Fab-FGF-2 conjugate was complexed with AdCMVLuc and was shown to mediate adenoviral infection of AIDS-KS cells that are refractory to transduction by native adenovirus. Since any gene therapy approach to the treatment of cancer is dependent on efficient and specific gene transfer to the disease cells, this retargeting strategy will be of utility in permitting infection of previously refractory cells.[6]

These experiments have therefore demonstrated that infection by adenoviral vectors can be retargeted via cellular receptors other than the native primary fiber receptor. This immunological approach to the modification of preformed adenoviral particles represents a versatile strategy for the rapid generation of adenoviral vectors targeted to a given population of cells. To date, the targeting moieties have comprised a vitamin, folic acid, and growth factors FGF-2 and EGF; however, our recent work indicates that antibodies and cytokines directed to specific receptors on the target cell surface can also be exploited. One limitation of the approach is the fact that the neutralizing Fab fragment is not covalently linked to the vector particle: The complex could therefore dissociate in the bloodstream following intravenous administration. We are therefore exploring methods to achieve the stable linkage of the targeting conjugate to the adenoviral particle.[7]

Achievement of Long Term Heterologous Gene Expression via Adenoviral Vectors

In addition to the issue of targeting, gene delivery via adenoviral vectors has been associated with the induction of an inflammatory/immunologic response when employed in vivo. This phenomenon has been understood to result from presentation of viral antigens via the major histocompatability complex (MHC I) pathway with induction of a cytotoxic T lymphocyte (CTL) response directed against genetically modified cells. A consequence of this phenomenon is immunological eradication of transduced cells. This process has been associated with the attenuation of expression of the transferred therapeutic gene based, at least in part, on loss of the transduced cell.[8,9] Based on an understanding of the biology of this phenomenon, specific strategies have been developed to mitigate this process. In this regard, methods have been developed to achieve more complete inactivation of viral components of the vector genome. Thus, although inflammatory/immunological issues have limited the overall utility of adenoviral factors for gene therapy applications, many of the aforementioned strategies appear promising and may allow this problem to be addressed.

An additional basis of limited transgene expression associated with adenoviral vectors derives from their non-integrative nature, such that vector sequences are not retained in the host genome. In this regard, after adenoviral vector-mediated gene transfer, the chimeric genome (transgene sequences and vector host genome sequences) is present epichromosomally in the target cells. Thus, with proliferation of transduced cells, vector sequences are lost, with the consequence of limited duration transgene expression. For utility in gene therapy strategies, it would be desirable to develop methods to achieve integration of adenoviral vector-delivered transgene sequences in infected cells.

A number of viruses have evolved mechanisms to maintain their genetic material in transduced target cells. To this end, some viruses have evolved methods to integrate their genomes into host chromosomes. This includes both DNA viruses, such as adeno-associated virus (AAV), as well as RNA viruses such as retroviruses. In addition, another mechanism for viral gene persistence is based on replication of the viral chromosome as an

extrachromosomal episome in synchrony with the host cell. This mechanism of persistence has been employed by Epstein-Barr virus. Based on these concepts, gene transfer vectors have been derived based on each of these parent virions to exploit these features of gene persistence. Based on their properties of gene persistence, some of these agents have also been exploited as vectors for in vivo gene delivery. Despite their utility for a variety of ex vivo gene transfer applications, retroviral vectors have been of extremely limited utility for in vivo gene delivery. This fact derives from several considerations, including the relatively low titers obtainable, the in vivo lability of the virion, and the requirement of target cell replication for integration. For AAV, very limited data regarding the in vivo utility of these agents has been available. This fact has also derived from technical issues related to preparation of material of sufficient titer for in vivo use. Thus, although it is clear that gene therapy approaches for metabolic diseases will require prolonged expression of therapeutic genes, the available vectors possessing integrative functions are not of sufficient utility for the mandated delivery approaches of direct in situ transduction of the liver. Conversely, while adenoviral vectors possess the basic in vivo gene delivery efficiency mandated for gene therapy approaches, they lack the ability to achieve prolonged gene expression in relevant target cells. Thus, the development of an integration capacity in the context of adenoviral vectors would address this key limitation of adenoviral vectors for this application. Such an approach to deriving a "chimeric vector" would favorably exploit the most desirable aspects of each system for gene therapy applications.

To address the issue of transient gene expression associated with adenoviral vectors, we developed a "chimeric" vector system that combines the high efficiency in vivo gene delivery characteristics of recombinant adenoviral vectors with integrative capacities derived from retroviruses. This was accomplished by rendering adenoviral vector infected target cells into transient "retroviral producer cells" via adenoviral vector-mediated delivery of retroviral packaging functions and retroviral vector sequences. In this manner, the locally elaborated retroviral vectors could infect neighboring parenchymal cells via an integrative vector. The conceptual basis of this approach in depicted in Figure 18.2.

As a first step toward implementing this strategy, we constructed adenoviral vectors encoding the requisite retroviral functions for in situ generation of retroviral producer cells. DNA segments encoding the retrovirus packaging functions gag, pol and amphotropic env genes derived from the retroviral packaging plasmid pPAM3, were cloned into the adenoviral shuttle vector pCA13. The resultant plasmid, pCAAmpg, was constructed to allow expression of all retroviral packaging functions under the control of the CMV intermediate/early enhancer-promoter. In addition, an adenoviral shuttle plasmid, pΔE1LNCMVGFP, was designed to contain the retroviral vector components. In this instance, a unit containing the neomycin resistance gene and the green fluorescent protein (GFP) expression cassette, flanked by retroviral LTRs and containing the MoMLV packaging signal, was cloned into the polylinker of the adenoviral shuttle vector pΔE1SP1A. A similar construct containing the LacZ reporter, pΔE1LNCMVLacZ, was derived in the same manner. Adenoviral vectors were derived by cotransfection of 293 cells with the shuttle vectors and the rescue plasmid, pBGHII.

We next demonstrated the ability of the adenoviral vectors to induce target cells to function as retroviral producers. Target cells were infected with a combination of the adenoviral vectors, AdCMVAmpg and AdLNCMVGFP, which we hypothesized would allow induction of retroviral particle production. As a parallel control, cells were also infected with AdLNCMVGFP only, which would not be predicted to generate retroviral particles. As a next step, we sought to directly demonstrate the production of transducing retroviral particles based on the process of retroviral producer cell induction. NIH-3T3 or W162 cells were infected with either a combination of AdCMVAmpg and AdLNCMVGFP, or AdLNCMVGFP only, then subsequently washed as before. The supernatants were harvested

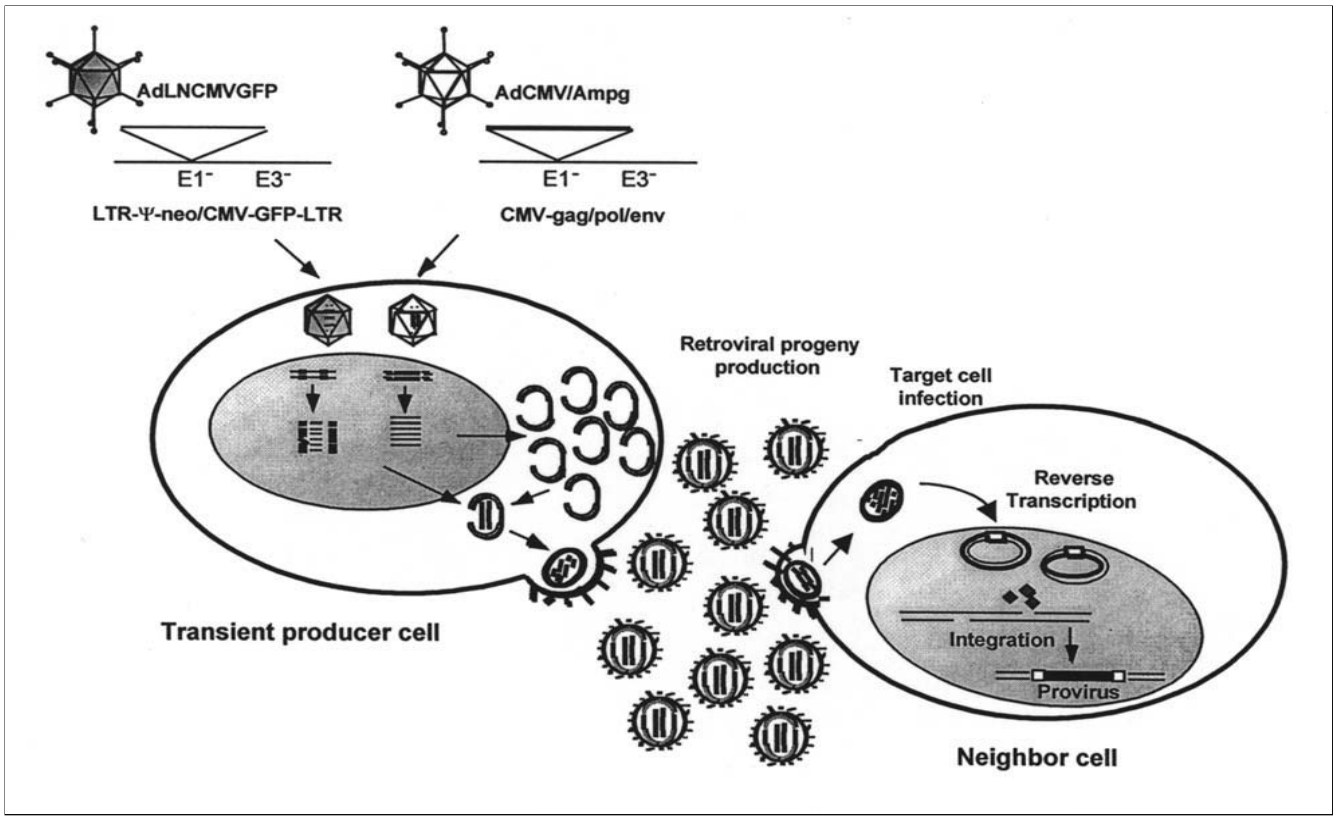

Fig.18.2. Schema of local generation of retroviral vectors at target organ site. Adenoviral vectors encoding retroviral vector and packaging functions accomplish in vivo gene transfer to target parenchymal cells at high efficiency, rendering them transient retroviral producer cells. The locally elaborated retroviral particles can thus directly infect neighbor cells.

at 48 hours post-infection and then employed to infect NIH-3T3 cells to determine retroviral titers. The supernatant-infected cells were maintained in culture for 20 days and analyzed as before for GFP expression. In this study, the supernatant derived from the AdLNCMVGFP virus-infected cells was not capable of inducing long term GFP expression in target cells. In contrast, cells infected with AdCMVAmpg plus AdLNCMVGFP supernatant demonstrated a high rate of GFP positivity at day 20.

This study provides confirmation that GFP expression resulted from infection with retroviruses derived from the original adenovirus-infected target cells. These long term GFP expression studies were designed to distinguish carry-over adenoviral transient gene expression (<2 weeks) from stable transduction mediated by retrovirus production. The results suggested that transducing retroviral particles had indeed been generated by adenoviral vector-delivered genes in target cells. In addition to this analysis, we sought to determine whether this methodology was associated with significant production of replication-competent retrovirus (RCR). For this study, we compared RCR generation derived via plasmid-based transfection methods versus the employment of the adenoviral/retroviral chimeric vector. HeLa cells were thus transfected with either pPAM3 plus pLNCLZ, or infected with AdCMVAmpg or AdLNCMVLacZ, or AdCMVAmpg plus AdLNCMVLacZ. These supernatants were then analyzed for the presence of RCR by a widely employed transformation assay. In this analysis, no RCR was noted with employment of an adenoviral/retroviral chimera component. Thus, the generation of RCR by this method does not appear to be in excess of conventional methods.

Next we sought to exploit this process in vivo. For these studies, the ovarian carcinoma cell line SK-OV3.ip1 was infected in vitro with either AdCMVAmpg plus AdLNCMVGFP, or AdLNCMVGFP alone. To confirm the in vivo generation of infected retroviral particles and infection of neighboring cells, we then mixed infected cells with uninfected cells at a ratio of 25% adenoviral vector infected cells with 75% untreated SK-OV3.ip1 cells and implanted them subcutaneously in athymic nude mice to allow tumor formation. Twenty days after implantation, both animal groups had palpable tumors that were harvested for analysis of GFP reporter gene persistence and expression. The group infected with AdLNCMVGFP only had rare, isolated fluorescent cells. In contrast, the tumors derived from the two virus group had large expansive clusters of GFP positive cells. Counting of positive cells in multiple fields allowed an estimate of transduced cells such that the one virus group had 15-20% positive cells whereas the two virus group had >80% GFP positive cells; thus in this group the number of positive cells was substantially greater than the proportion of adenovirally infected cells in the original implanted mixture. The extensive distribution of GFP positive cells in the two virus group suggested stable genetic modification of neighboring cells via in situ retroviral vectors.

As a more stringent test of our concept, we explored the potential to link in vivo adenoviral vector transduction to in situ retroviral producer generation. For these experiments, athymic nude mice were orthotopically transplanted with the human ovarian cancer cell line SK-OV3.ip1. Five days post-implantation, animals were treated intraperitoneally with either AdLNCMVGFP only, or AdLNCMVGFP plus AdCMVAmpg. Sixteen days post-adenovirus infection, the animals were sacrificed and tumors analyzed as before. In this analysis, no GFP positive cells could be demonstrated in the one virus group. In contrast, islands of GFP positive cells could readily be identified in the group which received both adenoviral vectors. Again, analysis of multiple microscopic fields demonstrated an overall transduction rate of <1% for the one virus group and 10-15% for the two virus group. The relative paucity of GFP expression in the animals given the AdLNCMVGFP is consistent with the temporal pattern of extinction of adenoviral vector-mediated gene transfer in this context. The persistence of GFP expression in vivo in the group receiving the

two adenoviral vectors, which allows full induction of retroviral packaging, is consistent with our in vitro findings whereby stable transduction had occurred based on secondarily elaborated retroviral vectors.

Acknowledgments

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CHAPTER 19

Adenovirus-AAV Combination Strategies for Gene Therapy

Krishna J. Fisher

Among the repertoire of eukaryotic viruses that have been genetically engineered for targeting therapeutic genes to somatic cells, adenovirus has captured considerable attention since the early 1990s. And, while much can be said about the strengths and weaknesses of adenovirus vectors for human gene therapy, a crossroads of sorts has been reached. Long since past are the days when deletion of the E1 region was hailed as a facile maneuver for disrupting the normal cascade of early and late gene expression, thereby rendering the virus tame. It now appears that these so-called first generation vectors exhibit many properties that are indicative of the wild type virus. The significance of this is best illustrated by the destructive immulogical forces that are mounted in response to adenovirus-mediated gene transfer, resulting in the purging of transduced cells.[1] With these findings at hand, adenovirus vector development is faced with the challenge of striking a balance between two critical parameters: ablating adverse cellular responses to de novo synthesis of viral proteins or other trans-acting factors, while promoting stability of the recombinant viral chromosome.

Adenovirus Vector Development

Generally speaking, there are two fundamental approaches to achieving an adenovirus vector design capable of attaining a state of equilibrium when delivered in vivo. The first and most widely recognized strategy involves the systematic removal of coding regions suspected of participating in the destruction of transduced cells,[2] or otherwise compromising the fidelity of transgene expression.[3] Various permutations of this theme have been explored, resulting in a cast of second generation vectors.[2] Despite measured success towards the development of genotypes with improved toxicity and persistence profiles,[4,5] the major limitation to this approach is the incessant molecular noise that emanates from coding regions that are retained. This outcome often confounds data interpretation, particularly when the ensuing cellular responses are not fully understood. The obvious solution to this problem is to completely "gut" the adenovirus genome, leaving behind the minimal cis-acting sequences needed for replicative synthesis (103 bp ITRs) and packaging (A repeats I-VII). The feasibility of a fully[6,7] or nearly fully[8] deleted adenovirus vector has been demonstrated by several groups, with the most intriguing results reported by Lieber et al[8] Using the Cre-lox P recombination system to excise E1, E2, and late gene domains, an adenovirus vector of 9 kb was produced and characterized. Although the deleted vector presented no evidence of hepatocellular injury in immunocompetent mice following intravenous administration, genome stability was severely compromised compared to a control E1-deleted first generation

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vector. It is obviously too early to extrapolate these findings; however, one interpretation suggests that a point of diminishing returns is eventually reached as DNA segments are systematically removed from the adenovirus chromosome.

The contrarian approach to vector design starts with the premise that the archetypical viral vector can be resolved into three functional moieties: a vehicle for uptake and delivery, cis-acting sequences for replication and packaging in production cells, and ancillary elements for promoting stability or maintenance of the recombinant genome once delivered to the nucleus. Applying this mold to adenovirus, two of the above three criteria are duly satisfied. First, the vehicle is an icosahedral protein capsid that is relatively stable, displays a remarkably efficient uptake mechanism, is amenable to protein modification, and can package large segments of DNA. Second, cis-acting elements for initiating replicative DNA synthesis and packaging are provided by the ITRs and A repeats, respectively. Yet, when the onus is shifted to identifying a mechanism for maintaining adenovirus vector integrity, the answer becomes less obvious. One conceivable option is to enlist the viral machinery encoded by the E2 region (i.e., 55 kDa terminal protein, 72 kDa DBP, and 140 kDa DNA polymerase) that normally mediates adenovirus replication. There is clearly merit to this scenario, as the adenovirus genome has been shown to persist as a replicon in its native wild type configuration[9] and as a recombinant vector.[8] Unfortunately, this action immediately reconstitutes the threat of stimulating cellular immunity and rejection, unless of course there exists anacceptable threshold. Still, there is the possibility that an as of yet characterized mechanism for episomal maintenance can be harnessed that does not involve replication[5] or viral gene expression.

An alternative strategy for stabilizing adenovirus mediated gene transfer seeks to recruit a heterologous hotspot capable of driving recombination with host cell DNA. A likely candidate for this role are the 146 nt inverted terminal repeats (ITRs) from adeno-associated virus (AAV).[10] These palindromic cis-acting elements are embedded with a set of instructions for efficient integration of the single-stranded DNA genome, thereby establishing non-pathogenic latent infections.[11] Structural analysis of AAV provirus reveals a mechanism that culminates in the formation of tandem concatomers preferentially targeted to specific sites (called AAVS1) on human chromosome 19q13.3-qter.[12,13] While the AAV genome also contains regulatory (*rep*) and structural (*cap*) genes, protein expression does not appear to be required for integration. This is supported by the biology of recombinant AAV vectors (rAAV) that have been deleted of all viral open reading frames in favor of a heterologous minigene.[14] Studies in cell culture[14,15] and animal models[16-20] suggest that rAAV gene transfer models the latent phase of the virus life cycle; however, site-specific integration appears to be lost due to the absence of AAV *Rep* genes.[21]

Adenovirus-AAV Blueprint

Important for the development of a hybrid Ad.AAV vector, attempts to sequester the gene transfer properties of the AAV ITRs in the context of plasmid DNA have yielded promising results; these constructs are essentially the same cis-acting reagents that serve as replication templates during lytic production of recombinant virus.[14] Shelling and Smith[22] characterized the arrangement of provirus DNA in transduced colonies that grew from cells transfected with an AAV plasmid or infected with recombinant virus. They found that the organization of integrated rAAV was not only similar between the two delivery methods, but recombination targeted the AAVS1 site on chromosome 19. Both the plasmid and viral vehicles expressed AAV Rep proteins, suggesting a recombination mechanism that likely models the wild type virus. More recently, Balague et al[23] studied the structural organization of integrated provirus derived from rAAV plasmids, and the role of AAV Rep78 expression. Stable colonies of transduced cells emerged at a significantly higher rate in cultures

transfected with rAAV plasmids, and this level was increased an additional 10-fold when supplemented with recombinant Rep78 protein. Southern blot analysis revealed integration was targeted to the AAVS1 site in more than 50% of the clones that grew from cultures transfected with rAAV plasmid in combination with Rep78. Furthermore, the provirus structure was predominantly organized in tandem concatamers. The significance of this work can be summarized as follows. First, the findings support the hypothesis that double-stranded rAAV vectors are templates for recombination with host cell DNA. Second, the mechanism appears to mirror viral latency based on the provirus structure. And, third, inclusion of Rep is not only important for high frequency integration, but it directs the recombinant AAV element to the AAVS1 site on chromosome 19.

Analogous to AAV plasmids, the underlying goal of a hybrid Ad.AAV vector is to harness the attributes of the AAV ITRs and donate them to recombinant adenovirus. By using adenovirus as a surrogate vehicle, AAV inherits a larger capsid for packaging and the prospect for improved production yields. Although largely conjecture at this stage, we envision a stratagem that models Odysseus' "Trojan Horse"; the vector enters the nucleus as a unit, yet only the rAAV domain is sequestered by the host cell chromatin. The proposed life cycle of the Ad.AAV vector is conceptually organized into three sequential phases, each of which is represented by a unique structural derivative of the nascent rAAV sequence. The cascade initiates with the rescue of the rAAV domain from the adenovirus chromosome (Phase I), an event that minimally requires the conversion of the linear duplex AAV ITR into a Holiday-like cruciform structure followed by symmetrical endonuclease cleavage. The product of this reaction is a linear, duplex rAAV episome with covalently closed, T-shaped ITRs at both ends. These theoretical predictions are based on in vitro replication assays using plasmid models of AAV provirus,[24,25] and possibly require expression of Rep proteins (Rep78/68) for hairpin formation and excision, although cellular proteins have also been implicated.[24,026]

The fate of the linear episomal duplex rAAV intermediate produced at Phase I of the Ad.AAV transduction mechanism is largely determined by the permissiveness of the cell. In the context of E1-expressing 293 cells, the prototype hybrid vector supplies a full complement of adenovirus helper functions sufficient to trigger an AAV lytic infection, contingent upon transcomplementation of deleted AAV *rep* and *cap* genes (Figs. 19.1, 19.2). Indeed, we[27] and others[28] have shown that the Ad.AAV system is a novel reagent for the production of high titer rAAV vector stocks (Fig. 19.2). For somatic gene transfer applications, however, it will be imperative to establish an intracellular milieu that favors integration of the mobilized rAAV element (Fig. 19.1). First and foremost, we anticipate that the adenovirus chromosome will have to be deleted of all (or most) viral open reading frames, leaving behind only the cis-acting domains for vector DNA synthesis (ITRs) and packaging (A repeats) (Fig. 19.3). This engineering advance will prevent the expression of adenovirus proteins capable of conditioning the cell for a lytic episode, reduce the chances for T cell activation or other adverse cellular response, and permit the packaging and delivery of large integrating DNAs. A second modification that will likely find its way into the composite Ad.AAV formula is a means for supplying regulated Rep protein expression during gene transfer. In addition to aiding rAAV rescue, Rep proteins should facilitate site-specific integration to AAVS1 sites on human chromosome 19.[22,23] Melding this ancillary function into the Ad.AAV vector is potentially complicated, however, due to the well described inhibitory effect of Rep proteins on adenovirus replication and production.[10] One strategy that was successfully exploited in our preliminary studies relied on poly-L-lysine conjugate technology, enabling a Rep plasmid to be electrostatically complexed to the Ad.AAV capsid.[27] Despite the high efficiency of this transfection system in cell culture, in vivo applications will be best served by cloning a Rep minigene directly into the adenovirus chromosome, possibly under the control of aninducible

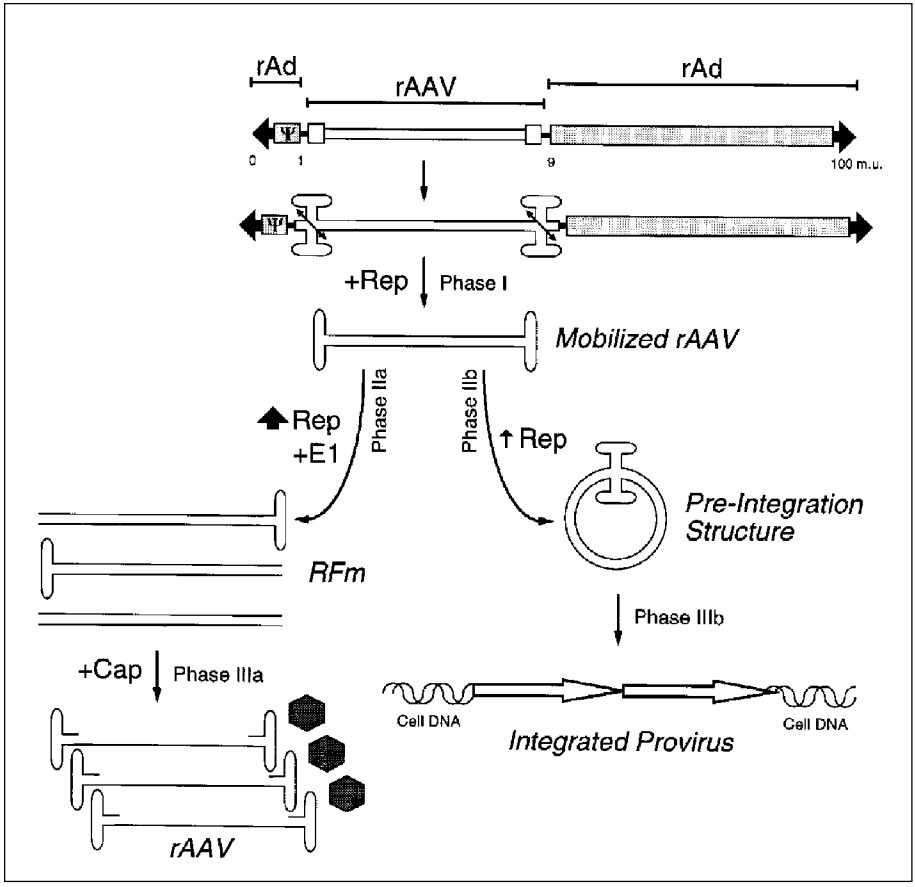

Fig.19.1. Life cycle of a hybrid Ad.AAV vector. A cartoon of the prototype Ad.AAV vector is shown at the top of the figure. The recombinant adenovirus chromosome (dl7001, labeled rAd) is deleted of E1 and E3 regions, but retains cis-acting sequences for replication (ITRs, solid arrowheads) and packaging (A repeats, shaded box labeled ψ). Recombinant AAV (labeled rAAV) is cloned into the E1 region and includes a heterologous minigene (open rectangle) flanked by ITRs (open squares). Shortly after being delivered to the nucleus of an infected cell, the linear duplex AAV ITRs are converted to a Holiday-like cruciform conformation. This structure is an effective substrate for symmetrical endonuclease cleavage, releasing the rAAV domain from the adenovirus chromosome (Phase I). In the presence of unregulated AAV Rep protein expression (Phase IIa), the covalently closed ITRs of the mobilized rAAV are resolved, allowing for replicative synthesis of duplex intermediates (labeled RFm); this event is greatly facilitated by adenovirus E1 proteins. Co-expression of AAV Cap proteins results in the accumulation of single-stranded rAAV genomes and progeny virus (Phase IIIa). If intracellular conditions are not permissive for AAV replication (i.e., limited expression of AAV and adenovirus regulatory proteins), the mobilized rAAV element is converted to a circular duplex pre-integration structure (Phase IIb), and eventually sequestered by host cell DNA (Phase IIIb). Circularization of episomal rAAV is predicted from the well established head-to-tail arrangement of integrated provirus (shown as tandem arrows in the figure).[29]

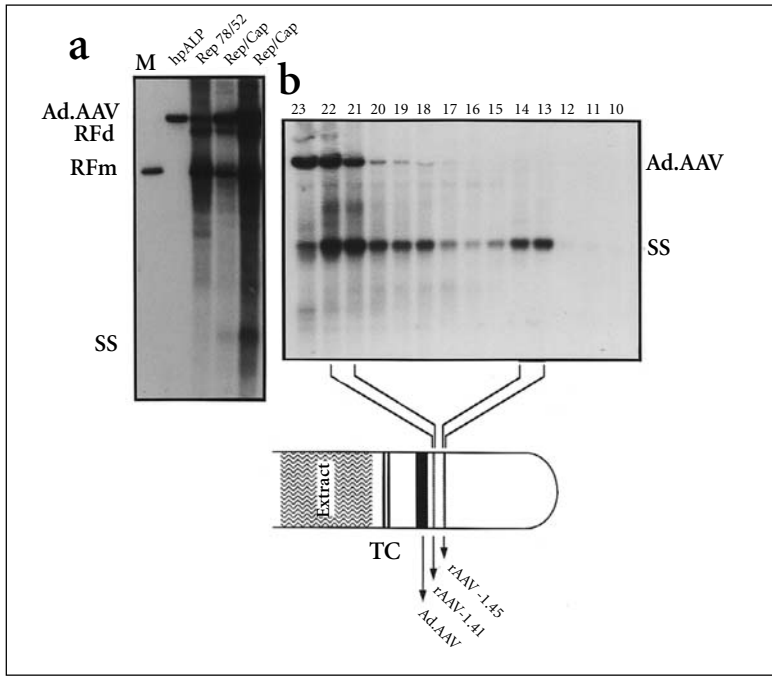

Fig 19.2. Rescue, replication, and production of rAAV in cells infected with a hybrid Ad.AAV vector. The capsid of a hybrid Ad.AAV vector was modified with poly-L-lysine, providing a means for condensing nucleic acids around the virion. The resulting conjugate was complexed with plasmid DNA and added to 293 cells (called trans-infection). **Panel (a)** Southern blot analysis of low molecular weight DNA isolated from 293 cells trans-infected with Ad.AAV and plasmid that encodes human placenta alkaline phosphatase (lane 1, second from left, control), AAV Rep 78/52 kDa (lane 2), or the entire AAV rep/cap open reading frame (lanes 3 and 4). The marker lane (labeled M) contains a sample of linear duplex rAAV (4.8 kb). The data suggest Rep proteins are important for efficient rescue and replication of duplex rAAV (RFm and RFd), while production of progeny single-stranded genomes (SS) is additionally dependent on AAV *cap* gene expression. **Panel (b)** Southern blot analysis of a fractionated extract from 293 cells trans-infected with Ad.AAV and plasmid that encodes AAV *rep/cap* genes. Cells were harvested 45 h post-trans-infection and lysed by sequential rounds of freeze-thaw. The extract was layer onto a CsCl step gradient, banded to equilibrium, and fractions (1.0 ml) collected from the bottom of the centrifuge tube. Samples (5.0 μl) of fractions 10-23 were treated with AAV capsid digestion buffer (50 mM Tris-Cl pH 8.0, 1.0 mM EDTA pH 8.0, 0.5% SDS, and 1.0 mg/ml Proteinase K) and analyzed by Southern hybridization. A cartoon of the centrifuge tube from which the samples were taken is shown below the autoradiogram. The banding position of infectious hybrid Ad.AAV vector, empty hybrid virions (TC), infectious rAAV (rAAV-1.41), and a denser species of rAAV (rAAV-1.45) are shown. In both Panels (a) and (b), bands corresponding to the Ad.AAV vector, linear duplex monomer (RFm) and dimer (RFd) forms of rAAV following rescue and replication, and single-stranded rAAV genomes (SS) are indicated. The blots were hybridized with a [^{32}P]-labeled restriction fragment of the rAAV domain. Reprinted with permission from Fisher KJ, Kelley WM, Burda JF et al. Human Gene Therapy 1996; 7:2079-2087. ©Mary Ann Liebert, Inc.

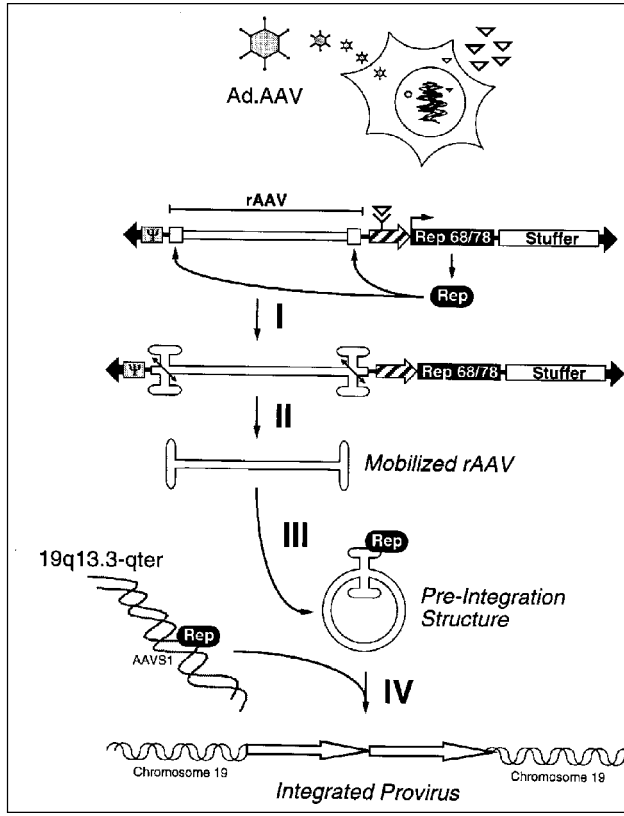

Fig.19.3. Proposed transduction mechanism of a composite Ad.AAV vector. The adenovirus chromosome is deleted of all open reading frames, retaining only cis-acting sequences for replication (ITRs; solid arrowheads) and packaging (A repeats; shaded box labeled ψ).[6] Recombinant AAV (labeled rAAV) in cloned into the E1 region and includes a heterologous minigene (open rectangle) flanked by ITRs (open squares). A minigene that encodes Rep 78/68 kDa proteins is positioned adjacent to the rAAV domain; the level of Rep expression is regulated by an inducible promoter (striped arrow). If necessary, stuffer sequence is inserted to yield a final recombinant chromosome that is $10<x<25$ kbp. The lower size requirement is important for the production of an infectious virion, while the upper limit facilitates removal of helper adenovirus during purification.[6] Starting at the top of the figure, a cell is infected with a composite Ad.AAV vector and subsequently treated with a compound (open triangles) that binds to response elements on the Rep heterologous promoter, thereby initiating transcription. Regulated Rep expression stimulates AAV ITR cruciform folding and cleavage (Phase I), liberating the rAAV domain from the surrogate recombinant chromosome (Phase II). The mobilized linear duplex rAAV intermediate is converted to a circular pre-integration structure (Phase III). An oligomeric complex of Rep 68/78 binds to the AAV ITRs and AAVS1 sites on human chromosome 19, acting to align the two molecules. Rep introduces a nick in the AAVS1 site initiating unidirectional cell DNA synthesis and strand displacement. Lagging strand synthesis begins on the displaced strand (which is attached to Rep), followed by a strand-switching step that attacks the circular AAV template. What ensues is a rolling-circle-like mechanism that displaces the lagging strand of the circular AAV. The newly synthesized strand, which contains an imprint of the AAV duplex, is eventually sequestered by host cell chromatin (Phase 1V). The above mechanism for AAV integration is adapted from Linden et al.[29]

promoter (Fig. 19.3). According to this arrangement, the Rep expression cassette is positioned outside the rAAV domain, limiting its persistence to that of the fully deleted adenovirus chromosome.

Conclusion

As with all emerging concepts in gene therapy vector development, a series of technical and theoretical hurdles challenges the efficacy of the composite Ad.AAV vector. Is efficient excision of the rAAV domain possible in the absence of adenovirus helper activity? Does the mobilized rAAV element contain the necessary information for undergoing recombination with host cell DNA? Although these and other aspects of the proposed transduction mechanism outlined in Figure 19.3 have yet to be experimentally validated, the fundamental premise that prompted the Ad.AAV concept remains intact. Adenovirus is an extremely versatile biological reagent that lends itself to extensive modification at both the protein and DNA levels. Our blueprint for an Ad.AAV vector seeks to exploit this property and in the process confer a mechanism for stable gene transfer. Adenovirus vectors have tremendous promise as gene vector drugs for the treatment of human diseases. However, in the absence of a defined mechanism for persistence, their role in clinical settings that demand long term transgene expression will remain a topic of debate.

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Chapter 20

Transcriptional and Promoter-Driven Control of Adenovirus-Mediated Gene Expression

Yoko Yoshida and Hirofumi Hamada

Transcriptionally targeted adenoviral vectors which are able to restrict and regulate the levels of expression of the therapeutic gene will have wide applicability for gene therapy. For cancer gene therapy, tumor-specific promoters could be used to drive drug sensitivity genes (e.g., HSV-tk, cytosine deaminase) for suicide gene therapy,[1] or E1A gene for restricted replication-competent adenovirus-mediated gene therapy. Candidate promoters are α-fetoprotein (AFP) promoter/enhancer, carcinoembryonic antigen (CEA) promoter, prostate-specific antigen (PSA) promoter, and so on (for review, see ref. 1). In addition to the tissue-specific promoter systems, inducible gene expression systems (for review, see ref. 2) are also applicable for the control of adenovirus-mediated gene expression. In this chapter, first we make a brief review of the applications of adenoviruses with the tissue-specific promoters, with an emphasis on cancer gene therapy. Then we demonstrate our successful application of the tetracycline-inducible system for adenoviral vectors.

Transcriptional and Promoter-Driven Targeting of Adenoviral Vectors

Candidate cis-acting sequences which could be used for cancer gene therapy are AFP for hepatoma, CEA for lung and gastric cancers, erbB2 for breast and pancreas cancers, DF3/MUC1 for breast cancer, tyrosinase for melanoma, PSA for prostate cancer, and so on.[1] Since some of these genes do not show a strict tumoral specificity, it is necessary to combine additional strategies to further restrict the expression to tumors, such as delivering with fiber-mutant adenoviral vectors which have altered tropism of target cells, or E1B55K-deficient adenoviral vectors which replicate preferentially in p53-defective tumor cells.[3]

The AFP gene is normally expressed in fetal liver and is transcriptionally silent in adult liver but overexpressed in human hepatocellular carcinoma (HCC). It has been shown that utilization of the AFP promoter/enhancer in an adenoviral vector can confer selective expression of a heterologous suicide gene in AFP-producing HCC cells in vitro as well as in vivo.[4-8] It has been demonstrated that the adenoviral vectors with the human AFP promoter/enhancer can be used to express the HSV-tk gene,[4-6] the *E. Coli* cytosine deaminase (CD) gene,[7] and the interleukin 2 gene[8] in AFP-producing HCC cell lines. Expression of the HSV-tk or the CD gene by the adenovirus with the AFP promoter/enhancer induced cells sensitive to ganciclovir (GCV) or 5-fluorocytosine (5FC), respectively, in the AFP-producing

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cells but not in the AFP-nonproducing cells. When the adenovirus for the β-galactosidase gene (lacZ) driven by the AFP promoter/enhancer was injected into established HCC tumors in vivo, expression of the β-galactosidase gene was confined to AFP-producing HCC xenografts.[7] Moreover, HCC xenografts regressed upon in vivo transduction with the CD gene followed by treatment with 5FC.[7]

Currently available in vivo gene transfer vectors are not capable of transferring a gene to all tumor cells. Therefore, successful application of suicide gene therapy in vivo relies on the bystander effect, where the active chemotherapeutic agent diffuses from the tumor cells in which it was produced to neighboring malignant cells in sufficient concentrations to suppress growth. An in vitro bystander effect was observed when only 10% of the cells were infected with the adenovirus for the HSVtk driven by the AFP promoter/enhancer.[6] Unlike the bystander effect in the HSVtk/GCV system, direct cellular contact is not necessary for the neighbor cell killing effect of the CD/5FC system.[9] The converted 5FU is able to diffuse across the cell membrane into adjacent cells and tumor tissues. In vivo adenovirus-mediated transfer of the CD gene to HCC tumors in nude mice effectively suppressed the growth of tumors, despite the fact that adenovirus infection was associated with the expression of the transferred gene in only a few percent of the tumor cells.[7]

Since HCC patients often have varying sizes of multiple tumors in the liver without extrahepatic metastasis, gene delivery to the liver tumor in vivo should be performed via hepatic artery or portal vein. Since toxicity was observed in experimental animal studies at high doses of recombinant adenovirus, it is important to determine whether we could use optimal amounts of the virus without hepatic damage. The elevated serum levels of AFP among these patients are mainly produced by tumors. However, surrounding normal hepatocytes may often express AFP to some degree. Cytotoxicity may be induced by HSVtk/GCV or CD/5FC treatment. In view of the clinical aspects of HCC, safety studies of these vectors should be performed.

CEA is expressed in a large percentage of colorectal carcinomas, other gastrointestinal carcinomas, breast and lung cancers. Unlike AFP promoter/enhancer, which has been thoroughly characterized, the CEA promoter needs more precise structural analysis. The group of Huber reported a DNA sequence and a functional analysis of 14.5 kb of CEA 5′ sequences.[10] Cis-acting sequences were identified that direct high level, specific expression of a reporter for the CD gene in CEA-positive colon carcinoma lines.[10] A CEA promoter sequence has been utilized to drive HSVtk, CD, or *E. Coli* phosphoribosyltransferase (UPRT) in recombinant adenoviral vectors.[11,12]

The prostate specific antigen (PSA) is a well characterized prostate-specific protein. A 5' 640 bp flanking sequence of the PSA gene has been studied and identified as a promoter sequence for the PSA gene.[13] Pang et al[14] have recently demonstrated that an 822 bp 5′ PSA gene fragment can dramatically increase gene expression when combined with the previously identified PSA promoter while preserving tissue specificity and androgen responsiveness. Rodriguez et al[15] reported a construction of an attenuated replication competent adenovirus CN706, in which the E1A gene was placed under the control of the PSA enhancer/promoter. The CN706 demonstrated a selective cytotoxicity toward PSA-expressing prostate cancer cells.[15]

Tetracycline-Inducible System for Adenoviral Vectors

From a technical point of view, it has been very difficult to generate recombinant adenoviruses encoding genes for cytotoxic products (e.g., vesicular stomatitis virus G-protein (VSV-G), rep gene of adeno-associated virus, and various cell cycle- and apoptosis-related genes) under the control of constitutively active promoters. Such foreign gene products are too toxic for the host cells to propagate the recombinant adenoviruses. One way to overcome

this technical difficulty is to use inducible promoters. In the absence of the inducer, the minimum "leaky" expression of cytotoxic products under the inducible promoter remains low enough for ample viral propagation. Once a high titer viral stock is obtained, high level gene expression is attained by simultaneous administration or expression of the inducer.

Gossen and Bujard developed tetracycline-controllable expression vectors for mammalian cells.[16] In this system, the tet repressor is combined with the C-terminal domain of VP16 from herpes simplex virus. The chimeric tetracycline-controlled transactivator (tTA) drives transcription of the Tet promoter (Tet) which consists of a minimal promoter (PhCMV*) fused to seven repetitive tetracycline operator (tetO) sequences.[16] Tetracycline prevents tTA from binding to the tetO sequences and the Tet promoter is silent. In the absence of tetracycline, tTA binding activates gene expression. The primary limitation of this system is the difficulty in expressing high levels of the tTA protein.[17] So far, the best results (i.e., tight regulation and large induction factors) have been obtained when cell lines stably expressing tTA were generated and tested for functionality.[17] Rigorous screening for tight tetracycline-regulatable expression is required, as individual clones may show wide variability in their expression pattern. Since transfected genes integrate at different chromosomal locations, some clones show considerable "leakiness" in the presence of tetracycline, while others may show low levels of expression or even be silent. The sequential transfection, cloning and screening steps take several months to generate a cell line for further study.[17] Instead of these laborious procedures to generate stable cell lines expressing tTA, we utilized recombinant adenoviruses for transient tTA expression. We attained tight tetracycline-regulatable gene expression by introducing tTA with NLS (NtTA).[18-19]

The transcriptional units of the recombinant adenoviruses generated in our study are schematically summarized in Fig. 20.1. We monitored gene transduction by the adenoviral vector AxTetZ at a MOI of 250 co-infected with AxCMtTA at various MOI (Fig. 20.2A). The basal "leaky" expression of lacZ by AxTetZ infection at a MOI of 250 was observed in less than 1.5% of the total cells, which was not affected in the presence or absence of 10 μg/ml tetracycline. When cells were co-infected with AxTetZ and AxCMtTA, a moderately high level expression of lacZ gene was achieved. The transduction efficiency was 29% at a MOI of 250 of each virus. Unexpectedly, the lacZ expression through the AxTetZ and AxCMtTA combination was not sufficiently suppressed by tetracycline. In the presence of 10 μg/ml tetracycline, 15% of the total cells expressed β-Gal; only 50% suppression of β-Gal expression was obtained (Fig. 20.2A).

Since the tTA fusion protein works in the nucleus, we speculated that including a nuclear localization signal (NLS) in the tTA protein might improve the tTA function. We utilized the NLS from SV40 T antigen[20] and generated a fusion protein, NtTA, which contains NLS at the amino-terminal of tTA. When cells were co-infected with AxTetZ and AxCM-NtTA, a remarkably higher level of lacZ gene expression was achieved (Fig. 20.2A). The transduction efficiency was 84% at a MOI of 250 of each virus. Moreover, the β-Gal expression through AxTetZ and AxCM-NtTA combination was fully suppressed by tetracycline. In the presence of 10 μg/ml tetracycline, β-Gal expression was at the minimal basal level obtained by the control AxTetZ infection without tTA coexpression. Thus, adding the NLS sequence to the N-terminal of the original tTA caused a remarkable improvement in the tetracycline-controllability.

tTA without NLS was reported to localize both in the cytoplasmic and in the nuclear extracts.[16] As shown in Fig. 20.2A, the efficiency of the lacZ gene transduction by AxCM-NtTA (i.e., tTA with NLS) in the absence of tetracycline was far better than that by AxCM-tTA (i.e., tTA without NLS). This may simply be due to the quantitative difference in the tTA molecules localized in the nuclei. In contrast, the lacZ gene expression by AxCM-NtTA was completely suppressed in the presence of tetracycline, while that by AxCM-tTA resulted in

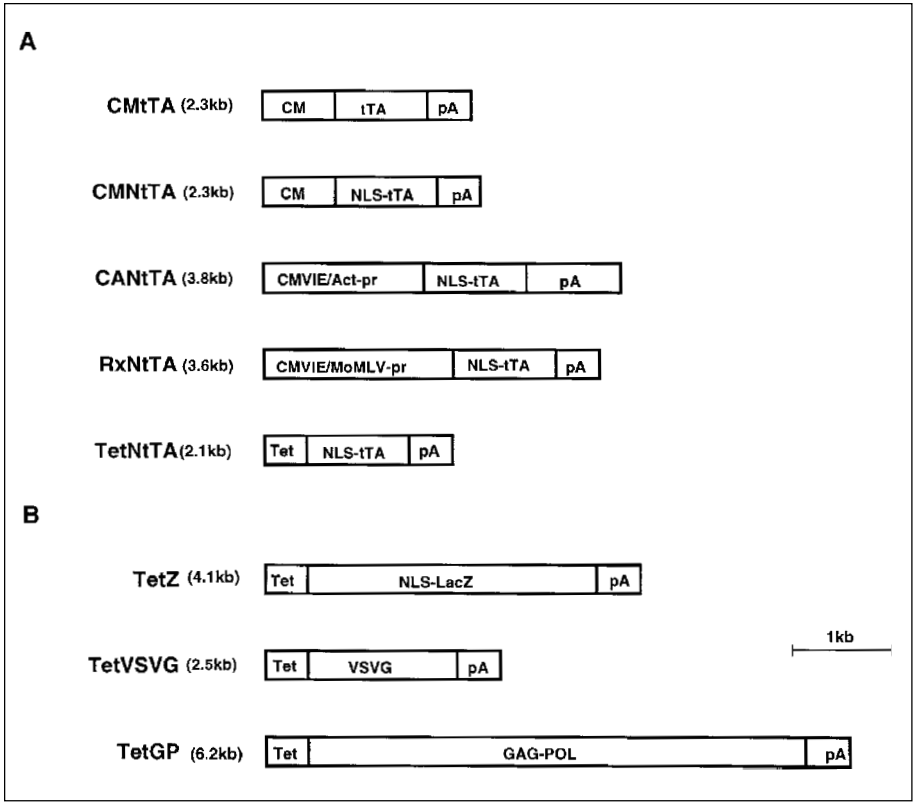

Fig. 20.1. Summary of transcriptional units constructed into recombinant adenoviruses.

only 50% suppression with tetracycline (Fig. 20.2A). We can not fully explain the qualitative difference in this result simply by the quantity of the tTA molecules localized in the nuclei. It is possible that the amino acid sequence of the NLS[20] may have produced some biochemical properties which result in more effective suppression of the tTA-binding with the Tet promoter. Although the precise mechanism of functional difference between tTA and NtTA remains to be studied, we concluded that the use of NtTA is advantageous for practical applications of the tetracycline-inducible system.

To choose an appropriate promoter for NtTA, we generated adenoviral constructs with NtTA driven by various promoters. Strong constitutive promoters such as CA[21] and Rx[22] showed very high levels of β-Gal expression. At a MOI of 250, the combination of AxTetZ with these NtTA expression viruses resulted in 80-85% lacZ gene transduction (Fig. 20.2B). Tetracycline administration effectively suppressed the reporter gene expression, down to the minimal basal expression (Fig. 20.2B). Schockett et al[23] reported that the autoregulatory expression system (pTet-tTAk) showed an improvement over the constitutive expression system (pcDNA-tTAk). We also tested the autoregulatory system in which the NtTA gene was driven under the control of the Tet promoter. In our system using adenovirus-mediated gene transduction, the autoregulatory expression system (AxTetNtTA) did not reveal any significant advantage over the constitutive expression promoters (i.e., CM, CA, and Rx).

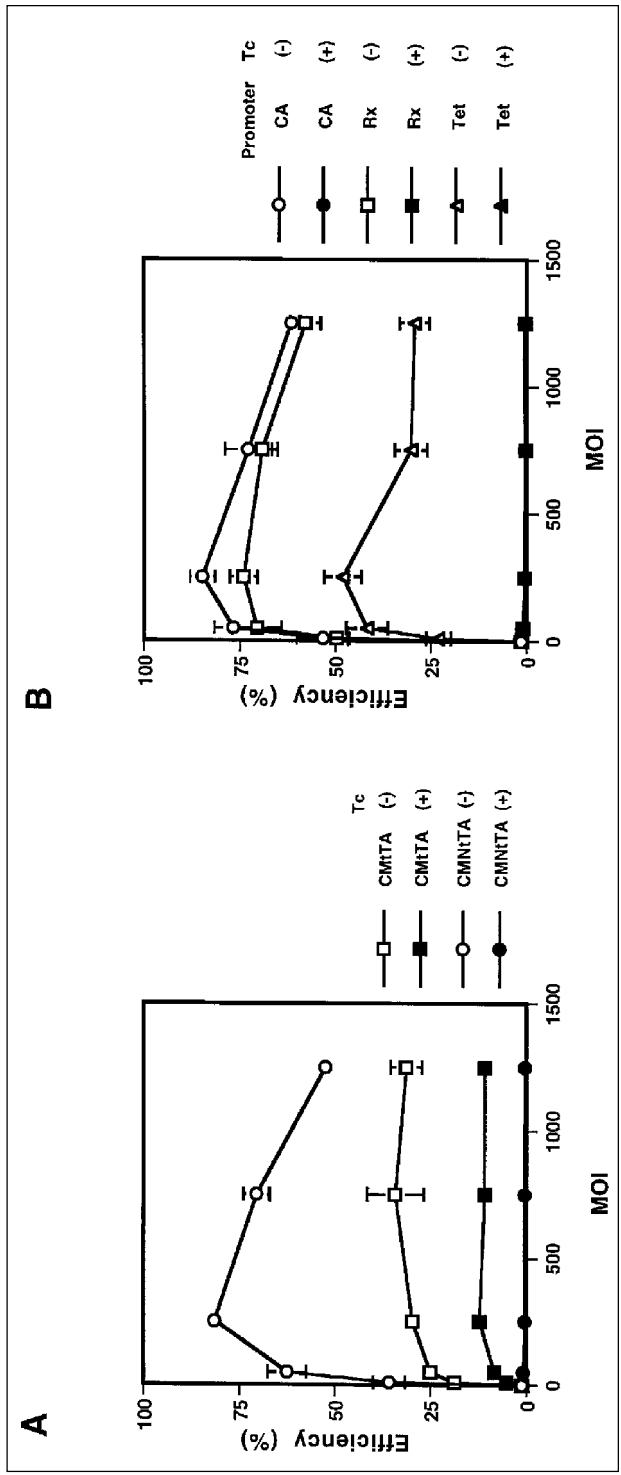

Fig. 20.2. Adenovirus-mediated inducible gene expression by tTA or NtTA (tTA with NLS). (A) Transduction efficiency of adenoviral vectors in HeLa cells was tested using the reporter adenovirus AxTetZ in combination with either AxCMtTA or AxCM-NtTA. Exponentially growing cells were seeded onto duplicate 12-well tissue culture plates at 5 x 10^4 cells per well. On the next day, serial dilution of adenoviruses (tTA or NtTA) were co-infected with AxTetZ at an MOI of 250, and cells were cultured in the presence or absence of 10 µg/ml tetracycline (Tc). Forty hours after infection, cells were fixed with 0.5% glutaraldehyde and stained with X-Gal. The number of LacZ-positive and -negative cells in three randomized fields of each well was recorded. Transduction efficiency is presented as % LacZ-positive cells. Experiments were repeated three times. (B) Comparison of various promoters for NtTA in the adenovirus-mediated inducible gene expression. Transduction efficiency of adenoviral vectors in HeLa cells was tested using the reporter adenovirus AxTetZ (moi 250) in combination with serial dilution of AxCA-NtTA, AxRxNtTA, or AxTetNtTA in the presence or absence of 10µg/ml tetracycline. Transduction efficiency is presented as % LacZ-positive cells. Experiments were repeated three times.

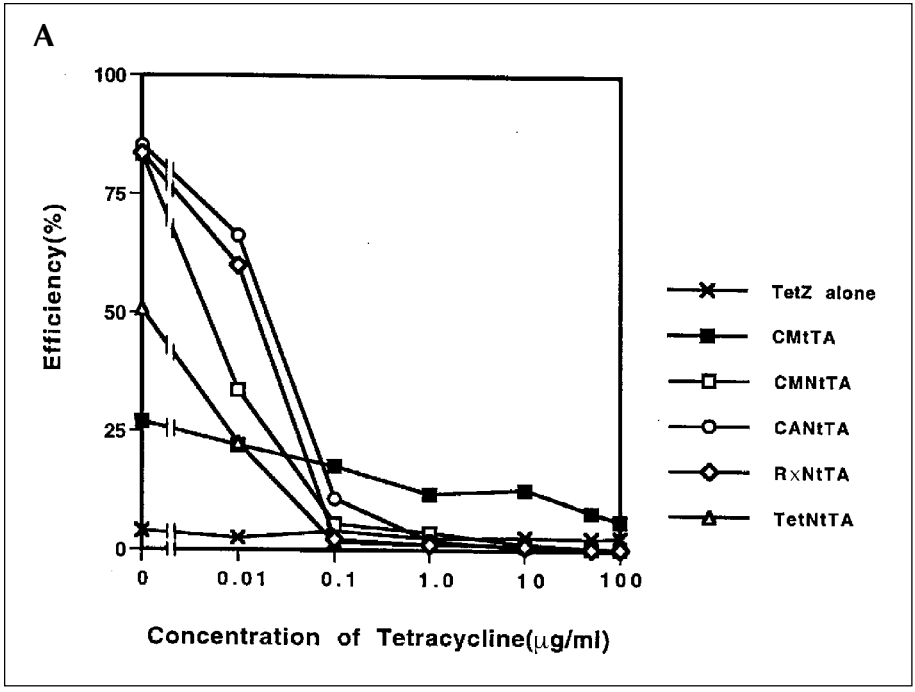

Fig. 20.3A. Tetracycline dependency of adenovirus-mediated gene expression by tTA or NtTA. (A) Tetracycline dose dependency. Transduction efficiency of adenoviral vectors in HeLa cells was tested using the reporter adenovirus AxTetZ (moi 250) in combination with AxCMtTA, AxCM-NtTA, AxCA-NtTA, AxRxNtTA, or AxTetNtTA (each at moi of 250) in the presence of serial dilution of tetracycline. Transduction efficiency is presented as % LacZ-positive cells. (B) (see opposite page) Representative X-Gal staining features. Transduction efficiency of adenoviral vectors in HeLa cells was tested using the reporter adenovirus AxTetZ (moi 250) alone (a, b) or in combination with moi 250 each of AxCMtTA (c, d), AxCM-NtTA (e, f), AxCA-NtTA (g, h), AxRxNtTA (i, j), or AxTetNtTA (k, l), either in the presence (b, d, f, h, j, l) or absence (a, c, e, g, i [ai], k) of 10 μg/ml tetracycline.

The reporter gene transduction of the autoregulatory system (TetNtTA) was significantly less efficient in the absence of tetracycline (Fig. 20.2B).

Tetracycline dose response relationship in the Tet/tTA system is shown in Figure 20.3A. With tTA (i.e., without NLS) as a transactivator, the transduction efficiency was less than 30%, and tetracycline downregulation was only 50% at 1 to 10 μg/ml. Even at 100 μg/ml of tetracycline, which was cytotoxic to mammalian cells, tetracycline-induced downregulation was not efficient (80% suppression). In clear contrast, we attained tight tetracycline-controllability by NtTA (i.e., tTA with NLS). Every promoter studied (i.e., CM, CA, Rx, and Tet) demonstrated a strict tetracycline controllability at 1 μg/ml of tetracycline and above (Fig. 20.3A). In Figure 20.3B, we demonstrate the gene transduction efficiency of the Tet/tTA system using reporter adenovirus AxTetZ at a MOI of 250 with various transactivator (i.e., tTA or NtTA) adenoviruses (each at a MOI of 250) in the presence or absence of 10 μg/ml tetracycline.

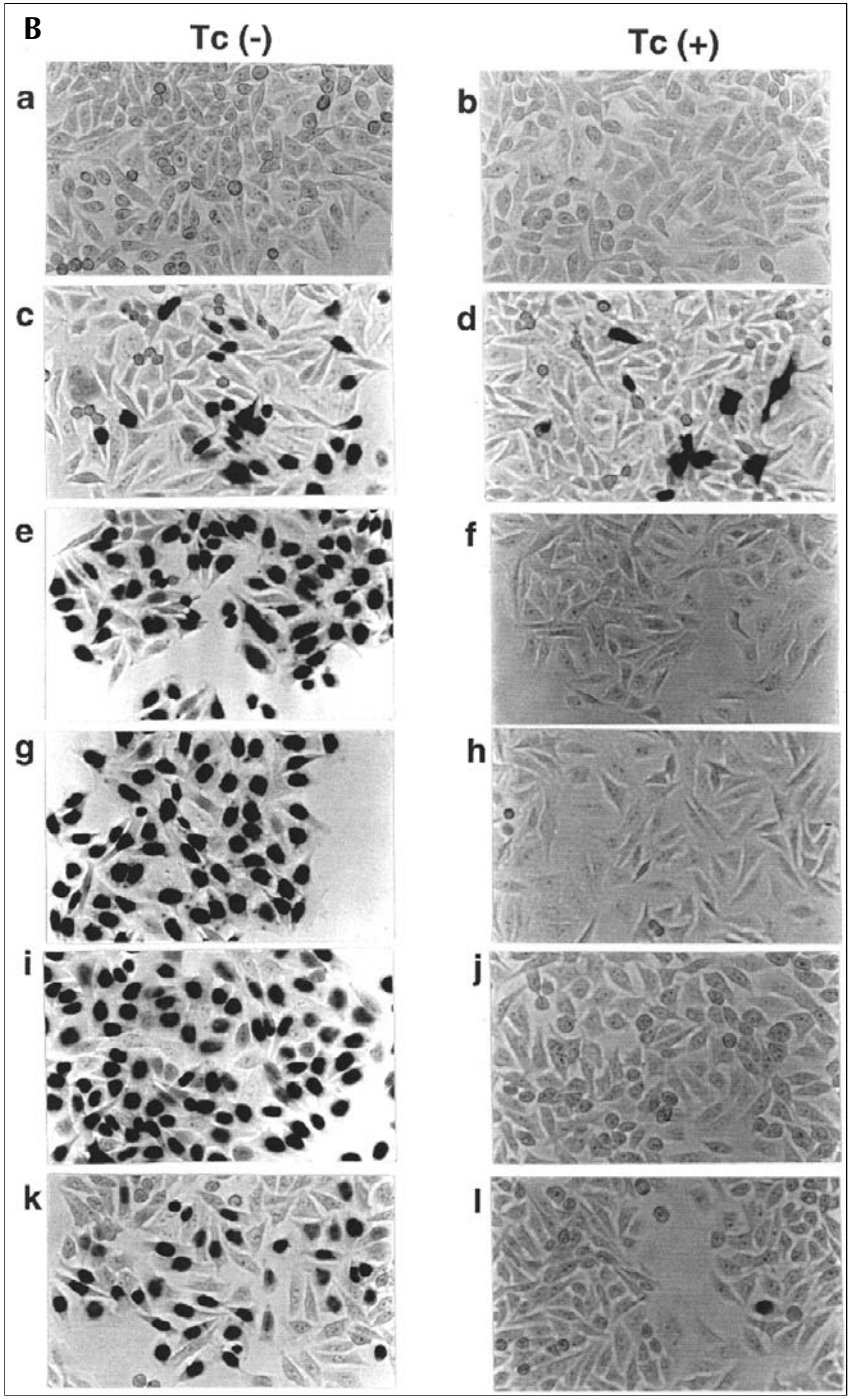

Fig. 20.3B. For detail see Fig 20.3 A

VSVG-Pseudotyped Retroviral Packaging System Through Adenovirus-Mediated Inducible Gene Transduction

VSVG-pseudotyped retroviral vectors are promising due to their high titer and broad host range (for review, see ref. 24). However, since VSV-G protein is toxic to cells, it is not possible to obtain stable cell lines with high level production of pseudotyped viral vectors.[25,26] To circumvent this technical difficulty, we used recombinant adenoviruses for inducible VSV-G gene transduction. Since we failed to get the adenovirus AxCA-VSVG with constitutive expression of the VSV-G gene, we applied the tetracycline-controllable gene expression system for VSV-G gene expression. The minimum "leaky" expression of VSV-G under control of Tet promoter apparently did not hamper the generation of recombinant adenovirus AxTetVSVG (VSV-G gene under the Tet promoter), since we obtained a high titer (4 x 10^9 pfu/ml) adenoviral stock solution of AxTetVSVG. Double infection of AxTetVSVG and AxRx-NtTA resulted in the expression of the VSV-G gene product in nearly 100% of the target cells, demonstrating the feasibility of a high level expression of the VSV-G gene driven by the Tet promoter.[19]

Although a number of retroviral packaging host cell lines have been used for basic and clinical studies for gene therapy, most of them are of either murine (i.e., NIH3T3) or human (i.e., 293) fibroblast origin. It remains to be studied systematically what cell lines are appropriate hosts of retroviral production for human gene therapy. In this study, we developed an adenovirus-mediated VSVG-pseudotype retroviral packaging system by using a tetracycline-inducible expression system. A recombinant adenovirus, AxTetGP, was generated to express MoMLV gag-pol gene under the control of the Tet promoter. AxCA-NtTA was used to transactivate the Tet promoter. We transfected packaging host cells with the reporter MFGlacZ retrovirus vector to monitor retroviral titers.[19] Simultaneous triple introduction of AxTetGP, AxTetVSVG, and AxCA-NtTA into human glioma cell lines U373 and T98G resulted in production of high titer (2-5 x 10^5 infectious units per ml) VSVG-pseudotyped retroviruses.[19] Much higher titers (more than 10^7/ml) were obtained by simple centrifuge concentration[26] of the virus from culture fluids at 50-80% yield efficiency.[19] Thus, the glioma lines U373 and T98G are good candidates for retroviral packaging hosts.

Future Applications

There are several advantages in the adenovirus-mediated tetracycline-inducible gene transduction system:

1. High titer (more than 10^9 pfu/ml) viral stock solutions are easily prepared;
2. A wide host range of adenoviral vector broadens applicability of the method;
3. Compared with conventional DNA transfection methods, adenovirus-mediated gene transduction is highly effective, often resulting in nearly 100% gene transduction of target cells;
4. The gene expression could be controlled at several steps (i.e., the introduced DNA copy number by adenoviral dosage, and the regulation of tTA function by tetracycline dosage).

The adenovirus-mediated inducible gene expression system will be useful for basic studies on protein functions in a wide variety of cells, including primary cultured cells, which are difficult to transduce by conventional transfection methods. Controllable expression of cytotoxic viral proteins by our method could also be useful for practical applications such as antiviral vaccine production and pseudotype viral vector generation, the latter of which we have demonstrated here.

The technology for adenovirus-mediated transient production of VSVG-pseudotyped retroviruses appears to have wide utility. It is useful for producing various pseudotyped

retroviral mutants, and for comparative studies using different cell lines as retroviral packaging hosts. Moreover, the method is applicable for rapidly testing retrovirus-related genes and gene products in various cells, including clinical samples from patients who receive gene therapy.

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CHAPTER 21

Development of an E1B, 55 kDa Gene-Deleted, Selectively Replicating Adenovirus for the Treatment of Cancer: ONYX-015

David H. Kirn

The vast majority of human cancers are incurable once metastatic. Chemotherapy and radiotherapy can induce tumor growth inhibition or regression in some cases, but solid tumor progression and resistance to these standard therapeutic modalities inevitably develops. Therefore, new agents with larger therapeutic indices between cancer cells and normal cells are needed. Viruses have been used as gene delivery vectors to cause cancer cell inhibition or killing.[1-3] One of the major difficulties with this approach, however, is the daunting goal of delivering genes to every cancer cell in the body.

In contrast, a replicating viral therapeutic can potentially overcome this limitation. Virus replication in a small fraction of the tumor cells can lead to amplification and spread of the antitumoral effect.[4] Cell killing can be due to viral replication and cell lysis exclusively, or this could be augmented by including additional immunostimulatory or toxin-producing genes.

Development of ONYX-015 (dl1520)

p53 is mutated in roughly fifty percent of all human cancers, including non-small cell lung (60%), colon (50%), breast (40%), head and neck (60%) and ovarian (60%) cancers in the advanced stages.[5] Loss of p53 function is associated with resistance to chemotherapy and/or decreased survival in numerous tumor types, including breast, colon, bladder, ovarian and non-small cell lung cancers.[5] Therefore, effective therapies for tumors that lack functional p53 are clearly needed.

p53 mediates cell cycle arrest and/or apoptosis in response to DNA damage (e.g., due to chemotherapy or radiation) or foreign DNA synthesis (e.g., during virus replication). Consequently, DNA tumor viruses such as adenovirus, SV40 and human papilloma virus encode for proteins that inactivate p53 and thereby allow efficient viral replication. For example, the adenovirus E1B-region 55 kDa protein binds and inactivates p53, in complex with the E4 ORF6 protein.[6]

Since p53 function must be blocked in order to allow efficient virus replication, it was hypothesized that an adenovirus lacking E1B, 55 kDa gene expression might be severely limited in its ability to replicate in normal cells; however, cancer cells that lack p53 function should support virus replication and resultant cell destruction. ONYX-015 (ONYX

Adenoviruses: Basic Biology to Gene Therapy, edited by Prem Seth.

Pharmaceuticals, Richmond, CA) is an attenuated adenovirus type 2/5 chimera (dl1520) with two mutations in the early region E1B, 55 kDa gene; this virus was created in the laboratory of Dr. Arnie Berk.[7] The cytopathic effects of wild type adenovirus and ONYX-015 were studied on a pair of cell lines that are identical except for p53 function: the RKO human colon cancer cell line with normal p53 function (the parent line), and an RKO subclone transfected with dominant-negative p53 (courtesy of Dr. Michael Kastan).[4] As predicted, ONYX-015 induced cytopathic effects identical to wild type adenovirus in the subclone lacking functional p53, while cytopathic effects with ONYX-015 were reduced by approximately two orders of magnitude in the parental tumor line harboring normal p53. Subsequently, a tumor cell line which was resistant to ONYX-015 due to normal p53 function (U2OS) became sensitive to ONYX-015 following transfection and expression of the E1B, 55 kDa gene. Therefore, this data supports the hypothesis that ONYX-015 is able to replicate selectively in p53-deficient cancer cells due to a deletion in the E1B, 55 kDa gene.

Subsequent experiments demonstrated that primary (non-immortalized) human endothelial cells, fibroblasts, small airway cells and mammary epithelial cells were resistant to ONYX-015 replication and cytolysis, in contrast to effects seen with wild type adenovirus (Fig. 21.1).[8] Replication-dependent cytopathic effects were demonstrated in human tumor cell lines of many different histologies following infection with ONYX-015. Tumor cells that lack p53 function through different mechanisms (p53 gene mutation and/or deletion, or p53 degradation by human papilloma virus E6 protein) were shown to be destroyed by ONYX-015.[8] In addition, several carcinoma lines with normal p53 gene sequence, including two chemotherapy-resistant ovarian cancer subclones, were efficiently lysed. ONYX-015 had significant in vivo antitumoral activity against subcutaneous human tumor xenografts in nude mice following intratumoral or intravenous injection. Efficacy against intraperitoneal carcinoma was documented following intraperitoneal virus administration (Heise C, Ganley I, publication pending). Due to the lack of efficient replication in rodent cells, however, immunocompetent (syngeneic) tumor models have not been useful for studying replication-dependent effects. Therefore, the role of the antiviral and antitumoral immune responses may only be determined in cancer patients until a novel model is developed. Definitive proof of selective antitumoral activity, however, required controlled clinical trials.

Combination Therapy with a Replicating Adenovirus and Chemotherapeutics

In order to study potential interactions between ONYX-015 and chemotherapy in vivo, experiments were carried out with cisplatin and 5-FU (two chemotherapeutic agents commonly used to treat head and neck cancer patients) with ONYX-015 in the nude mouse-HLaC (head and neck) human tumor xenograft model.[8] Tumors were treated with intratumoral ONYX-015 followed by intraperitoneal cisplatin or 5-FU on days 8-12 . Four groups of mice were treated with one of the following regimens: ONYX-015 plus chemotherapy (cisplatin or 5-FU), chemotherapy alone (cisplatin or 5-FU), ONYX-015 alone, or vehicles alone. All treatment groups received identical injections of the active agent or vehicle control into both the tumor and peritoneum. Unlike cisplatin or 5-FU alone, treatment with ONYX-015 alone significantly increased survival times versus placebo ($p=0.01$). The combination of cisplatin or 5-FU with ONYX-015 was more effective than chemotherapy or virus treatment alone. Similar results have been reported with other tumor types in vivo. Subsequent studies of ONYX-015 in combination with cisplatin and 5-FU have demonstrated that simultaneous administration of ONYX-015 and chemotherapy, or ONYX-015 first, is superior to chemotherapy followed by ONYX-015 (D. Kirn, unpublished data).

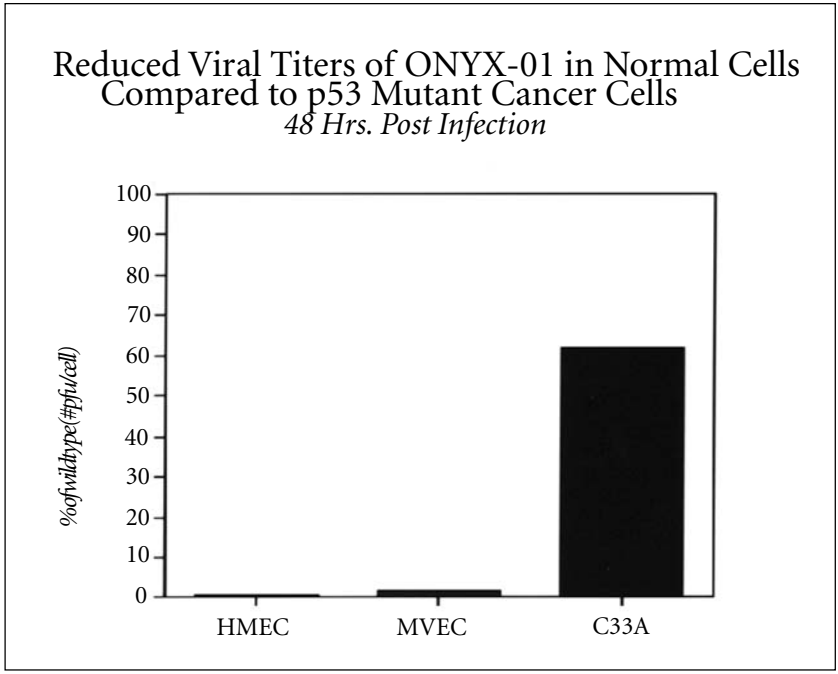

Fig. 21.1. Mammary epithelial cells (HMEC), microvascular endothelial cells (MVEC) and human cervical carcinoma cells (C33A) were infected with either ONYX-015 or wild type adenoviruses. 48 hours post-infection the virus was released from the cells by three freeze/thaw cycles and the resulting supernatant was titered on HEK293 cells. The amount of ONYX-015 produced 48 hours post-infection was normalized against the amount of wild type virus produced in the same cell line during the same time period.©*Nature Medicine* , 1997; 3(639):45.

Clinical Development of ONYX-015

Approach

ONYX-015 is a novel agent with a novel mechanism of action. We predicted that both toxicity and efficacy would be dependent on the intrinsic ability of a given tumor to replicate the virus, to the location of the tumor to be treated (e.g., intracranial vs. peripheral) and to the route of administration of the virus. In addition, data on viral replication, antiviral immune responses and their relationship to antitumoral efficacy were critical in the early stages of development. We therefore elected to treat patients with recurrent head and neck carcinomas initially.

Phase I Trial: Head and Neck Cancer

Patients enrolled onto the Phase I trial had recurrent squamous cell carcinoma of the head and neck that was not surgically curable and had failed either prior radiation or chemotherapy.[9,10] p53 gene sequence and immunohistochemical staining were determined on all tumors but were not used as entry criteria. Other baseline tests included lymphocyte subsets (CD3, CD4, CD8), delayed-type hypersensitivity skin testing (including mumps and Candida) and neutralizing antibodies to ONYX-015. Six patient cohorts received single

intratumoral injections of ONYX-015 every four weeks (until progression) at doses from 10^7 to 10^{11} pfu per dose. Two additional cohorts received five consecutive daily doses of 10^9 or 10^{10} per day (total dose 5 x 10^9 or 5 x 10^{10}) every four weeks (multidose cohorts). Following treatment, patients were observed for toxicity and for target (injected) tumor response. Additional biological endpoints included changes in neutralizing antibodies, the presence of virus in the blood (PCR days 3, 8), viral replication within the injected tumor (in tumor biopsies on days 8 and 22) and associated immune cell infiltration.

No significant toxicity was seen in any of the 32 patients treated. Flu-like symptoms were noted in approximately two-thirds of patients. No toxicity occurred in the adjacent normal tissues. Neutralizing antibodies were positive in approximately 70% of the cases prior to treatment. Following treatment, all patients had positive antibody titers, and all but one patient had an increase in antibody titer. Replication was identified infrequently on day 8 tumor biopsies in patients on the single injection protocol, while day 8 biopsies (n=5) were uniformally positive in tumors from patients on the multidose regimen (Fig. 21.2).

Three of the 23 patients on the single dose regimen had partial clinical responses (PR) of the injected tumor, and three patients with stable disease had ≥50% necrosis of the injected tumor. Two of nine patients on the multidose regimen had clinical PRs and an additional five had tumor stabilization (4 of whom had significant necrosis); only two patients had progressive disease. One patient received seven treatments over seven months while maintaining a partial remission. Responding patients included some with positive baseline neutralizing antibodies and tumors with a normal p53 gene sequence. However, definitive correlations between these variables and the degree of tumor response cannot be made until larger Phase II trials are completed.

Phase II Trials: Head and Neck Cancer

Trial Design and Preliminary Results

Based on these results, two Phase II trials in head and neck cancer patients were initiated. In the first study, ONYX-015 was injected intratumorally daily for 5 consecutive days (10^{10} plaque-forming units per day) in patients with recurrent, refractory squamous cell carcinoma of the head and neck. Patients had unresectable tumors that had progressed on chemotherapy and/or radiation following tumor recurrence. p53 sequencing was performed on all tumors. Treatment cycles were repeated every 3 weeks. Primary endpoints include the target (injected) tumor regression rate, time to target tumor progression and safety.

Preliminary data demonstrate that both complete and partial tumor regressions can be achieved. Data on the duration of tumor regressions are unavailable pending study completion. Correlations of regression with p53 gene sequence and neutralizing antibody levels will be performed. Mild flu-like symptoms have been seen in approximately 90% of patients; no other toxicity was seen. No normal tissue toxicity was reported, despite direct injection of normal tissue adjacent to the tumor bed. ONYX-015 was well tolerated and had antitumoral activity in refractory, recurrent head and neck cancer patients. Enrollment on this study continues.

ONYX-015 in Combination with Chemotherapy

In a second Phase II trial, patients are being treated simultaneously over five days with ONYX-015 intratumorally (as above) and standard chemotherapy intravenously: cisplatin (day 1 bolus) and continuous infusion of 5-fluorouracil (days 1-5). This is based on preclinical results showing additive or synergistic efficacy in vivo when ONYX-015 and cisplatin, 5-FU are co-administered.[8] These patients are all chemotherapy naïve in the setting

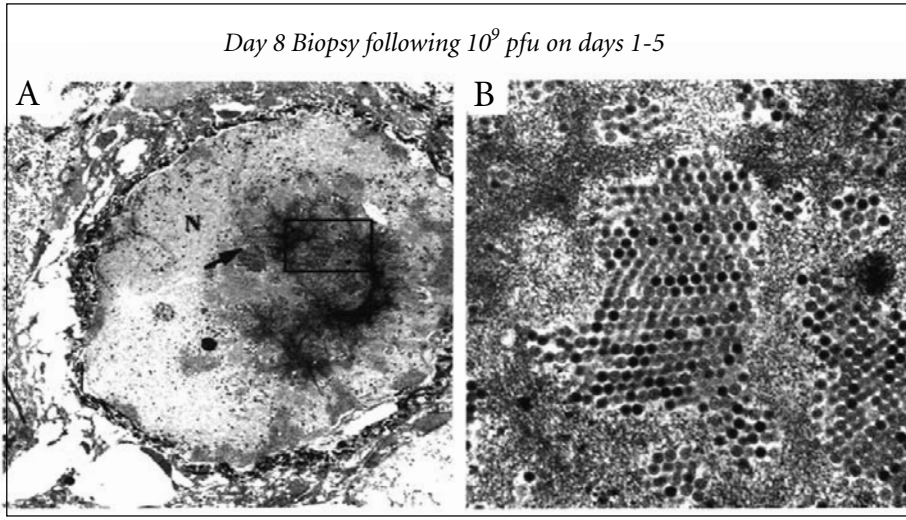

Fig. 21.2. ONYX-015 head and neck cancer studies: electron microscopy demonstrates replicating virus particles in tumor cells. (A) Squamous tumor cell with replicating adenovirus in nucleus. (B) Higher power magnification of ONYX-015 particles.

of recurrent disease. Planned enrollment of patients into this study will continue through the end of 1998.

Other Tumor Targets for Clinical Development

Additional local or regional tumor targets include ovarian cancer (Phase I intraperitoneal injection trial underway since 1997), pancreatic cancer (Phase I intratumoral injection trial underway since 1997), colorectal liver metastases (hepatic arterial infusion trial to be initiated in 1998), superficial recurrent bladder cancer (intravesical administration) and malignant astrocytomas (including glioblastoma multiforme).

Conclusion

Selectively replicating viruses may offer a new approach to cancer treatment. If successful in clinical trials, these agents will constitute a new category in the antitumoral armamentarium. Many viruses are currently being studied, and an adenovirus (ONYX-015) entered clinical trials in 1996; herpesvirus agents are scheduled to enter clinical trials in 1998. Critical issues need to be addressed if the utility of these agents is to be optimized. For each virus, the effect of antiviral immunity on antitumoral efficacy must be better understood. For all viruses, physical barriers to spread within tumors (e.g., fibrosis, pressure gradients) must be overcome. Although proof of concept experiments with chemotherapy and ONYX-015 have been encouraging, further studies are required to determine optimal treatment regimen sequencing. Combination studies with radiation therapy are also underway with ONYX-015. Finally, these agents may require modification (e.g., coat modification) in order to be maximally effective against systemic metastases following intravenous administration.

Second generation virus constructs will be developed based on clinical and preclinical data. Enhanced replication and virulence against tumor cells will be a major goal. The necessary degree of selectivity for tumor cells versus normal cells will depend on the route of administration; normal tissue will be exposed to much higher doses of virus following intravenous injection than intratumoral injection, for example. Finally, replicating viruses have been constructed that carry genes encoding prodrug activating enzymes (e.g., cytosine deaminase or thymidine kinase) or immunomodulatory cytokines, for example. This approach will allow the beneficial attributes of gene therapy agents to be combined with the advantages of selectively replicating vectors.

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CHAPTER 22

Adenoviral Vectors for the Manipulation of Human Hematolymphoid Cells: Purging and Other Applications

Timothy C. Meeker, Joanne M. Wroblewski and Prem Seth

The human bone marrow produces a complex array of specialized cells designed to function in a myriad of ways. The pluripotent hematopoietic stem cell (HSC) gives rise to committed progenitors for the myeloid and lymphoid lineages. Myeloid progenitors ultimately give rise to granulocytes, monocytes/macrophages, red cells and megakaryocytes/platelets, while lymphoid progenitors give rise to B and T lymphocytes.

Cells derived from the bone marrow have been viewed as an attractive gene transfer target since the earliest days of gene therapy. A number of diseases are manifested in these cells, ranging from immunodeficiency states (including AIDS) to cancer. In addition, it has been appreciated that the transfer of genes to these cells in an effort to augment immunologic responsiveness can lead to an enhanced immune response against a broad range of cancers. Thus, gene delivery to bone marrow-derived cells in order to augment the immune response, repair or replace a defective gene, or eradicate malignant cells, is an important goal. However, other vectors are generally considered to have advantages over adenoviral vectors for gene transfer to lymphocytes and myeloid cells.

Recently, several groups have further explored the potential utility of adenoviral vectors in the manipulation of cells derived from the bone marrow, in a way that addresses the multiple lineages. The prevalent concept that cells from the blood and bone marrow are simply refractory to transduction using adenoviral vectors, is being reexamined. A major new evolving concept is that adenoviral vectors mediate gene transfer with differing efficiencies among different subpopulations of bone marrow-derived cells.

Our goal in this chapter is to briefly summarize what is known about the efficiency of gene transfer using adenoviral vectors into subpopulations of cells derived from the bone marrow. Based on this information, experimental approaches with potential clinical utility can be envisioned. Because HSC are relatively refractory to gene transfer using adenoviral vectors, the selective elimination of cancer cells from HSC autografts (also called purging) may be possible. The growing body of work that supports the use of adenoviral vectors for purging will be summarized.

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Gene Transfer to Bone Marrow-Derived Cells: Lymphocytes

For several decades it has been known that adenoviruses can infect human lymphocytes, although the efficiency of infection is rather low. For example, a study from 1974 indicated that adenovirus type 5 was able to infect 3.2-6.5% of cultured Jiyoye lymphoma cells and 1.0-1.9% of cultured Raji lymphoma cells.[1] In a report from 1982, adenovirus strains 5 and 6 were shown to persist for months in cultures of EBV-transformed B lymphocytes from human cord blood.[2] This type of data supported the use of adenoviral vectors in occasional studies of lymphocyte cell biology and gene expression.[3] However, many workers concluded that the use of adenoviral vectors for gene therapy of human lymphoid diseases would not be fruitful.

Recently, more efficient gene transfer to human lymphocytes was documented. This increased efficiency is largely the result of improved technical advances in the field, allowing investigators to use a higher multiplicity of infection (moi). In one report, activated human peripheral blood T lymphocytes could be transduced using a vector encoding β-galactosidase (Ad.RSV.β-Gal). For best results cells were treated with phorbol myristate acetate and phytohemagglutinin, vector was used at an moi = 1,000 and cells were evaluated at 72 hours. Under these conditions, approximately 16% of T lymphocytes from peripheral blood were transduced.[4]

In another report, chronic lymphocytic leukemia (CLL) cells harvested from the peripheral blood of patients were studied.[5] CLL cells have been considered refractory to gene transfer using standard methods such as lipofection, electroporation, and calcium phosphate precipitation. These cells could be transduced to high efficiency, although in some instances an moi as high as 10,000 was required. It was suggested that adenoviral vectors expressing CD80 (a surface marker involved in immune responses) may be introduced into CLL cells ex vivo. The injection of these transduced cells into patients might augment the host immune response against the CLL.

Using an adenoviral vector encoding β-galactosidase (Ad-β-Gal), a large panel of lymphocytic cell lines was studied by us. It was shown that lymphocytic cell lines, representing anaplastic large cell lymphoma, Hodgkin's disease, Burkitt's lymphoma and multiple myeloma, could be transduced effectively.[6] These results were confirmed in killing assays using Adtk plus ganciclovir and Ad-p53. Similar data for multiple myeloma cell lines has been presented by others.[7]

It has also been shown that engineering the adenoviral penton base or fiber protein, which function in adhesion and internalization, can enhance gene transfer to lymphocytes. In one report Wickham and coworkers redirected virus binding by introducing seven lysine residues into the fiber protein of the vector called AdZ.F(pK7). They observed a 40-fold increase in transduction of T lymphocytes. In another report they used a bispecific antibody to target adenovirus to T lymphocytes and found that 25-90% of T cells were transduced using an moi between 20 and 100.[8,9]

Thus, improvements in adenoviral vector technology are allowing gene transfer to lymphocyte populations in selected situations. Such lymphocyte populations remain relatively refractory to adenoviral vector gene transfer when compared to epithelial cell targets. However, adenoviral vectors represent a promising gene transfer tool for lymphocyte gene delivery.

Gene Transfer to Bone Marrow-Derived Cells: Myeloid Cells

In 1985, adenoviral vectors were used to transduce the human K562 erythroleukemia cell line.[10] This work provided the first indication that myeloid cells might be receptive to gene transfer using adenoviral vectors. Since then, notable sucesses have come in two major areas.

First, human monocytes were shown to be efficiently transduced when stimulated with GM-CSF or M-CSF.[4] This gene transfer was dependent on expression of α_V integrins, to mediate virus entry. Using an moi = 1,000, approximately 53% of monocytes incubated with M-CSF were transduced after 3 days in culture.

Second, dendritic cells, which are derived from the bone marrow after incubation with GM-CSF and IL-4, can be transduced by adenoviral vectors.[11,12] In one set of experiments, 20% of dendritic cells were transduced using an moi = 5,000. Liposomes enhanced this delivery. Dendritic cells play a key role in the presentation of antigen during an immune response. Therefore, it is now possible to use adenoviral vectors to introduce large amounts of antigen directly into dendritic cells. These cells can then process this antigen for presentation. By this approach, an augmented immune response, perhaps to key tumor antigens, can be stimulated. This approach has the potential to assist in the treatment of many forms of cancer.

HSC are Relatively Resistant to Transduction Using Adenoviral Vectors

A number of groups have shown that adenoviral vectors do not damage HSC or hematopoietic progenitor cells under appropriate conditions. In an initial report an adenoviral vector expressing the *Bcl-xs* gene (Adbcl-xs) was utilized.[13] Bcl-xs expression favors apoptosis by blocking the action of Bcl-2 family members that inhibit apoptosis. Adbcl-xs caused no toxicity to CFU-GM derived from normal bone marrow at mois up to 5,000, but some toxicitiy (50% decrease in colony number) was observed at an moi = 10,000. In the same report, the effect of Adbcl-xs on engraftment of human hematopoietic cell in SCID mice was reported. At an moi as high as 100,000, no evidence of toxicity was observed.

Since adenovirus-mediated gene transfer depends upon the successful entry of adenoviral particles into cells, Seth and coworkers directly measured receptor number for human bone marrow cells. In contrast to the high receptor number on breast cancer cells, bone marrow cells had no detectable receptors. They reasoned that such cells would be poor targets for adenovirus-mediated gene transfer. They proceeded to show that bone marrow mononuclear cells and $CD34^+$ cells from mobilized peripheral blood (mPB) were refractory to transduction using Ad.RSV.β-Gal (moi up to 500).[18] Subsequent experiments using Ad-p53 showed no toxicity for $CD34^+$ cells from mPB and no significant alteration of CFU-GM (colony-forming unit-granulocyte/macrophage) until an moi of 1,000 was reached.

Wroblewski and coworkers showed no alteration of CFU-GM using an adenoviral vector expressing herpes simplex thymidine kinase (Adtk) at an moi = 200 plus ganciclovir. Similar experiments using Ad-p53 confirmed that this vector also spared CFU-GM under standard conditions. More recent work from this group has demonstrated no effect of Ad-p53 (moi =200) on BFU-E (burst-forming unit-erythroid), CFU-GEMM (colony-forming unit-granulocyte/erythroid/macrophage/megakaryocyte), or LTC-IC (long-term culture-initiating cell) assays (Fig. 22.1).

There is some disagreement as to whether adenoviral vectors are capable of gene transfer to HSC under some selected conditions. An early indication of this controversy was a report using a long term bone marrow culture system stimulated by multiple cytokines.[14] Hematopoietic cells were exposed to an adenoviral vector expressing adenosine deaminase (AdADA) and ADA activity was followed. In two of four cultures, sustained ADA activity was detected for 10 weeks. Unfortunately, attempts to define the cell types expressing ADA were unsuccessful, so it remained unclear whether hematopoietic progenitors were transduced (as opposed to stromal cells, fully differentiated cells, etc.).

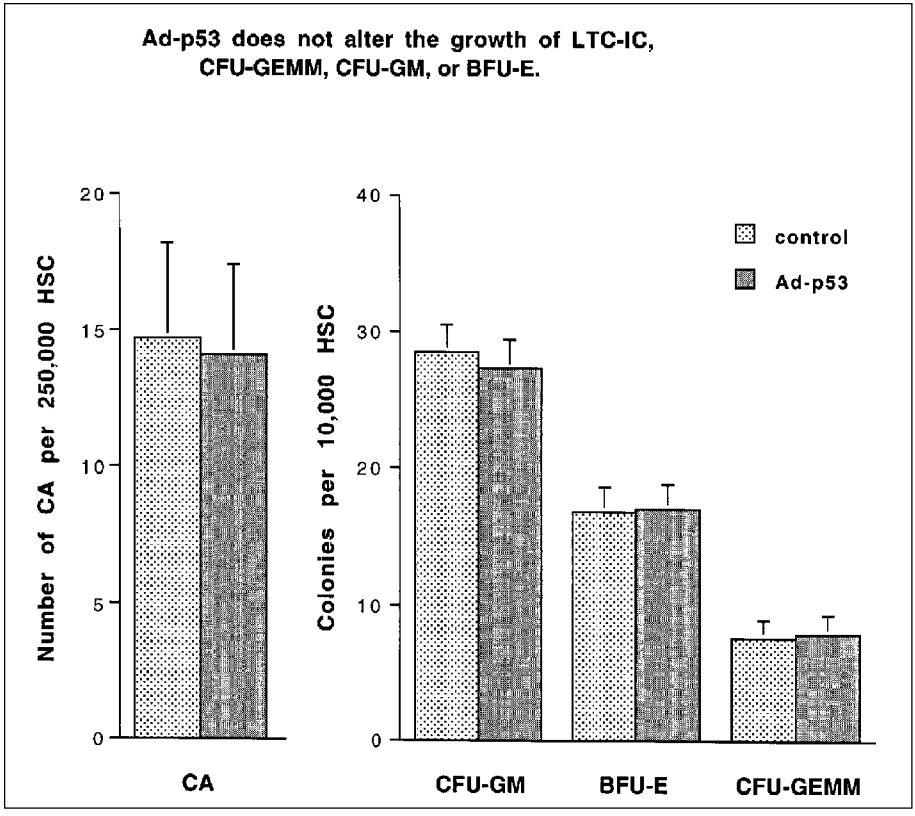

Fig. 22.1. Ad-p53 does not alter the growth of LTC-IC, CFU-GEMM, CFU-GM or BFU-E. Mononuclear HSC were incubated with PBS (control) or adenovirus at an moi = 200 for 2 hours at 37°C in 5% CO_2. For LTC-IC assays (n = 5), cells were washed 3 times before culturing on FBMD-1 feeder layers in 6-well plates. Initially, 2.5 x 10^5 HSC were aliquoted per well and cultured at 32°C in 5% CO_2. Cobblestone areas (CA) were enumerated after 6 weeks in culture.[23] CFU-GM, BFU-E and CFU-GEMM assays (n = 11) were scored after 2 weeks of culture at 37°C in 5% CO_2. The data are presented as the mean ± the standard error of the mean. There were no significant differences observed between control conditions (stippled bars) and Ad-p53 (shaded bars) for any of the colony types examined. Thus, the Ad-p53 vector did not exhibit toxicity toward HSC.

Subsequently, two groups demonstrated that bone marrow-derived myeloid progenitor cells can be transduced by adenoviral vectors. When Adβ-Gal was incubated with cells for 24 hours at an moi = 500, one group found 34.5% of bone marrow mononuclear cells and 20% of $CD34^+$ cells stained with X-Gal.[15] It was further shown that the transduced cells included CFU-GM, BFU-E and $CD34^+CD38^-$ progenitors. Another group cultured bone marrow-derived $CD34^+$ cells for 12 to 16 hours with IL-3, IL-6 and SCF (stem cell factor), and then adenoviral vectors encoding β-Gal or alkaline phosphatase (AP) were added for a 48 hour culture period. Transduction of hematopoietic progenitors was achieved with both Adβ-Gal and AdAP.[16]

At one level these latter papers seem to conflict with the work indicating that HSC are resistant to transduction using adenoviral vectors. The details of the methodology used in each of the papers is key, however. Conditions emphasizing long incubation times with concentrated vector (18-48 hours) and exposure to multiple growth factors correlated with HSC transduction.[17] Resistance of HSC to transduction correlated with short times of incubation (1-4 hours) and culture without supplemental growth factors. Thus, using easily achievable conditions, HSC can be exposed to adenoviral vectors without detectable gene transfer or toxicity.

Purging: Exploiting the Resistance of HSC to Transduction

As noted above, gene transfer to HSC can be minimized using conditions that easily transduce epithelial cells. Thus, the selective elimination of cancer cells from HSC autografts as part of high-dose chemotherapy and autologous hematopoietic stem cell transplantation (HDC/AHSCT) might be useful. HDC-AHSCT is an increasingly common approach to the treatment of cancer. In this approach, an aliquot of HSC is removed from the patient before therapy (Fig. 22.2). The patient is then treated with chemotherapy and/or radiotherapy in high doses in an effort to eradicate all remaining cancer. Without HSC reinfusion, the doses of chemotherapy and/or radiotherapy used would generally be lethal, as a result of irreversible damage to the bone marrow and the resulting depletion of key elements from the blood. The reinfusion of HSC is then critical to reconsitute hematopoiesis.

In HDC/AHSCT, the HSC have the potential to be contaminated with cancer cells, because they escape the high-dose chemotherapy and/or radiotherapy. Thus, untreated cancer cells might be reinfused into a recipient that otherwise had been rendered cancer-free. Purging, or the selective elimination of cancer cells from the HSC autograft, represents one solution to the problem of contaminated HSC autografts.

Clarke and coworkers first showed the potential of adenoviral vectors for purging. To mimic the clinical situation, they mixed 1.5 x 10^4 SHSY-5 human neuroblastoma cells with one million low density human bone marrow mononuclear cells. This mixture was incubated with vector and then colonies of SHSY-5 cells and hematopoietic progenitor cells were assayed. Using Adbcl-xs at mois ranging from 2,000 to 10,000, the ability of SHSY-5 human neuroblastoma cells to form colonies was abolished (see Table 22.1).[13] CFU-GM colony number was not altered until an moi = 10,000 was reached, at which point CFU-GM colony numbers were decreased by 50%. Thus, Adbcl-xs purged neuroblasoma cells from bone marrow mononuclear cells.

Seth and coworkers were interested in the ability of adenoviral vectors to purge human breast cancer cells from autografts. As noted above, they showed that breast cancer cell lines exhibited a substantial number of adenoviral receptors, while bone marrow cells exhibited no detectable adenoviral receptors. A mixed population of breast cancer cells and $CD34^+$ cells was exposed to a replication-deficient adenovirus (moi = 100) and a plasmid encoding bacterial Pseudomonas exotoxin for 24 hours. There was complete destruction of the cancer cells, while only a minimum reduction in the CFU-GM colonies was observed.[18] In the same study, they mixed the MDA-MB-231 cell line with mPB and purged with Ad-p53. They were able to demonstrate the selective elimination of MDA-MB-231 breast cancer cells starting at an moi = 1. At an moi of 10 or 100, 100% of MDA-MB-231 cells were killed, while no alteration of CFU-GM was noted. Using an moi = 1,000, 100% of MDA-MB-231 cells were eliminated, but a small decrease in CFU-GM (approximately 20%) was noted.[18] Thus, MDA-MB-231 cells were estimated to be at least 3 orders of magnitude more sensitive to killing than were HSC cells. They suggested the possible utility of adenoviral vectors for purging of breast cancer cells.

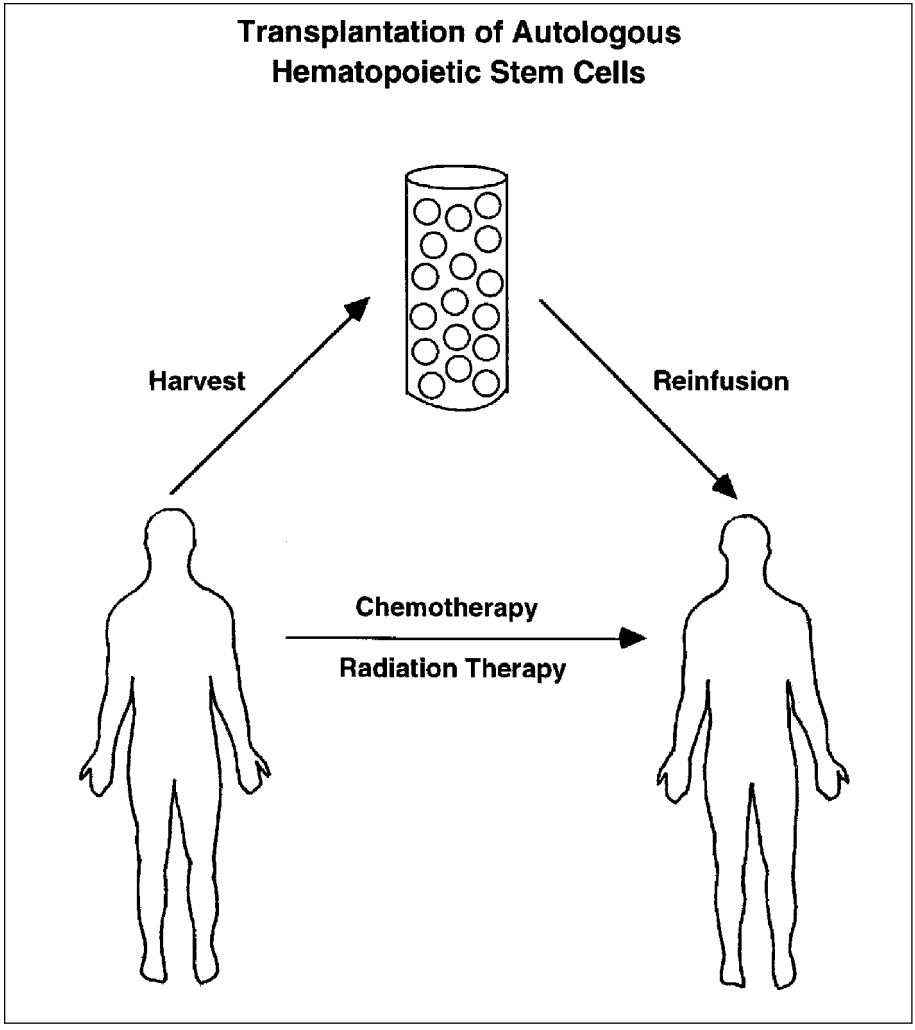

Fig. 22.2. Transplantation of Autologous Hematopoietic Stem Cells. In this approach, HSC are removed from a patient with cancer and stored. The patient is intensively treated with chemotherapy and/or radiotherapy, after which the HSC are reinfused.

Wroblewski and coworkers studied purging of a cervical carcinoma cell line (HeLa), a breast cancer cell line (MCF-7) and a lymphoma cell line (JB6). In their assays, cell lines and HSC were mixed, exposed to virus and then plated for colony enumeration. The principle of purging was tested using Adtk and HeLa. HeLa colonies were reduced to 4% while no alteration of CFU-GM size or number was observed. Similar results were obtained for MCF-7 and JB6. The authors then studied Ad-p53 in similar experiments. This vector killed the tumor cells very efficiently and no reduction in CFU-GM was observed. Thus, the ability to purge greater than 2 logs of cancer cells using Ad-p53 was demonstrated.[19]

Table 22.1. Summary of purging approaches

Author	Promotor/ Gene	Stem Cells	Conditions	Target Cells	Log Purge
Clarke (13)	RSV-Bcl-xs	BM MNC	4hr., moi= 2-10,000	Neuroblastoma	4
Seth (18)	CMV-p53	BM MNC mPB-CD34$^+$	2hr., moi=200	Breast	>3
Wroblewski (19)	CMV-tk CMV-p53	mPB	2hr., moi=200	Cervical Breast Lymphoma	>2
Chen (20)	CMV-tk DF3/MUC1-tk	PB	2 hr., moi= 10-100	Breast Prostrate Lung Glioblastoma	>6
Kim (22)	sFv-erbB2	BM MNC	2 hr., moi=100	Prostate	>2

BM = bone marrow; mPB = mobilized BM; MNC = mononuclear cells

Newer approaches have been developed to improve the ability to purge cancer cells from HSC cells. Chen and coworkers designed an adenoviral vector expressing tk under the control of the DF3/MUC1 promotor.[20] This led to selective tk expression in epithelial cells. In some experiments they documented a 6 log purge. Toxicity for HSC was not observed. The importance of this work was emphasized in an accompanying editorial.[21]

Kim and coworkers developed a vector to target a cancer-specific genetic change.[22] A vector, expressing an sFv that binds to erbB2 and is directed to the endoplasmic reticulum, was produced. Purging of prostate cancer cells by 2 or more logs was observed. Again, the vector was found to have little toxicity for HSC.

Thus, an expanding body of work has clearly demonstrated that cancer cells can be effectively removed from HSC cells using adenoviral vectors. Furthermore, in all instances, it was apparently easy to identify conditions to spare HSC.

Purging: Future Directions

The potential of adenoviral vectors for purging has been shown. Efforts are in progress at a number of centers to accumulate sufficient preclinical data to support a clinical trial, perhaps in breast cancer. Breast cancer is an attractive disease to target because outcomes after HDC/AHSCT are often disappointing. Further, recent data suggest that more than 50% of patients with stage II, III or IV breast cancer have metastatic cancer cells which contaminate HSC autografts. This type of contamination correlates with decreased survival after HDC/AHSCT. In the next few years work is needed with carefully defined HSC samples

from appropriate patients to assess purging efficacy in vitro. Animal models must be exploited to assist in defining the optimal design of clinical trials.

New vectors will continue to improve the potential for purging. The benefit of tissue-specific promotors has already been demonstrated and will lead to better vectors. Adenoviral vectors are amenable to better selective targeting. Targeting may be improved by engineering the adenoviral fiber gene and protein, by altering the penton base gene and protein or by using antibodies.[8,9] Using adenoviral vectors to express genes that are preferentially toxic to cancer cells (such as the anti-erbB2 sFv) also represents a concept for additional exploitation.

Conclusion

Cells derived from the bone marrow are wonderfully diverse. The use of adenoviral vectors in the manipulation of these myriad cell types is a fertile, underexplored area. Nonetheless, several areas for further translational research have been clearly identified. Among these, purging represents an application in which the lack of gene transfer to HSC can be exploited.

Acknowledgment

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CHAPTER 23

Adenovirus Transformation and Tumorigenicity

Robert P. Ricciardi

In natural infection of humans, adenoviruses generally enter differentiated, quiescent epithelial cells. For successful propagation of virus to occur, these infected cells need to be growth stimulated. This function is performed by the concerted actions of the viral E1A and E1B gene products, which bind key cellular proteins, causing activation of the cell cycle and inhibition of growth arrest and apoptosis. These same E1A and E1B functions are responsible for the ability of each of the approximately fifty human adenovirus serotypes to transform rodent cells in culture. Rodent cells fail to support lytic infection but become transformed through the chance integration and expression of the early region E1A and E1B genes. In addition, rodent cells as well as human cells can be transformed by transfecting E1A and E1B plasmids. Adenovirus-transformed cells exhibit immortalization, proliferative growth capacity and morphological alteration, all of which are hallmarks of cancer cells. However, only a subset of adenoviruses—or the cells which they transform—are actually able to generate tumors in immunocompetent adult rodents. The mechanism responsible for this tumorigenic phenotype is mediated by E1A and is postulated to be a manifestation of the means by which the highly oncogenic viruses escape immunosurveillance during persistent infection in humans. This review briefly describes currently held notions as to how E1A and E1B collaborate to transform cells and then examines the mechanism of E1A-mediated tumorigenesis. The importance of these oncogenic mechanisms as related to the use of adenovirus in gene therapy is discussed. The influence of the E4 ORFs on oncogenesis is reviewed elsewhere[1] and in this volume.[2] Due to space limitations, most of the numerous studies which support the findings and mechanisms described in this chapter are mentioned in recent reviews, where they are covered more extensively.[3-10]

E1A Activates the Cell Cycle and Induces Proliferation in Transformed Cells

The well-studied E1A gene of adenovirus-5 (Ad5) encodes two major proteins of 243 amino acids (aa) and 289 aa which are identical except that the larger protein contains an internal 46 aa domain referred to as CR3 (Fig. 23.1A). The E1A coding region comprises multiple discrete domains that physically target different cellular factors. The CR3 domain directly stimulates transcription of viral and cellular promoters by acting as a bridge that contacts both DNA binding proteins and basal transcription factors but is dispensable for transformation. By contrast, the CR1, CR2 and amino-terminal domains of E1A are essential for transformation and indirectly regulate transcription of cellular growth genes by targeting critical factors, namely the pRb and p300 family proteins, that govern their expression. [3,7,9]

Adenoviruses: Basic Biology to Gene Therapy, edited by Prem Seth.

The CR1 and CR2 domains of E1A bind to the retinoblastoma protein pRb (p105) and pRb-related family members (p107 and p130), prohibiting their abilities to govern the cell cycle; pRb regulates progression from G_1 to S and p130 regulates G_0 to G_1 (reviewed in refs. 3, 5, 6). Interestingly, CR2 contains an LXCXE motif which is required for these associations and which is conserved in dissimilar viral oncoproteins that also bind pRb proteins, i.e., SV40 T antigen and HPV E7. Likewise, the pRb proteins contain a 'pocket sequence' which is able to bind E1A, T antigen and E7. In the G_1 phase of the normal cell cycle, hypophosphorylated pRb complexes with E2F transcription factors, which prevents them from stimulating the promoters of genes involved in DNA synthesis and the cell cycle, e.g., dihydrofolate reductase, DNA polymerase-α, thymidine kinase and cyclin A (Fig. 23.1B). As the cell cycle progresses into the S-phase, pRb becomes increasingly phosphorylated, which causes it to release from the E2F factors which now can stimulate genes necessary for DNA synthesis. However, by sequestering the pRb family of proteins, E1A essentially coerces and maintains an active cell cycle by enabling the E2F factors to function constitutively.

Binding of E1A to p300 interferes with the ability of this cellular protein to conduct its dual, yet related, activities of promoting differentiation and restricting the cell cycle (reviewed in refs. 5, 6, 8). The cellular p300 protein binds to the E1A amino-terminus and a portion of CR1 which is distinct from that required to bind pRb proteins (Fig. 23.1A). Other p300 family members which bind to E1A include the very homologous CBP and a newly identified 400 kDa protein.[5] p300/CBP regulate transcription initiation of many tissue specific promoters by serving as adaptors that integrate signals from upstream DNA binding proteins (e.g., cyclic AMP response element binding protein (CREB) and nuclear hormone receptors) and basal transcription factors (TBP and TFIIB). p300/CBP can also function to arrest the cell cycle by functioning as coactivators of p53-mediated transcription of p21, the cyclin-dependent kinase inhibitor. Thus, by associating with E1A, the p300/CBP induction of tissue specific gene expression and growth arrest are abolished (Fig. 23.1B). This contributes to the viral reprogramming of a differentiated cell from a state of quiescence to a state of active DNA synthesis. In addition to stimulating promoters by functioning as a transcriptional adaptor, p300/CBP can also increase gene expression through its association with a histone acetyltransferase (pCAF) (reviewed in refs. 8, 11). This histone acetyltransferase activity is responsible for remodeling chromatin such that the accessibility of nucleosomal DNA to transcription factors is increased. Binding to E1A disables p300/CBP from recruiting pCAF[12] (Fig. 23.1B). Interestingly, an intrinsic histone acetyltransferase activity has also been described for p300/CBP[11] and shown to function in the acetylation of a non-histone protein, specifically, p53.[13] The acetylation of p53 increases its ability to bind DNA, which consequently increases p21 expression. Therefore, because of its ability to bind p300/CBP, E1A has the potential to block the multiple ways in which these versatile cellular proteins drive the cell into quiescence.

Thus, there is a clear functional link between complex formation of E1A with the pRb proteins and E1A with p300/CBP. In each case, the end-point is to remove blocks to DNA synthesis in order to attain an active cell cycle. It is fascinating to consider how E1A has evolved distinct coding domains that target proteins which are related only by their capacities to regulate cell growth.

E1B Blocks Growth Arrest and Apoptosis Induced by E1A in Transformed Cells

E1A is able to immortalize cells by conferring constitutive DNA synthesis. However, cells transformed by E1A alone are not stable in culture because their steady proliferation leads to an accumulation of DNA damage that can trigger either p53-induced growth arrest or apoptosis. Growth arrest provides an extended time needed for DNA repair, but apoptosis

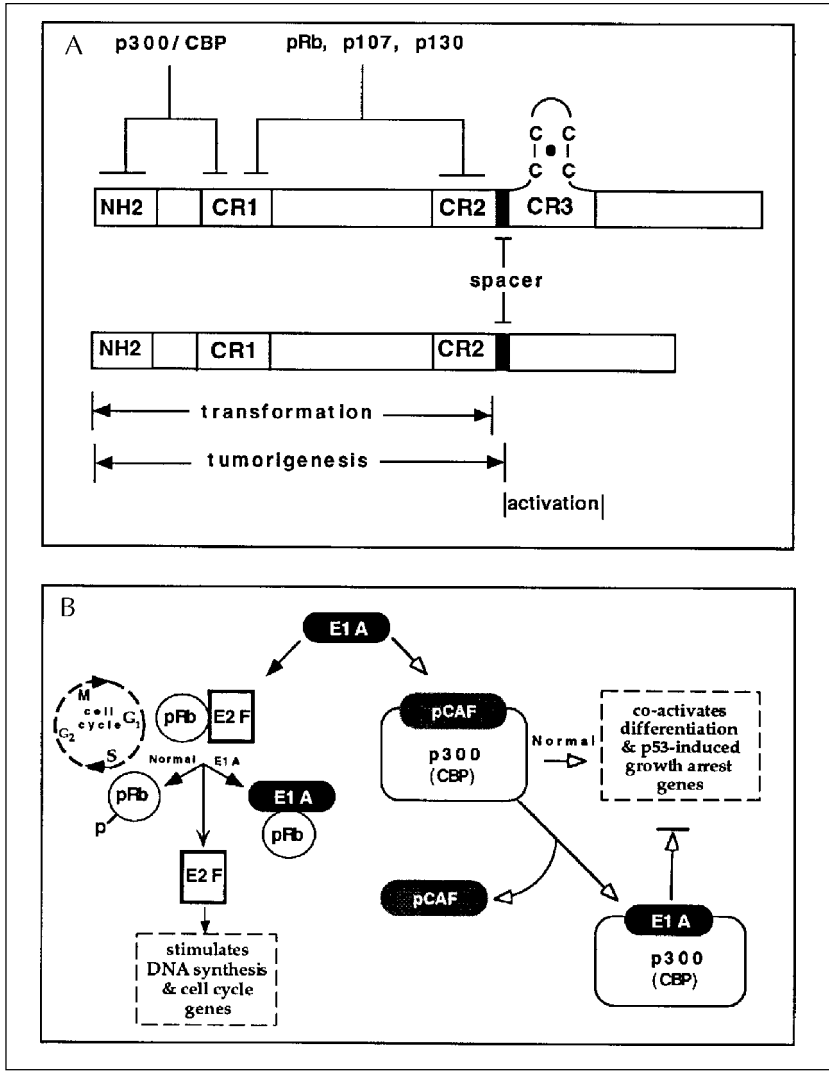

Fig. 23.1. E1A proteins associate with key cellular proteins to constitutively activate the cell cycle in transformed cells. (A) The E1A coding domains required for function and binding to cellular proteins. Both E1A proteins are identical except that the larger protein (289 aa in Ad5) contains an activation domain with a Cys-4 zinc finger. The activation domain (46 aa) stimulates transcription by binding factors of the RNA PolII initiation complex and upstream DNA binding proteins, but is not essential for transformation. Transformation requires the CR1 and CR2 domains for binding to the retinoblastoma family of proteins (pRb, p107 and p130), and the NH2 domain and a different portion of CR1 for binding to p300/CBP. The highly tumorigenic Ad12 E1A contains a 20 amino acid 'spacer' between CR2 and CR3 which is necessary for tumorigenesis. (B) E1A disrupts the normal cell cycle by sequestering pRb proteins, which allows E2F factors to constitutively stimulate promoters of genes needed for cellular replication and cell cycle progression. E1A also binds to the transcription cofactor p300/CBP to prohibit activation of genes involved in differentiation and growth arrest. One way E1A blocks p300 function is to prevent binding of pCAF, a histone acetyltransferase.

will occur if the repair is insufficient or if the DNA damage is too extensive and threatens the stability of the cell and consequently the host. Each of these cellular responses are counteracted by the E1B 55 kDa and 19 kDa proteins (Fig. 23.2A). The E1B 55 kDa (55K) and 19 kDa (19K) proteins act independently to inhibit growth arrest and apoptosis, respectively (Fig. 23.2B). In this way, E1B cooperates with E1A to produce a completely transformed cell. Essentially, E1A pushes on the accelerator and E1B cuts the brake cables.

The E1B 55K protein binds to p53 and prevents it from stimulating the promoters of growth arrest genes, such as p21 and GADD45 (reviewed in refs. 3, 5). Mechanistically, the E1B 55K protein complexes with the amino-terminal activation domain of p53 while allowing p53 to remain bound to its DNA recognition site on the promoter (Fig. 23.2B). Interestingly, E1B 55K may function as a dimer which can actually increase the DNA binding affinity of p53 to the promoter.[14] Even in the absence of p53, the E1B 55K protein exhibits an inherent transcriptional repression activity, which may be regulated by phosphorylation, when it is tethered to a promoter as a GAL4 fusion protein.[15] Thus, E1B 55K may not only block p53-mediated transcription by masking its activation domain, but also may use the surface of DNA-bound p53 as a strategic site to exert active repression. The interaction of E1B 55K with p53 may potentially have another repressive effect that involves p300. As mentioned above, one function of p300/CBP is to increase p53-mediated transcription of the p21 growth arrest gene; this effect is blocked by the binding of E1A to p300/CBP (Fig. 23.1B). Interestingly, a physical interaction between CBP and the activation domain of p53 has also been described recently.[16] Therefore, it is possible that the binding of E1B 55K to the p53 activation domain could simultaneously prevent p300/CBP from binding to p53 and inhibit it from functioning as a transcriptional adaptor and as an acetylase (Fig. 23.2B). Thus, in addition to its direct role in repression, E1B 55K may also act in a redundant fashion with E1A to prevent p300/CBP from stimulating p53 mediated transcription.

The function of the E1B 19K protein is to block p53-dependent apoptosis that stems from E1A-induced cellular proliferation (Fig. 23.2B ; reviewed in ref. 10). p53 activates expression of Bax, which is one of several apoptotic proteins which promotes cell death. E1B 19K and its cellular homolog Bcl-2 function as inhibitors of apoptosis by heterodimerizing with Bax as well as other proteins engaged in programmed cell death, including Bak and Bik. In this way, E1B 19K blocks the cascade pathway that generates a family of cysteine proteases referred to as caspases. These caspases serve as executioners of cell death by cleaving a variety of cellular proteins at specific aspartate residues. Some of the caspase substrates include ADP-ribose polymerase (PARP) and the nuclear mitotic apparatus protein (NuMA). In order to block apoptosis, E1B 19K needs to localize to the nuclear/ER membranes. This localization of E1B 19K is probably mediated through its association with lamin A/C which are, interestingly, also caspase substrates.[17]

It is thus apparent why adenovirus transformation requires the collaboration of E1A and E1B proteins. E1A products deregulate cellular growth through their associations with pRB and p300/CBP proteins, while E1B products prevent growth arrest and apoptosis by blocking the normal p53 responses to DNA damage that accompany E1A mediated stimulation of DNA synthesis. But what makes transformed cells of only certain adenovirus serotypes tumorigenic?

Adenovirus Tumorigenesis—MHC Class I Downregulation as a Means of Immunoescape

Transformed cells need to evade the immune system as one of their prerequisites for being able to proliferate into tumors. This is underscored by the fact that transformed cells of the so-called nontumorigenic serotypes (e.g., Ad5) can actually form tumors in animals that are athymic or depleted of T cells but not in those which are immunocompetent

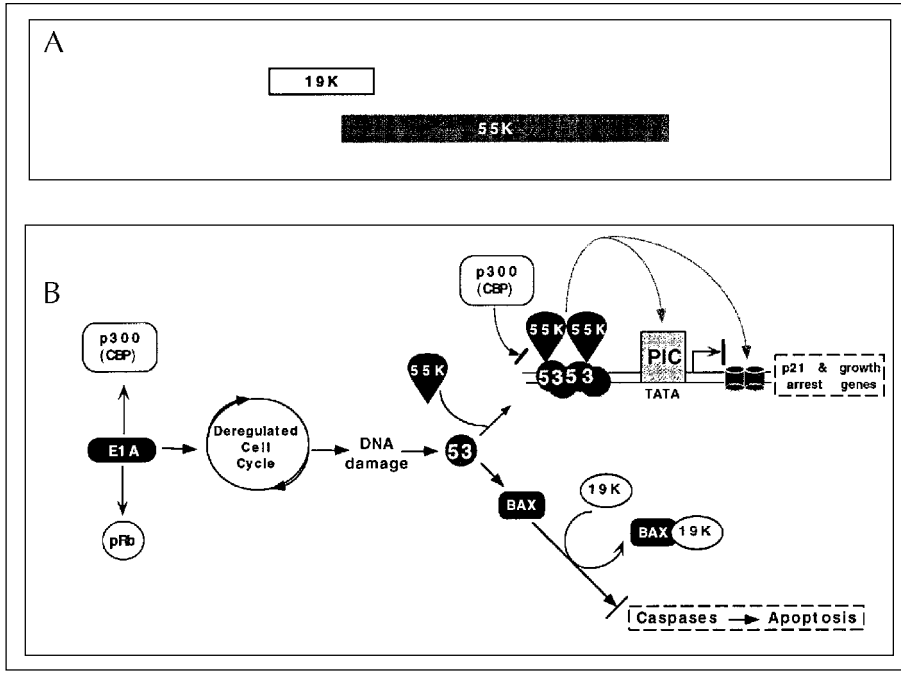

Fig. 23.2. The two E1B proteins use independent mechanisms to block p53 mediated growth arrest and apoptosis. (A) E1B encodes two major proteins of 19 kDa (19K) and 55 kDa (55K) which have partially overlapping, but completely different, open reading frames. (B) The E1B proteins block p53-mediated growth arrest and apoptosis in response to DNA damage that results from deregulation of the cell cycle by E1A. In this model, dimeric E1B 55K is shown to bind tetrameric p53 on the promoter, and to repress transcription of p21 and growth arrest genes by blocking the activation domain of p53 and actively repressing the RNA PolII preinitiation complex (PIC), as well as possibly interfering with the binding of p300/CBP cofactor to p53. The 19K protein complexes with p53-induced cell death proteins such as Bax, to prohibit activation of the caspase pathway that leads to apoptosis.

(reviewed in refs. 3, 4). Thus, immune evasion is an underlying feature that distinguishes the tumorigenic from the nontumorigenic serotypes.

Insight into this phenomenon has been furnished largely by Ad12, the most highly studied of the tumorigenic serotypes (reviewed in ref. 3). Either Ad12 virus or cultured Ad12 E1-transformed cells generate tumors when injected into immunocompetent adult syngeneic rats. In cells transformed by Ad12, the surface levels of the major histocompatibility complex (MHC) class I antigens are greatly diminished, which provides the Ad12-transformed cells with a means of escaping immunosurveillance by cytotoxic T lymphocytes (CTLs). Significantly, cells from tumors retain the same low levels of class I antigens as the preinjected parental cells. Consistent with the connection between low class I levels and immune escape is the resistance of Ad12-transformed cells to lysis by syngeneic CTLs in vitro, as compared to CTL susceptibility of non-tumorigenic Ad5-transformed cells, which have comparatively high levels of class I expression. Treatment of Ad12-transformed cells with IFN-γ, a known stimulator of class I expression, abrogates their resistance to CTL lysis. Moreover, in vivo

there is increased survival of rodents injected with Ad12-transformed cells that either are pretreated with IFN-γ or that express exogenous class I genes.

The *E1A* gene of Ad12 is solely responsible for the reduced class I antigen expression which can be observed in rat, mouse or human cells transformed in culture (reviewed in ref. 3). Ad12 E1A actively and dominantly mediates downregulation of class I expression as substantiated by stable Ad5/12 somatic cell hybrids that retain the same diminished levels of class I antigens as the parental Ad12-transformed cell fusion partner. The antigens encoded by all class I alleles (murine H-2 K, D and L or human HLA A, B and C) are diminished in Ad12-transformed cells. Importantly, the block in class I antigen expression occurs at the level of transcription. Mutational analysis of the class I promoter established that the enhancer is the target of E1A mediated downregulation of class I transcription. What it is that makes this transcriptional element a target was revealed upon detailed examination of the binding activities at two distinct DNA recognition sites (R1 and R2) within the enhancer (reviewed in ref. 3).

The R1 site of the class I enhancer contains a DNA recognition sequence for NFκB, a potent activator of transcription. Importantly, in Ad12-transformed cells there is negligible binding of NFκB to the R1 site, which is in contrast to Ad5-transformed cells, where there is strong binding of the activator (refer to Fig. 23.3; reviewed in ref. 3). This negligible NFκB binding in Ad12-transformed cells is not due to a lack of p50 or p65, the two subunits which compose this activator. In fact, the amounts of nuclear p65 and p50, respectively, are nearly equivalent to those in Ad5-transformed cells. This and other findings have ruled out an involvement of IκBs,[18] the regulated gate keepers of NFκB nuclear translocation. Experiments using detergent suggested prima facie that a nuclear inhibitor might associate with NFκB and prevent it from binding DNA,[18] but this is now ruled out largely by the fact that Ad12 nuclear extracts do not contain an inhibitory activity that is transferable to Ad5 nuclear extracts.[19] Rather, it is now thought that in Ad12-transformed cells the block is due to chemical modification of NFκB. When denatured-renatured p65 and p50 subunits from Ad5 and Ad12-transformed cells were combined to reconstitute hybrid NFκBs, only the $p50_{Ad12}/p65_{Ad5}$ combination exhibited reduced DNA binding activity, indicating that the p50 subunit from Ad12-transformed cells is defective.[19] Moreover, reduced phosphorylation of the $p50_{Ad12}$ subunit corresponds with the block in DNA binding of NFκB.[19] It is relevant to note that the activation domain of p65 is not an Ad12 E1A target.[19]

The R2 site of the class I enhancer contains a DNA recognition sequence for members of the nuclear hormone receptor family, such as RARs, RXRs, and certain orphan receptors. In Ad12-transformed cells there is strong homodimeric binding of the orphan receptor COUP-TF, a transcriptional repressor.[20] By contrast, in nontumorigenic Ad5 cells there is minimal binding of COUP-TF at this site. The differential COUP-TF binding activity between Ad5 and Ad12-transformed cells correlates with the ability of the R2 element to negatively regulate transcription only in Ad12-transformed cells.[20] Recent evidence suggests that in Ad12-transformed cells the mechanism by which COUP-TF functions as a transcriptional repressor is through its association with a histone deacetylase which may facilitate compaction of chromatin (Fig. 23.3).[21] This of course does not exclude the potential of COUP-TF to directly repress the transcription initiation complex as well. Of note, RAR/RXR heterodimers in the liganded state associate with a histone acetylase activity that results in active transcription, but in the unliganded state associate with a histone deacetylase activity that results in repressed transcription. Perhaps COUP-TF is an apparent constitutive repressor, because this orphan receptor truly lacks a natural ligand.[21]

Thus, Ad12 E1A mediates global downregulation of the MHC class I enhancer by affecting the binding activities at both transcription factor recognition sites (Fig. 23.3). The positive-acting transcription factor NFκB is inhibited from binding to the R1 site, while the

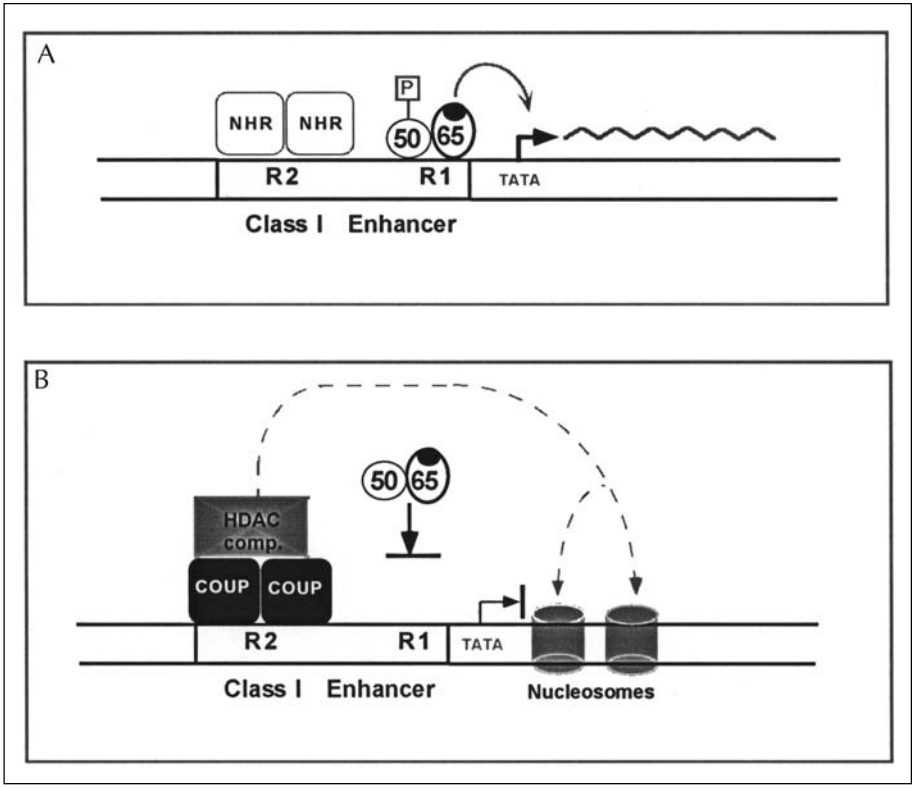

Fig. 23.3. Model for Ad12 E1A-mediated global downregulation of the MHC class I enhancer. (A) Activation of the class I promoter requires binding of the activator NFκB (p65/p50) to the R1 site of the enhancer. The R2 site is capable of binding different nuclear hormone receptors (NHR) such as RAR/RXR that contribute to activation, or—as occurs in non-tumorigenic Ad5-transformed cells—negligible amounts of the repressor COUP-TF, which has minimal influence on transcription. (B) In Ad12-transformed cells, transcription from the class I promoter is prevented by the inability of NFκB to bind to the R1 site of the enhancer. Inability of NFκB to bind DNA correlates with reduced phosphorylation of the p50 subunit. The repressor COUP-TF binds very strongly to the R2 site of the enhancer and represses transcription through an associated histone deacetylase which may create greater compaction of chromatin. COUP-TF might also repress transcription from the RNA PolII initiation complex (not shown). These two modes by which Ad12 E1A mediates inactivation of the enhancer assures that MHC class I transcription remains downregulated.

repressor COUP-TF—amongst all of the known nuclear hormone receptors—exhibits essentially exclusive binding to the R2 site. The differential binding activities of NFκB and COUP-TF map to the first exon of Ad12 E1A excluding CR3.[22] It remains to be determined if Ad12 E1A mediates differential binding of COUP-TF and NFκB by directly acting on a common cellular target such as a kinase. This global downregulation of the enhancer probably assures shut-off of class I transcription under varied physiological conditions, e.g., COUP-TF may override a temporary increase in NFκB due to physiological fluctuations of inducing

cytokines. In addition, the bidirectional NFκB site-containing promoter of the TAP1 and LMP2 genes which function in transport and processing of MHC class I antigens is also downregulated in Ad12-transformed cells;[23] the binding activity of NFκB is also greatly reduced in this bidirectional promoter.[24] By these mechanisms, reduced class I levels are maintained and avoidance of CTL lysis is maximized in tumorigenic Ad12-transformed cells.

A Connection Between Tumorigenesis and Viral Persistence

Diminished class I expression in Ad12-transformed cells likely reflects a means of achieving viral persistence during Ad12 infection of humans. The fact that the class I enhancer is inactivated by an E1A-mediated affect on the binding activities of two such dissimilar transcription factors underscores the potential importance of this mechanism in viral persistence of Ad12. I previously proposed that the difference in tumorigenic potential between Ad5 and Ad12-transformed cells relates to a difference in their mechanisms of immunoescape required for viral persistence.[3] In nontumorigenic Ad5, the E3 gene encodes a protein which physically interferes with the transport of class I molecules to the cell surface (reviewed in ref. 25). Because E1A and E1B are the only viral genes required by all adenoviruses to transform cells, then accordingly only Ad12 (but not Ad5)-transformed cells will be reduced in class I surface antigens. But why should Ad5 and Ad12 employ these different CTL escape mechanisms entailing E3 and E1A, respectively? It may be related to their having evolved different tropisms, Ad12 being enteric and Ad5 respiratory. Some credence for this proposal is consistent with the fact that E1A/E1B-transformed cells of two other enteric strains (Ad40 and Ad41) are reduced in class I expression and that neither Ad12, Ad40 nor Ad41 encodes the functional counterpart of E3 that physically interferes with transport of class I antigens to the cell surface (reviewed in ref. 3).

E1A-Mediated Tumorigenesis Involves More than MHC Class I Downregulation

E1A of tumorigenic Ad12 contains a unique segment of 20 aa located between CR2 and CR3 which is referred to as the 'spacer' (Fig. 23.1A). This spacer segment, which does not reside in non-tumorigenic Ad5 E1A and has no role in transformation, is essential for tumorigenesis by Ad12 E1A, along with a more amino-terminal region of Ad12 E1A (reviewed in ref. 4). Significantly, while tumorigenesis is abrogated upon point mutation of the spacer segment, downregulation of MHC class I transcription is not affected.[4,26] Thus, a lowering of the levels of class I antigens can be considered an immunoescape strategy that is essential, but not sufficient, for tumorigenesis. It remains to be established whether the spacer provides another immune escape mechanism, such as evasion from natural killer (NK) cells, especially since cells which have low class I antigen levels are more susceptible to natural killer (NK) cells. Of direct relevance, Ad12-transformed cells have greater resistance (or less susceptibility) to NK killing than do those of Ad5.[3,4,27,28] Other unexplained tumorigenic mechanisms may be operating also. For example, it is intriguing that C-terminal deletion mutants of Ad5 E1A can cooperate with Ras to generate transformed cells which form invasive tumors in syngeneic rats, whereas wt Ad5 E1A and Ras-transformed cells are incapable of generating tumors (reviewed in ref. 5). A short motif that resides near the extreme C-terminus of the coding region of Ad5 E1A has been shown to bind to a cellular protein, CtBP, whose function—at least in this context—may be to restrain metastasis.[29] It has yet to be determined if Ad5 E1A and Ad12 E1A might show a difference in binding to CtBP that would correspond with their difference in tumorigenic potential.

Conclusion

The mechanisms used by adenovirus E1A and E1B proteins to usurp control of the cell cycle, growth arrest and apoptosis are employed in both infected and transformed cells. The subversion results as a consequence of the abilities of E1A and E1B proteins to bind and disable the function of key cellular proteins that govern these cellular processes. Moreover, the E1A-dependent tumorigenic phenotype observed with Ad12 could reflect the particular mode of immune escape and viral persistence utilized by the enteric subset of adenoviruses. These transforming and tumorigenic properties have posed important considerations for the usage of adenovirus vectors in gene therapy. Arguments that favor their application include the fact that the nontumorigenic Ad5 has been employed as a live vaccine for military recruits for many years. In addition, adenoviruses are generally acquired during childhood and are largely ubiquitous in the population. Furthermore, in most vectors the transforming region is deleted. Also, large surveys have indicated no evidence for involvement of any of the adenovirus serotypes in different human cancer tissues.[30] Arguments on the side of caution remind us that these surveys were conducted about two decades ago and perhaps should be repeated using more sensitive techniques. In the future, the use of different seroptypes which might be useful for tissue targeting based on their natural tropism e.g., enteric tropism[31] will require a full understanding of their immune escape strategies. In an interesting twist, knowledge of E1A and E1B function has served as the rationale for employing an E1B deletion virus that is intended to selectively destroy tumor cells of the head and neck (reviewed in refs. 32, 33).

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CHAPTER 24

Homologous Recombination Between Exogenous and Integrated Adenovirus DNA Sequences

C. S. H. Young and Gregory J. Duigou

The recent dramatic increase in the use of adenovirus as a transduction vector has led to a renewed interest in all phases of its life cycle. Among the issues that have received increased attention are those concerning the genetic integrity of the viral genome after infection into either permissive or nonpermissive cells. Integrity can be compromised in two ways. In one, the viral genome undergoes random integration into the cellular genome using host cell nonhomologous recombination and repair mechanisms.[1] Integration may result in alterations to the sequence or expression of genes located in the viral sequence. Alternatively, the viral genome can be modified by homologous recombination between the free viral DNA and viral sequences previously integrated in the cellular genome. The latter phenomenon, which was first observed in semipermissive rat F4 cells transformed by adenovirus serotype 2 (Ad2) and then infected by Ad5,[2] is of considerable practical importance. Most adenovirus vectors proposed for gene therapy are designed to be replication-incompetent by virtue of the presence of a transgene cassette in essential regions of the genome. However, during amplification in complementing cell lines, there is the potential to produce replication-competent viruses by homologous recombination. In circumstances where the virus preparation is to be administered to human patients for purposes of gene therapy, the presence of such replication-competent adenoviruses (so-called RCA), capable of establishing a productive infection in the patient, is of obvious clinical importance. This review will focus on some of the publications that have documented the production of RCA, the circumstances that may favor its occurrence and detection, and some speculations about the mechanisms that underlie it.

A Comparison of Extrachromosomal Homologous Recombination with that Occurring Between Extrachromosomal and Integrated Sequences

It has been known for many years that recombination between homologous extrachromosomal DNA molecules occurs at very high frequencies. This is true of viral genomes, including those of adenoviruses,[3] and of DNA molecules transfected into cells.[4] In contrast, recombination between homologous sequences in mitotic chromosomes and targeting of chromosomal sequences by exogenous DNA is usually very rare (reviewed in ref. 5). In addition, the targeting efficiency is locus-dependent. These contrasting observations suggest that the biochemical machinery necessary for homologous recombination is present

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in mitotic cells, but that the DNA targets themselves differ in their intrinsic recombination potential. The possible reasons behind the much lower frequencies observed in chromosomal-exogenous DNA recombination include poor accessibility to the DNA in the chromatin structure, low copy number of the integrated sequences as compared with the exogenous DNA levels, and, in the case of viral DNA recombination, possible induction of recombination enzymes encoded either by the virus or by the cell. There is no evidence to date that adenoviruses encode any components of the enzymatic machinery necessary for homologous recombination,[6] but there is no compelling evidence for or against the idea that infection might induce cellular recombination functions.

Recombination Between Exogenous Viral DNA and Sequences Integrated Into the Cellular Genome

Recombination between viral DNA genomes and integrated cellular sequences has been observed in both SV40 and adenovirus-derived cell lines.[7-9] Although the occurrence of homologous recombination between viral and integrated sequences is generally very low, the first observations with adenovirus were an exception to this rule.[2] A clone of semi-permissive rat cells transformed by Ad2 was infected with Ad5. The viruses emerging from the infected cells were purified, and the mixed population of DNA was analyzed for *Bam*HI restriction site polymorphisms. Because the presence of Ad2-specific fragments could be detected readily against the background of Ad5-specific fragments, the results suggested that homologous recombination had occurred at high frequency between the free genome and the integrated sequences. As discussed below, such recombination in human cells is extremely rare and often difficult to detect.

The creation of primate cell lines capable of complementing deficient human adenovirus mutants[10,11] was among the most important developments in adenovirus genetics. For the first time, reliable methods to create and propagate virus containing deletions in essential viral functions were available. The most famous example, the human 293 cell line, was created by Graham and his colleagues by transfection of randomly sheared Ad5 DNA into human embryonic kidney fibroblasts.[10] The cells express E1A and E1B functions, are highly transfectable, can be used in standard plaque assays, and can be adapted to suspension culture. As a consequence, they have been of paramount value in the creation of adenovirus mutants and vectors. More recent examples of complementing cell lines include those containing and expressing one or more of the *E1A*, *E1B*, *E2A*, *E2B* and *E4* genes.[12-23] All of these cell lines not only allow the propagation of viruses with deletions of the respective genes, but also allow foreign transgenes and associated transcription elements to be embedded in the viral genome in their place. Large quantities of transgene-containing virus can be obtained and used to infect nonpermissive cells for investigative or therapeutic purposes. As mentioned above, one of the safety issues with this strategy is the production of replication-competent viruses capable of infecting and replicating within human cells. Because the original virus usually has large deletions within essential viral coding sequences, suppression of the mutant phenotype by mutation in the remaining viral genes is unlikely. A more likely source is recombination between the viral genome and the integrated viral sequences present in the complementing cell line. In order for this to occur, the cellular genome must contain adenoviral sequences flanking both sides of the deletion in the vector. As discussed below, this is the case with many of the complementing cell lines.

As an example of the possibility of RCA formation, we shall consider the case of human 293 cells and the propagation of recombinant vector viruses with major deletions in E1A and E1B. Recently, the integrated sequences have been characterized at the nucleotide level, and it is now known that the viral sequences extend from the left hand terminus of the

adenoviral genome to nucleotide 4344.[24] Early reports suggested that there might be at least some level of recombination between the cellular sequences and the deleted virus genomes, because viruses with wild type phenotypes (what would now be called RCA) could be obtained from stocks of replication-deficient virus.[25] Similarly, adenovirus vectors expressing herpes virus ICP0, when tested on non-permissive human cells, were found to contain a very low level of RCA.[26] The genotype of the virus was consistent with recombination with the integrated sequences because individual clones of RCA had sequences that could have been derived from both the cellular integrated E1 region and from the right hand end of the vector. However, inadvertent contamination with the wild type parent to the vector was not ruled out by these observations, and it was not until the publication of the careful studies of Hehir et al in 1996 that this issue was finally resolved.[9] In this report, the E1-deleted vector viruses were derived from Ad2, and nucleotide differences between Ad2 and the Ad5 DNA integrated in the 293 cell could be used to determine unambiguously that recombination had generated RCA (see Fig. 24.1). Sequence analysis showed that information must have been derived from the cellular sequences to generate an Ad2/Ad5 chimeric virus. The authors also demonstrated that the production of RCA could be reduced to very low levels (~2 RCA in 10^{11} infectious units) by shortening the length of available homology on the right of the transgene from 836 nt to 315 nt (recalculated from the recent data concerning the precise Ad5 sequence present in 293 cells). The question of how little homology is necessary for recombination to take place has been addressed for extrachromosomal recombination between transfected DNAs (reviewed in ref. 27), but the minimum length necessary on either side of the insert for RCA generation has not been determined. Recent efforts to eliminate RCA include the creation of cell lines, derived from human lung carcinoma A549 cells, which contain integrated Ad5 DNA lacking left hand end flanking homology available for recombination to remove the transgene.[28] In principle, this construction method could be applied to any new cell line to be developed. Of the currently available cell lines, only those expressing partial E4 regions,[20] or E4 ORF6 alone,[21-23] can complement major deletions and substitutions in E4 and will not be able to generate RCA by homologous recombination.

As yet there have been no attempts to establish a reliable rate of RCA formation. Because it is likely that the intrinsic rate of recombination is within an order of magnitude of typical mutation rates, a reasonable method of measurement would be to perform a fluctuation test. Nevertheless, a careful set of analyses, using several different methods of detection, showed that the proportion of RCA arising from a vector expressing β-galactosidase increased with the number of passages.[29] This proportion is determined both by the intrinsic rate of recombination per cell per generation and also by the selective advantage of the two genotypes. In general, where a selective advantage exists, it is the wild type RCA that outgrows the original vector. Thus, measurement of rates must avoid the contribution of selective advantage. This issue becomes of greater theoretical interest if the transgene itself has effects on recombination rates. Recent efforts in our own laboratory have been directed at influencing recombination and repair mechanisms by expressing known or suspected eukaryotic repair genes from adenovirus vectors. These include human and budding yeast *RAD51* genes, the human *REC2* gene and the putative kinase domain of the human ATM gene. None of these vectors has had a noticeable increase in the generation of *RCA*, but a true measure by fluctuation test has not been performed to confirm these preliminary observations. It is relevant that most of these vectors have significantly lower replication rates than would the equivalent RCA, yet levels of RCA are below 1 in 10^7.

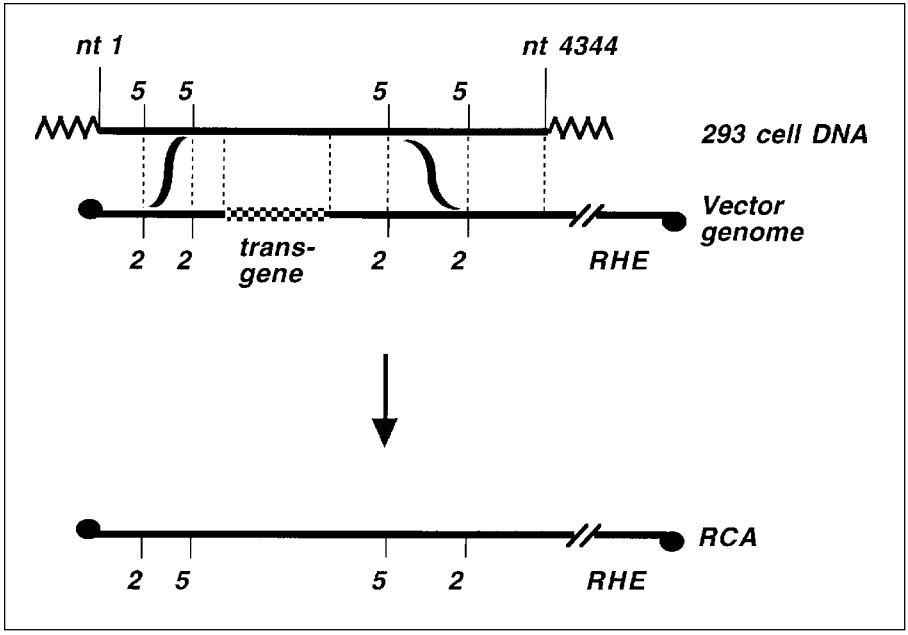

Fig. 24.1. The generation of RCA from an Ad2-based vector and the integrated Ad5 DNA present in 293 cells. The diagram is based on information from refs. 9 and 24. The 293 cell DNA is represented by adenovirus DNA from nucleotide 1 to nucleotide 4344 (solid line) flanked by cellular sequences on either side (serrated lines). The Ad2-based vector contains a CFTR transgene (patterned line) substituting for most of the E1 region, and the terminal protein covalently attached to both 5' ends of the duplex is designated by the solid ellipses. A representative set of sequence differences between Ad5 and Ad2 in the common DNA is indicated by the numbers 5 and 2, and the vertical dotted lines connecting them. The vertical dotted lines without associated numbers represent major sequence discontinuities between the vector and the 293 cell adenovirus sequences. Crossovers, which can be detected within any adjacent pair of vertical lines, are indicated by the curved lines. The diagram is not to scale, and there are many more sequence polymorphisms between Ad2 and Ad5 than the four shown.[9] Note that if the embedded left hand terminus of adenovirus DNA in the 293 cell can be liberated from the cellular genome, for example by DNA replication initiation or termination, there is no formal requirement for the left hand end crossover. The crossover would, however, be required in human 911 cells,[12] in which the adenovirus sequence begins at least 79 nt inward of the terminus. The crossovers shown are deduced from the genetic structure of the RCA depicted in the bottom line. RHE indicates the right hand end of the vector and of the derived RCA genomes. Many vectors will have alterations in the E3 and/or the E4 regions that distinguish them from wild type virus genomes. These alterations will be present in the RCA genome.

Can Adenovirus be Used to Target Homologous Sequences for Purposes of Gene Modification?

The demonstration that RCA is a true manifestation of homologous recombination between the extrachromosomal and integrated adenovirus sequences makes it plausible to suggest that adenovirus could be used as an aid in gene targeting. So far, only three reports

have appeared that address this issue.[30-32] In one case[30] the target was a bovine papillomavirus-based extrachromosomal plasmid. The target contained a truncated neomycin resistance gene, and a replication-defective adenovirus vector supplied the missing sequence. Selection of a functional neomycin resistance gene demonstrated very high levels of correction of the target mutation. Although this is an encouraging result, it remains to be demonstrated that this level of enhanced correction could be achieved with mutations located in the mammalian genome. Indeed, in one of the other reports,[31] in which a replication-competent adenovirus was used to correct a mutant *aprt* gene in Chinese hamster cells, targeting was much lower, although homologous events predominated over nonhomologous integrations. Similarly, adenovirus targeting of the endogenous Fgr locus in mouse embryonic stem cells was inefficient, comparable to levels seen previously with plasmid-based electroporation, although the proportion of homologous to nonhomologous targeted events was somewhat increased.

Potential Investigation of RCA Formation

The practical issue of how to prevent RCA formation is clearly solvable by eliminating, or at least severely limiting, the homology available for flanking recombination. From a theoretical standpoint, although RCA formation in human cells is a rare event, it remains a good model for the more general problem of targeting of exogenous DNA to the mammalian chromosome. Even with the enhanced selective methods available for gene targeting,[5] treatments that increased the levels of homologous recombination would be of great value, especially for those genes that have proved to be recalcitrant to targeting. As mentioned earlier, different genes have very different rates of interaction with exogenous DNA, sometimes making it almost impossible to modify a specific target. The study of RCA formation has the potential to shed light on the factors that could increase recombination rates and perhaps to give insight into mechanism.

The first requirement, as mentioned above, is to obtain accurate values for the rate of RCA formation measured by fluctuation tests. If the length of homology is one of the rate-limiting steps in RCA formation, initial experiments should be conducted with vectors with extended adenovirus sequences surrounding the transgene (or with simple E1 deletions) rather than with currently used vectors, in most of which homology is limited to a few hundred nucleotides to the left and right of the transgene. The severely defective E1A mutant *dl*312, in which nt 448 to 1349 are deleted,[33] would be a good candidate. If this proves to be a useful experimental approach, progressive reduction in flanking homology could be attempted to test the homology requirement in a rigorous way. Second, it would be informative to see if DNA damaging agents administered to either the virus or the cell alter the rate of RCA formation. An inductive effect of cellular treatment would suggest that factors necessary for high rates of recombination are absent in the untreated cell, or that inhibitors of recombination are removed upon treatment. Third, as adenoviral vectors expressing known or suspected recombination or repair genes become available, the intrinsic rate of RCA can be measured. As mentioned above, no instances of this have been seen with vectors expressing a variety of the suspected eukaryotic repair genes, but very recently we have noted that vectors expressing any of the several E2F transcription family genes have high levels of RCA soon after plaque purification. If this high frequency can be shown to result from an inductive rather than a selective mechanism, an avenue is open to explore the induction of repair and recombination genes. Many years ago, Capecchi reported that levels of recombination are highest in early to mid-S-phase,[34] and because E2F induces many genes involved in DNA replication, the presence of high levels of RCA may also result from this induction. Whether the high levels of RCA in stocks of E2F vectors reflect a greater

accessibility of the recombining partners or a true induction of recombination proteins remains to be determined, but regardless of the outcome, study of the mechanism of RCA is likely to yield important insights into the mechanism of recombinational repair in mammalian cells.

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Chapter 25

Adenovirus-Induced Pathogenesis

Harold S. Ginsberg

With the discovery of antibiotics during World War II, the use of tissue culture soon became relatively easy after the war had ended. These findings led to the discovery of many new viruses. One of these, as described in chapter 1, was adenoviruses, in 1953[1] and 1954.[2] During the period since World War II, one has only to compare the books on virology that T. Rivers and F. Horsfall edited in 1959,[3] and that B. Fields et al edited in 1996,[4] to understand the number of viruses discovered during this period.

Since the time of their discovery, 49 different types have been isolated from patients, and the diseases they induce are multiple (Table 25.1). Serum neutralization titers indicate that there is some cross-reactivity with a few other adenoviruses, but that it is not excessive. Thus,with homolgous, type-specific sera, type 3 virus has a titer of 2048 and type 48 has a titer of only 16; type 30 has a titer of 512 and type 49 a titer of 16; type 44 has a titer of 64 and type 48 a titer of 4096; and type 46 has a titer of 32 and type 49 a titer of 2048. It is critical to note, however, that type 48 and 49 have restriction enzyme analysis different from each other and from all other adenovirus types.[5]

Despite extensive clinical and laboratory knowledge of adenoviruses (i.e., structure, mechanism of replication, identification of structure of genome and genes contained therein) and the pathogenesis of the diseases, the different adenoviruses produced related to how the virus enters the body, how it spreads in the body to reach the organ(s) affected in the disease, and the course of the infection, little or nothing was known of the molecular mechanism by which the virus produced any of its multiple diseases. When, however, Pacini, Dubovi, and Clyde discovered that type 5 adenovirus could replicate in lungs and produce pneumonia in *Sigmodon hispidus* cotton rats that was pathologically identical to that produced in humans,[6] a new, exciting field was opened to us. *Sigmodon fulviventer*, another strain of cotton rats, was also tested, but they were not nearly as sensitive a host, either relative to viral replication or to pathogenicity. This finding initiated our investigation of the molecular pathogenesis of adenovirus pneumonia, which will be the content of this chapter.

Molecular Pathogenesis of Adenovirus Pneumonia

Replication of Type 5 Adenovirus and Effect of Size of Inoculum on Pathological Response

Intranasal inoculation of 10^2 to 10^{10} pfu clearly demonstrates that the virus is not adapted to the cotton rat lung, since the larger the infectious dose the higher the titer attained and the faster the maximum is reached. Moreover, the larger the infectious dose, the more extensive the pathological response.[7] Indeed, an infectious dose of 10^{10} pfu uniformly produced a fatal disease and a viral titer of $10^{9.4}$ was attained. Immunoflourescent and electron microscopic studies revealed that the virus primarily infected bronchiolar epitheial

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Table 25.1. Diseases due to adenoviruses

Diseases	Those at Risk	Serotypes
acute pharyngitis	young children, including infants	1-3; 5-7
pharngoconjunctival	school children	3,7,14
pneumonia	infants, young children	1-3; 7
acute respiratory disease (ARD)	military recruits—without pneumonia	3,4,7,14,21
acute respiratory disease (ARD)	military recruits—with pneumonia	4,7
epidemic keratoconjunctivitis	all ages	8,11,19,37#
pertussis-like syndrome	young children, including infants	5
acute hemorrhagic cystitis	young children, including infants	11,21
gastroenteritis	young children, including infants	40,41
hepatitis	children and infants with liver transplants	1,2,5
continued shedding of virus in unrinary tract	immunosuppressed persons	34,35
no symptoms; from stools	AIDS patients	48,49

types 11, 19, and 37 less frequent
Modified from Horowitz MS. Adenoviruses. In Fields BN, Knipe DM, Howley Pm, eds. Fundamental virology. Philadelphia: Lippincott-Raven Publishers, 1996:2149-2171.

cells, although an occasional bronchial epithelial cell was also infected. A rare infected cell was seen within the alveoli, and these were undoubtedly cells shed from infected alveoli. It is critical to note that no matter how extensive the pathological response, the infected cells died, but they were not lysed; hence, it is totally incorrect to label the adenovirus replication as being a lytic infection.

The pathological inflammation is divided into early and late phases. Depending upon the administered infectious dose, the early phase reaches its maxium in 3 to 5 days after infection and consists of diffuse infiltration of the peribronchiolar and alveolar regions with lymphocytes, monocytes-macrophages, and neutrophils.[8] The infected bronchiolar epithelial cells, depending upon the infectious dose, contain cytoplasmic vacuolation and loss of cilia, but they are not lysed. The late phase of the pathological response to infection consists almost entirely of a peribronchial and perivascular response of primarily lymphocytes. The late phase reaches its peak 2 to 3 days after the peak of the early phase, around day 7.[8,9]

The Gene Functions Responsible for Pathogenesis

Cell culture studies indicated that the virion capsid fiber was responsible for the major pathogenic response.[9] The cotton rat investigations clearly indicated that these in vitro studies are not accurate, for when the cotton rats were infected with H5ts125,[10] a gene containing a defective DNA-binding protein so that DNA synthesis is inhibited and, therefore, activation of the late genes producing viral capsid proteins does not occur since the cotton rat body temperature is greater than 39°C, the pathological response was indistinguishable from that which wild type Ad5 effected.[10] In addition, infection with H5ts149, a temperature-sensitive mutant affecting the viral DNA polymerase gene so that DNA synthesis could not occur and late genes could not be expressed, the pathological inflammation was also indistinguishable from that Ad5 wt virus induced.[11] Moreover, infection with a mutant in the fiber gene did not reduce the inflammatory response (unpublished data).

Since only early genes are required to induce Ad5 pneumonia in cotton rats, the first set of genes investigated were those contained in early region 3 (E3), since this region encompasses about 10 percent of the entire genome but is totally unessential for viral replication. It seemed unlikely that a viral genome would persist, probably for many generations, without this region serving a beneficial function for the virus. Hence, experiments were initiated employing mutants in which the entire E3 region or just individual genes were deleted. Experiments were done with a set of mutants in which each of the E3 genes were individually deleted. Only the mutant that contained a deletion of the 19 kDa gene showed a marked increase in the inflammatory response to infection.[11] Previous research had demonstrated that this E3 19 kDa glycoprotein (gp19K) combines with the class I major histocompatibiliy antigen (MHC) in the endoplasmic reticulum and inhibits its glycosylation, which reduces its transport to the surface of infected cells.[12] It was then demonstrated that, when cells were infected with a mutant in which only gp19K had been deleted, the transport of class I MHC to the infected cell surface was greatly increased, and, therefore, a markedly increased lymphocytic inflammation ensued. It was also observed that when only the gene encoding a 14.7 kDa protein, which resides at the very 3′-end of the E3 region, was deleted, although the extent of the inflammatory response was not significantly increased, the exudate contained considerably more polymorphonuclear leukocytes, which resembled the response that tumor necrosis factor alpha (TNF-α) induces. Unfortunately, reagents for cotton rats were not available to determine whether TNF-α was indeed the causative agent.

Data also clearly indicated that the E1B 55 kDa gene plays an important role in pathogenesis. This gene has been shown to be essential to shut off host protein synthesis.[12] When the cotton rat was infected with H5dl110, in which a 5′-end portion of the gene encoding the E1B 55 kDa protein was deleted, the virus replicated like wt Ad5, but the pathogenic, late phase of the inflammatory response was markedly reduced (manuscript in preparation). The role that the Ad5 E1B 55 kDa protein plays in the pathogenic process is not yet clear, but the data suggest that this effect on the late phase of the inflammatory process may be due to this, and probably other, viral genes expressed on the infected cell surface. Other data show that the E1A domains 1 and 2 are expressed on the surface of infected cells in culture.

A Mouse Model for Investigation of the Mechanism of Adenovirus Pathogenesis

As described earlier, studies using the cotton rat *S. hispidus* suggested that the inflammatory response to infection during the early phase was, at least in large part, due to a response to TNF-α and that the late phase was the result of a major cytotoxic T-cell response[11] to the amount of class I MHC expressed on the infected cell surface. It was then impossible to test these hypothses because cotton rat reagents were not available, and they still are not. It was

known, however, that adenovirus early gene functions are expressed in cultured mouse cells and, therefore, experiments were initiated to determine whether, if these early genes were expressed in mice, they would be the Ad5 early viral genes that induce the inflammatory response in cotton rat lungs. Four mouse strains were tested in the initial experiments: C57Bl/6,C57Bl/10, C3H, and CBA.[13] Pneumonia developed in all four strains, but it was most extensive in C57Bl/6 mice. Moreover, it is critical to note that the early and late phases were identical to those noted in cotton rats. Since the virus cannot replicate in mouse lungs, however, it was essential that very large infectious inocula be employed, i.e., 10^{10} pfu per mouse, so that the number of pulmonary bronchiolar cells infected and the degree of early gene expression was similar to that attained with a productive infection of cotton rats.[13] These findings made it possible to determine whether the data on the mechanism of adenovirus-induced pulmonary inflammation obtained in *S. hispidus* experiments were, indeed, the result of the hypotheses expressed above.

Cytokines

It was a critical finding that, one day after infection, TNF-α, interleukin-1 (IL-1) and interleukin-6 (IL-6) were present in homogenates of Ad5 wt-infected lungs, but only IL-6 was present in the bloodstream. IL-6 was detectable as early as six hours after infection and attained maximum levels one day after infection, wherteas TNF-α and IL-1 attained their highest levels 2-3 days after infection.[13] It is important to note that none of the interferons was detectable at any time in these lung homogenates (Havel E, Trudeau Institute, personal communication).

To determine whether these cytokines were, indeed, responsible for the inflammatory response to Ad5 wt pulmonary infection, specific antibodies to each cytokine (obtained through the generosity of Dr. L. Moldawer) were used in separate experiments. The immune serum was injected prior to infection and daily thereafter. It is striking that only anti-TNF-α reduced the extent of the early inflammatory response to infection. The TNF-α antibodies, however, only reduced the inflammatory early phase by 50 to 75 per cent, which implies that some other cytokine or other factor(s) plays a role in effecting the inflammation (unpublished data).

It should be noted that in Ad2- or Ad5-infected cultures the E3 14.7 kDa and the 10.4/14.5 gene products protect infected cells from lysis by TNF-α.[14] It must be emphasized again, however, that in in vivo animal models the infected epithelial cells are not lysed. Of course they do die, probably owing to the shut-off of their DNA and protein syntheses.[9,11,12]

Effect of Steroids on Ad5 wt Pulmonary Pathogenesis

Steroids markedly reduce the pathological response to cytokines, as well as to immunological reactions. Therefore, the effect of hydrocortisone on the pulmonary pathogenesis of Ad5 wt infection was investigated. Hydrocortisone was administered subcutaneouly, employing two different courses: One began one day before infection and the other one day after infection, and both had continuing daily subcutaneous injections. Both regimes essentially eliminated both the early and late phases of pulmonary inflammation so that histologically the treated mice did not show any differences from the uninfected controls. It is important to note, however, that the cytokine response was also significantly reduced, but not entirely eliminated (unpublished data).

Role of the Cellular Immune Response to Ad5 wt Pulmonary Infection

As noted previously, Ad5 wt pulmonary infection clearly demonstrated that the E3-encoded 19 kDa glycoprotein markedly suppresses expression of the class I MHC on the infected cell surface, and that when the encoding gene was mutated, the late phase of the

pathogeneic response in cotton rats was markedly enhanced, which implied that the late phase of the inflammatory response is an immune reaction.[11] The early inflammatory response to infection in C57Bl/6 mice consisted primarily of CD4 and natural killer cells but, in the late inflammatory phase, the majority of cells were CD8 cells; however, some B cells were present (unpublished data).

C57Bl/10ScN parent and congenic Nu/Nu mice were available (but C56Bl/6 NuNu mice could not be obtained) to test the data implying that the late inflammatory phase resulted from a cellular immune response to the Ad5 virus. It was striking that the pathological response to Ad5 wt infection with 10^{10} pfu produced a marked decrease in the peribronchial lymphocytic infiltration, and there was essentially no peribronchial infiltration in the nude mice, whereas the usual inflammatory response occurred in the parent strain.[13]

Discussion

The value of cell cultures for isolating viruses, understanding the mechanism by which viruses replicate, revealing the nucleic acid composition of the genome and discovering the genes contained within the genome, the function of each gene within the genome, and producing and isolating genetic mutants is tremendous. Cell culture research, on the other hand, at least in the case of adenoviruses, has been misleading and of little assistance in revealing the actual mechanism by which adenoviruses produce disease, particularly in the example of how type 5 adenovirus produces pneumonia. The same will probably be true for understanding the pathogenesis of other adenovirus diseases in the future. The discovery that the cotton rat, if inoculated intranasally with Ad5 wt virus, effected a pneumonia that was pathologically identical to that in humans[6,7,10] led to the investigations revealing an understanding of the molecular pathogenesis of adenovirus pneumonia. As described, the finding that only early gene functions are required to produce the pneumonia was, indeed, a surprising discovery,[10,11] and this finding led to the realization that mice could also be used to study the molecular pathogenesis of adenovirus pneumonia.[13] The use of mice for this research was of particular value, since the reagents are available for determining whether cytokines play a critical role in production of the inflammatory response to Ad5 pulmonary infection and finding that, indeed, they do. Furthermore, it permitted the discovery of the role of the E3 gene encoding the 19 kDa glycoprotein in inhibiting transport of the class I MHC to the surface of infected cells, which markedly reduces the attraction of cytotoxic T cells to attack the infected cells, and, therefore, permitting Ad5 and viruses of the same group of adenoviruses to establish latent infections.[13] Moreover, the E3 region contains a gene that encodes a 14.7 kDa protein that plays an important role in suppressing elaboration of TNF-α.[11] It has been reported that the E3 10.7 kDa and 14.5 kDa proteins inhibit TNF-α from lysing infected cells.[14] It must be emphasized again, however, that these studies were done in cell cultures, and that in vivo, in humans or in the animal models described, infected cells are not lysed, i.e., adenoviruses do not produce a lytic infection.

The discovery that genes encoded in the E3 region actually reduce the inflammatory response to Ad5 infection is of great importance in the construction of adenovirus vectors for gene therapy or vaccine production. Unfortunately, the first generation vectors contained deletions of the entire E3 region to help make space to permit insertion of the desired gene for expression. It is highly recommended that adenovirus vectors retain the entire E3 region and delete E4 instead. The E4 region plays important roles in viral replication, but deletion of the entire E4 region does not increase the inflammatory response (unpublished data) and permits space for insertion and expression of the desired gene.

Animal models may also have their failings. Thus, adenoviruses transform cells in culture, and some types of adenoviruses, especially types 12, 18, and 31, produce malignacies in animal models. Extensive studies, however, have been unable to detect any malignancies that adenoviruses produce in humans.

This research on molecular pathogenesis of adenovirus pneumonia is only an important beginning that hopefully will lead the way to discovering the molecular pathogenesis of many other adenovirus-induced diseases. It is clear that different adenovirus types produce different diseases. As one example, type 5 adenovirus can induce acute pharyngitis, as do types 1, 2, 3, 6, and 7. Yet, of these viruses, only types 3 and 7 induce acute pharyngoconjunctival fever, but type 14 also induces this acute, severe disease (Table 25.1). Also, type 5 adenovirus replicates extremely well in the gastrointestinal tract but does not produce any disease, whereas types 40 and 41 induce severe gastroenteritis, particulary in children. Many such examples can be given, but the need for much more research in this area is essential. It is to be hoped that our animal models, or if necessary others to be discovered, will permit investigation into these critical fields. Undoubtedly, as more is learned about the mechanisms of molecular pathogenesis of these viral diseases, new approaches to prevention and therapy will be discovered.

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Chapter 26

Adenovirus-Host Interactions to Subvert the Host Immune System

William S. M. Wold and Ann E. Tollefson

Viruses have evolved ingenious mechanisms to evade the host antiviral response. The first to be recognized is antigenic variation, which allows viruses to escape preexisting antibodies, and is exemplified by influenza virus epidemics that occasionally sweep across the world. Essentially every antiviral response that we can imagine is targeted by different viruses, including inhibition of cytokine and chemokine responses, killer immune cell activity, apoptosis (programmed cell death), shut-off of cellular protein synthesis, and regulation of cellular gene expression. These viral anti-host proteins are fascinating because they teach us about the immune system, cell and molecular biology, and viral pathogenesis. They also teach how to design gene therapy vectors which, of course, face many of the same obstacles as wild type viruses. Further, it may be possible to use viral proteins in vectors to blunt the host response to the vector. In this article we will discuss adenovirus (Ad) proteins that counteract host responses. Most of these proteins are coded by the E3 transcription unit (Fig. 26.1), a cassette of genes expressed throughout Ad infection.[1] These proteins are presumed to prolong acute Ad infections, thereby providing more opportunity for the virus to replicate, and to permit long term persistent Ad infection.

Serotype 5, the best understood Ad, causes a relatively mild upper respiratory tract infection in young children, and may form persistent infections in leukocytes. Most of what has been learned about Ad infections in vivo has come from studies with mice.[2] The initial infection activates the innate immune system, leading to the synthesis of tumor necrosis factor (TNF) and other cytokines, probably by activated macrophages. This early inflammatory response is associated with the infiltration of monocytes, neutrophils, and Natural killer cells. Later, specific immunity develops, including Ad-specific cytotoxic T lymphocytes (CTL) and antibodies.

CTL are believed to be a major mechanism by which Ad-infected cells are eliminated.[3] Ad encodes at least four polypeptides that appear to inhibit killing by CTL[1] (Fig. 26.2). In order for CTL to kill virus-infected cells, the T-cell receptor must first recognize viral peptides complexed with major histocompatibility complex (MHC) class I antigens expressed on the infected cell surface. Ad encodes a protein named E3 gp19k that prevents this from occuring. gp19K is a membrane glycoprotein, localized in the endoplasmic reticulum (ER), which forms a complex with newly synthesized class I antigens and prevents their transport to the cell surface. Accordingly, Ad-infected cells are not killed by Ad-specific or alloreactive CTL. The lumenal domain of gp19K mediates binding to the class I heavy chain. Retention of

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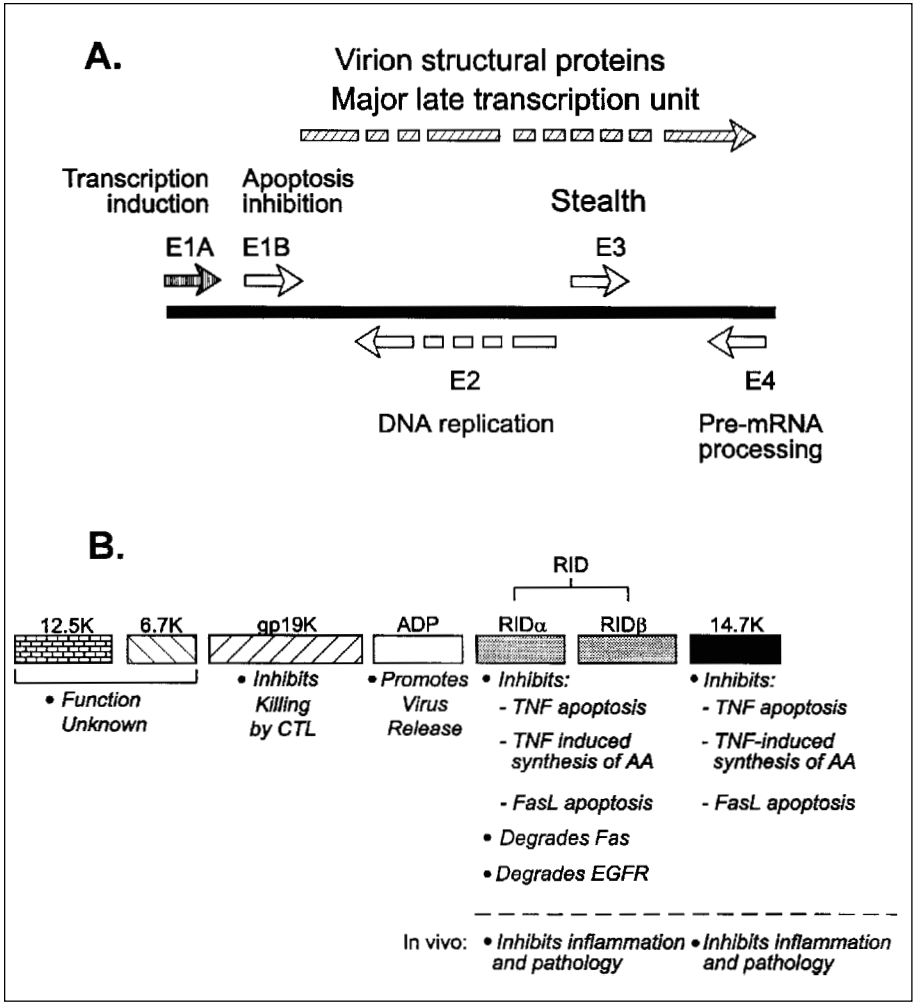

Fig. 26.1. Schematic of the adenovirus genome and the E3 transcription unit. (A) The genome consists of a linear duplex DNA molecule of 36,000 base pairs and about 36 genes (bar). The transcription units are shown by arrows. The E1A, E1B, E2, E3, and E4 regions are expressed during "early" stages of infection, prior to viral DNA replication; the genes are somewhat grouped according to their functions, as indicated. The "major late" transcription unit, which is expressed following DNA replication, encodes primarily viral structural proteins. (B) The E3 transcription unit contains seven known genes, shown by the bars. The functions associated with each protein are indicated. RIDα and RIDβ function as a protein complex named RID, an acronym for "Receptor Internalization and Degradation". RID was previously named E3 10.4K/14.5K, and RIDα and RIDβ were named E3 10.4K and E3 14.5K, respectively.[1]

both gp19K and class I antigens in the ER is mediated by an ER-retention signal, KKXX, found at the extreme C-terminus of gp19K. This signal was discovered in gp19K, and serves as an ER retention signal in many cellular ER membrane proteins. gp19K has different

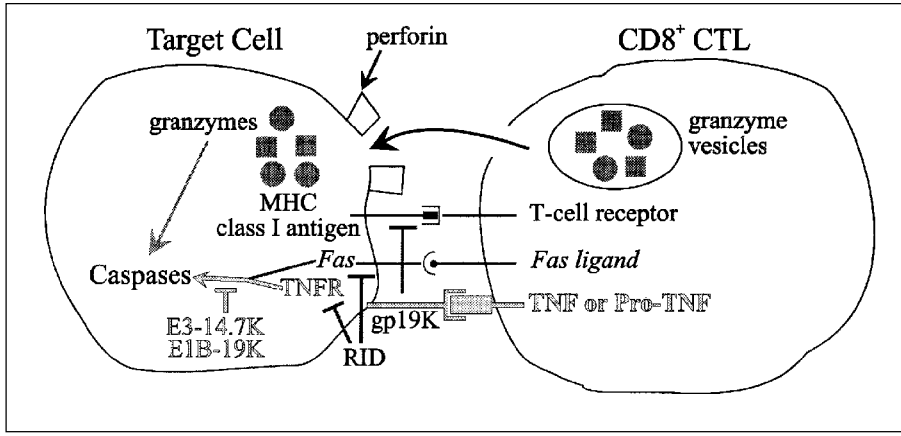

Fig. 26.2. Adenovirus proteins that inhibit apoptosis induced by cytotoxic T lymphocytes. Target cells express MHC class I antigens complexed with peptides on their cell surface. When this complex engages the T-cell receptor, the CTL become activated and express cytotoxic molecules such as Fas ligand and pro-TNF. TNF is also secreted in an active form (this is not true for Fas ligand). Fas ligand and TNF interact with their receptors on target cells and induce apoptosis by activating caspases. The Ad gp19K protein blocks CTL killing by preventing transport of class I antigens to the cell surface. RID blocks CTL killing by removing Fas and TNFR1 from the cell surface. E3 14.7K and E1B 19K independently inhibit TNF- and FasL-induced apoptosis by interfering with protein-protein interactions that lead to activation of the pro-apoptotic caspases; the ability of E3 14.7K and E1B 19K to block killing by CTL is hypothetical and has not been shown experimentally.

affinities for different class I antigens. It binds to all human class I antigens, but its affinity can vary by two orders of magnitude.[1] In the mouse, it binds best to H-2 D^b and K^d, next best to L^d, and it does not bind to D^d, K^b, D^k or K^k.

Once activated, CTL kill targets through three pathways.[4] The major one involves perforin and granzymes, where perforin forms holes in the target cell and granzymes are introduced into the cell. One of the granzymes, granzyme B, induces apoptosis by activating the caspases, pro-apoptotic, Asp-directed proteases that mediate apoptosis. The other pathway is mediated through the receptor named Fas, which is expressed on the target cell. A third pathway, which is observed in long term cell lysis assays, is mediated through the type I TNF receptor (TNFR1). TNF is the prototype for the TNF family of cytokines. It is expressed on the surface of, and is secreted by, activated monocytes and T cells. These cells become activated during the innate and immune-specific phases of the immune response to infection. Fas ligand (FasL) is another member of the TNF family which is expressed on activated leukocytes. TNF and FasL interact with their respective receptors, TNFR1 and Fas, which are expressed on most cell types. This interaction triggers a series of protein-protein interactions that leads to apoptosis.[4] With FasL, Fas trimerizes, then binds the protein named FADD. Binding occurs through a protein domain called the death domain (DD) present in both Fas and FADD. FADD contains a second domain called the death effector domain(DED), which binds to a DED in caspase 8 and results in activation of caspase 8. Activated caspase 8 cleaves and activates downstream caspases. The mechanism for TNF is similar except that TNFR1 binds through its DD to another DD-containing protein, TRADD; this is followed

by binding to FADD and caspase 8. Ad encodes several proteins that modify the cellular response to TNF and Fas.

Most cells are not killed by TNF, probably because TNF induces genes, via the NFκB transcription factor, that prevent apoptosis. Ad infection sensitizes cells to TNF, a property that has been mapped to the Ad E1A proteins.[5] There are two E1A proteins, named 289R and 243R, that are coded by alternatively spliced RNAs. The 289R protein functions primarily in turning on Ad gene expression, whereas the 243R protein deregulates the cell cycle, forcing cells from G0 into S-phase. The 243R protein binds to two distinct cellular proteins, pRb and p300/CBP (and their family members).[6] In quiescent cells, pRb exists in a complex with members of the E2F family of transcription factors. This complex is bound to promoters containing E2F sites, and the promoters are repressed.[7] E2F sites are found on promoters for genes that function in S-phase, e.g., thymidine kinase, ribonucleotide reductase, etc. The 243R protein forms a complex with pRb, disrupting the pRb/E2F complex and liberating E2F, which is then free to activate promoters with E2F sites. The p300/CBP protein is a transcription adaptor protein complex, and also a histone acetyltransferase, that represses certain enhancer-linked promoters.[7] These promoters are thought to regulate genes that induce differentiation and inhibit exit from G0. The 243R protein binds and disrupts the p300/CBP complex, allowing expression of genes that permit cells to enter S-phase. Ad-infected cells must be in S-phase in order for Ad DNA to replicate. The ability of the 243R protein to sensitize cells to TNF also requires that it disrupt both the pRb and the p300/CBP complexes.[5]

Destruction of Ad-infected cells by TNF and FasL before Ad has a chance to replicate is clearly not to the advantage of Ad, and, indeed, the virus has proteins that inhibit killing by these ligands.[8,9] One of these proteins is named E3 14.7K (a 14,700 dalton protein coded by the E3 transcription unit). E3 14.7K is a fairly abundant non-membrane protein. In 1988 we showed, in collaboration with Linda Gooding, that E3 14.7K inhibits TNF-induced apoptosis of Ad-infected cells.[10] This was the first report of a viral protein that blocks the cellular response to a cytokine. A recent study indicates that E3 14.7K also inhibits apoptosis induced by FasL,[11] although this is not true in all situations.[12] We have some insight into the mechanism of action of E3 14.7K. It is reported to bind to caspase 8 and inhibit apoptosis induced when caspase 8 is transfected into cells.[11] Thus, 14.7K may inactivate caspase 8 directly, or it may inhibit a downstream caspase. In this sense, 14.7K is similar to the cowpox CrmA and the baculovirus p35 proteins, both of which are caspase inhibitors. E3 14.7K interacts with at least two other proteins, FIP-2 and FIP-1, as first revealed in yeast two-hybrid experiments.[13] FIP-2 reverses the ability of 14.7K to inhibit apoptosis induced by transfection of the cytoplasmic domain of either TNFR1 or RIP, a protein involved in one of the death pathways induced by TNF. Thus, 14.7K may block two pathways of apoptosis from TNFR1. E3 14.7K also binds FIP-1, which is a small GTPase related to Ras; the significance of the 14.7K-FIP-1 interaction is not known.

RID (receptor internalization and degradation) is another Ad E3 coded protein that blocks the effects of apoptosis-inducing cytokines. RID is a protein complex consisting of two polypeptides, RIDα and RIDβ.[14] Both RIDα and RIDβ are integral membrane proteins that localize in part to the plasma membrane. RID has a number of interesting functions. It inhibits apoptosis in Ad-infected cells treated with a monoclonal antibody that triggers apoptosis through the Fas pathway.[12,14] The mechanism by which this occurs is very novel: RID stimulates the internalization of Fas into endosomes which are transported to lysosomes where Fas is degraded.[14] Since Fas is not on the cell surface, it cannot interact with the Fas agonist antibody (or FasL). RID also causes a similar internalization and degradation of the epidermal growth factor receptor (EGFR),[15] as well as the receptors for insulin and insulin-like growth factor.[16] However, not all cell surface receptors are affected, including transferrin

receptor, HER2, MHC class I, and lymphotoxin β.[12] A possible reason why Ad may wish to eliminate these growth factor receptors will be discussed later.

We have a few clues to the mechanism of action of RID. Most receptors, e.g., EGFR, are known to be internalized into endosomes following interaction with their ligand. A subclass of receptors is then sorted to lysosomes where the receptors are degraded, resulting in the attenuation of the signal. Sorting of these receptors is mediated by specific signals, the dileucine and tyrosine-based motifs, found in the cytoplasmic domains of the receptors. Our working model holds that RID acts as surrogate ligand for Fas, EGFR, and other receptors, causing them to be sorted from the cell surface to lysosomes where they are degraded. There is some circumstantial evidence supporting this model. First, when examined by immunofluorescence, RID can be seen in the plasma membrane, ER, and Golgi.[14] When transiently transfected into cells, RID can also be found in vesicles that appear to contain Fas.[14] Second, RIDα and RIDβ both are oriented in the membrane with their C-terminal domains extending into the cytoplasm.[1] Both RIDα and RIDβ contain motifs in their cytoplasmic domains that resemble those in EGFR.[1] RIDα has two potential dileucine motifs as well as a tyrosine-based motif, and RIDβ has a tyrosine-based motif. Third, RID is very stable, whereas Fas and EGFR are very unstable in the presence of RID. We propose that RID coerces these receptors to enter into endosomes, and then to be targeted to lysosomes via the sorting signals in RID. After RID dumps the receptors in the lysosomes, RID recycles back to the cell surface to pick up additional receptors and repeat the process.

A number of years ago we reported that RID inhibits apoptosis induced by TNF.[17] Our preliminary results indicate that TNFR1 is cleared from the cell surface as a function of RID in Ad-infected cells, but that the process is less efficient than that with Fas (unpublished results). We do not know whether RID affects TNFR1 and Fas (and EGFR) by the same mechanism. Regardless, TNFR1 is removed from the cell surface, and that could explain how RID prevents TNF-induced apoptosis.

Still another Ad protein inhibits killing of cells through the Fas and TNFR1 pathways.[14,18,19] This protein, named E1B 19K, is a homolog of Bcl-2, the cellular protein that inhibits apoptosis.[9,19,20] In common with the anti-apoptotic members of the Bcl-2 family, E1B 19K binds and inactivates pro-apoptotic Bcl-2 family members such as Bax, Bak, and Bik-1/Nbk. E1B 19K was recently reported to inhibit apoptosis induced by FADD but not by caspase 8, apparently by disrupting the oligomerization of FADD and sequestering a component of the multiprotein complex that mediates apoptosis.[19] E1B 19K also binds other cellular proteins, named Nip-1, -2, and -3.[20] Some of these proteins are involved in apoptosis.

In addition to inhibiting apoptosis, RID and E3 14.7K also have at least one other effect on cells, namely they prevent TNF-induced release of arachidonic acid (AA). The AA is synthesized by cytosolic phospholipase A_2 (cPLA2), a cytosolic enzyme that, when activated by TNF, translocates to membranes and cleaves AA from membrane phospholipids.[21] RID inhibits the TNF-induced translocation of $cPLA_2$ to membranes.[22] This happens before RID has cleared TNFR1 from the cell surface, so it probably occurs by a mechanism different from the downregulation of cell surface TNFR1. The mechanism by which E3 14.7K prevents TNF-induced release of AA is not known.

Most information available on these Ad proteins and their functions has been obtained in cell culture studies. However, there are animal data consistent with the conclusions. When cotton rats were infected in the lung with wild type Ad or Ad mutants lacking certain E3 genes, increased pathology and inflammation were observed with a gp19K-negative mutant.[23] A mutant lacking RID and E3 14.7K was also more pathogenic. When mice were infected in the lung with Ad wild type virus or E3 mutants, RID and E3 14.7K were found to play independent roles in reducing inflammation and pathogenesis.[24] In another type of

study, when gp19K and the β-glucuronidase reporter were expressed from a typical Ad gene therapy vector, i.e., with the E1 region deleted such that Ad genes cannot be expressed, gp19K prolonged β-glucuronidase expression in the livers of mice with class I molecules able to bind to gp19K.[25] E3 14.7K expressed in the alveoli of E3 14.7K transgenic mice reduced the infiltration of inflammatory cells, especially lymphocytes, it reduced pathology when these mice were infected with an Ad vector expressing luciferase as a reporter, and it prolonged expression of luciferase.[26]

The ability of RID and E3 14.7K to inhibit inflammation in vivo and to inhibit TNF-induced AA release in vitro may offer a reason why RID downregulates growth factor receptors. That is, growth factor signal transduction activates the Ras-MAP kinase pathway, which can result in phosphorylation and activation of $cPLA_2$, release of AA, and an inflammatory response. RID, by getting RID of the growth factor receptors, would prevent this inflammatory response.

In summary, Ad has elaborate, and in some ways redundant, tools to overcome the DD-containing receptors Fas and TNFR1. RID gets rid of Fas and TNFR1, thereby precluding activity through these receptors. E1B 19K inhibits apoptosis at the level of FADD, it blocks the pro-apopotic Bcl-2 family members, and it interferes with the functions of the Nip proteins. E3 14.7K inhibits caspase 8 or perhaps downstream caspases, and it may interfere with TNF-induced apoptosis through RIP. In many if not all of these cases, the net effect is that the caspases do not become activated.

The Ad proteins would be expected to keep infected cells alive throughout the course of infection. During the early inflammatory stage, the proteins should prevent killing by macrophages and NK cells, which secrete TNF and express TNF and FasL on their surface. The proteins could inhibit the infiltration of inflammatory cells by preventing the synthesis of AA. During the late immune-specific stage, the proteins should inhibit killing by CTL, with E3 gp19k blocking expression of class I antigens on the cell surface, and RID, E3 14.7K, and E1B 19K inhibiting apoptosis through the TNFR1 and Fas pathways (Fig. 26.2). We anticipate that RID, E3 14.7K, and E1B 19K will also inhibit apoptosis induced by TRAIL and other TNF family members that activate DD-containing receptors.

These anti-immune Ad proteins are not only fascinating in terms of their biology, they also have the potential to be useful in treating disease. As mentioned, synthesis of gp19K by an Ad vector increased transgene expression.[25] This was also observed with a vector engineered to express all the Ad E3 genes.[27] E3 14.7K made in transgenic mice also improved the persistence of a vector. Thus, incorporation of E3 genes into Ad vectors may hide the vectors from the immune system, prevent apoptosis, inhibit inflammation, and allow the vector to persist for longer periods. E3 genes may also be useful out of the context of the Ad genome when the immune response and apoptosis are an issue. Horwitz and coworkers have engineered transgenic mice to express E3 genes in the pancreatic β-cells. These β-cells were successfully transplanted into allogeneic strains of mice.[28] Thus, E3 genes could possibly be used in human tissue transplantation.

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Chapter 27

Implications of the Innate Immune System for Adenovirus-Mediated Gene Transfer

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Adenovirus is one of the most well-studied viruses, partly due to its use as a model of eukaryotic gene expression and partly due to efforts to develop a vaccine against outbreaks of adenovirus infection. The potential use of adenovirus for in vivo gene therapy has renewed interest in the immunological responses elicited to in vivo administration of adenovirus and to recombinant, replication-deficient adenovirus vectors (Ad). Adenoviruses were initially selected for development of virus-based gene replacement therapy for cystic fibrosis lung disease, because of their ability to transfer a functional recombinant gene (transgene) to the airway epithelium of the lung in vivo (reviewed in ref. 1). Widespread interest in this and other broad potential clinical applications led to extensive evaluations of the safety and efficacy of in vivo Ad-mediated gene transfer to the lung in a variety of animal models and Phase I human clinical trials. Such studies have greatly improved our practical knowledge of the potential uses and current limitations of these vectors for gene transfer.

While Ad-mediated gene transfer is very efficient in most in vitro cell culture models, in vivo gene transfer to the intact nasal and lung airway epithelium is much less efficient in both animal models and humans.[2] Although the reasons for this low efficiency of in vivo gene transfer are not yet completely understood, there are two main determinants: 1. A low rate of initial gene transfer into airway epithelial cells of immunologically naive animals with virtually no effective gene transfer on repeat administration; and 2. An active elimination of transgene expression.

Numerous preclinical studies in a variety of animal models, as well as several Phase I human clinical trials, have established the critical role of the adaptive immune responses directed at specific Ad epitopes (or, in some cases, transgene epitopes). Briefly, studies in normal and athymic (nude) mice established that MHC class I-restricted CD8 cytotoxic T lymphocytes (CTL) directed at Ad epitopes destroy transduced cells, thus decreasing the duration of transgene expression.[3,4] A second adaptive immune response, the formation of specific neutralizing antibody directed at Ad capsid epitopes, is known to be an important determinant of the low efficiency of repeated Ad-mediated gene transfer.[3] Furthermore, development of this antigen-specific antibody response is enhanced by MHC class II restricted, Ad-specific CD4 T helper (T_h) cells.[3] Thus, adaptive immune responses provide an explanation for the short duration of Ad-mediated transgene expression and, in part, the difficulty of repeated gene transfer.

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Despite the importance of adaptive immune responses in other aspects of in vivo Ad-mediated gene transfer, they fail to explain the low efficiency of initial gene transfer to lung epithelium. Experiments using immature or well differentiated epithelial cells and intact airway epithelium have established the presence of several barriers to in vivo Ad uptake, thus providing a partial explanation for the low efficiency of initial gene transfer. These barriers include diminished expression of adenovirus receptor (CAR) and coreceptor (integrin $\alpha_V\beta_5$) on the apical membrane (e.g., luminal) surface of mature, well differentiated airway epithelial. Separately, several lines of evidence have now established an important role for nonspecific or innate immune host responses in determining the initial rate of Ad-mediated gene transfer in vivo.[4-6] Because adaptive immune responses to Ad have been reviewed in detail elsewhere (see chapter 28 of this book), this chapter will focus on the recent data regarding the innate immune system in lung and its role in Ad-mediated gene transfer in the lung.

Definition of Innate Immunity

Immunity to microorganisms consists of two systems: innate or natural immunity, and acquired or adaptive immunity. Both systems have evolved in parallel over the last 400 million years since the appearance of the lymphocyte-based adaptive immune system. However, the innate immune system is phylogenetically older and components of innate immunity can be found, presumably, in virtually all Mesozoic organisms. In contrast, adaptive immunity is present only in cartilaginous and bony fish, amphibians, reptiles, birds and mammals.[7] The principal difference between innate and adaptive immunity is the distinct means by which each recognizes and directs responses to microorganisms.

Innate immunity can be defined as a system of predefined or fixed responses that identify and detoxify potentially noxious substances. Proteins mediating innate immune defenses are encoded by genes expressed without internal gene rearrangement to alter the specificity of recognition as occurs in adaptive immunity. For example, a macrophage confronted by a gram-negative bacteria binds the cell surface receptor for lipopolysacharide (LPS) within the bacterial wall and responds by secreting a variety of inflammatory mediators such as proinflammatory cytokines, chemoattractive chemokines etc. Subsequent events are triggered in target cells by these cytokines and chemokines. In this context, the macrophage, its LPS receptor and the various cytokines and chemokines are all part of the innate immune response that also includes phagocytosis of the bacteria by the alveolar macrophage. As illustrated by this example, components of the innate immune system often interact with carbohydrate moieties via lectin-like domains to recognize and bind to components of the cell walls of pathogens.

Adaptive immunity is a fundamentally distinct system, encoded by genes which undergo structural rearrangements in lymphocytes to generate proteins that bind to foreign antigens. The adaptive immune response creates a seemingly infinite diversity of antibody and cellular receptors that recognize the substance to which the lymphocyte was exposed. On repeated exposure to the substance, memory cells of the adaptive immune system proliferate and mediate subsequent downstream immunological events, e.g., antibody production, generation of CTL responses etc.

Innate Immunity to Infection in the Lung

A vast array of functionally integrated cellular and molecular components mediate innate immune protection against viral and other infections. While a detailed discussion of each of these is beyond the scope of this article (for a comprehensive review see ref. 8), the major components of innate immunity against virus infection will be discussed briefly prior to reviewing the current data regarding innate immunity and Ad-mediated gene transfer to

the lung. Inhaled microorganisms first encounter a series of innate defenses and if these are successfully penetrated, adaptive defenses are engaged (Fig. 27.1). The innate lung defenses include physical or mechanical barriers and a variety of nonspecific inflammatory molecules, nonspecific inflammatory cells and the respiratory epithelium itself. The molecular components include soluble factors of natural immunity, cytokines, interferon (IFN)s. The cellular component includes macrophages, neutrophils, natural killer (NK) cells, and non-MHC restricted cytotoxic lymphocytes.

Inhaled agents that penetrate the physical defenses may interact with either soluble or cellular factors and can initiate an inflammatory response. The type and magnitude of the response that ensues depends both on the nature of the inhaled agent and which component of innate immunity is engaged. For example, many carbohydrate-containing infectious organisms (e.g., bacteria, some viruses) as well as some inorganic materials (e.g., silica, asbestos) result in intense inflammation or pneumonia with accumulation of release of numerous proinflammatory cytokines, chemoattractive chemokines and inflammatory cell infiltration. Importantly, because the lung is constantly exposed to a plethora of diverse inhaled agents, many of which are not noxious, a critically important aspect of innate immunity in the lung is the ability to recognize the vast array of inhaled agents, but to respond only to potentially noxious materials so as to minimize toxicity to the lung.

Physical and Mechanical Barriers

In Mesozoic organisms, the necessarily large alveolar surface required for adequate gas exchange is topologically an "exterior" surface and thus is exposed to a broad range of inhaled debris and microorganisms. Through evolution, invagination of this tissue within the body to form the lung has created a primary physical barrier that protects the fragile alveolar surface. The mucociliary escalator present within airways creates a second line of mechanical defense based on production of a biochemical barrier (e.g., mucous) and a means for ejecting foreign material from lung tissue (e.g., via fluid secretion and the action of cilia). Thus, these two physical/mechanical barriers comprise a constitutive, less "reactive" innate immune defense that protects against inhaled particulate matter regardless of whether it is noxious or innocuous.

Cellular Innate Immune Responses

Macrophages are of particular importance in natural resistance to infection because of their ability to accumulate invading pathogens by phagocytosis and to restrict the pathogens' replication or degrade them. The antiviral activity of macrophages is usually considered in terms of two mechanisms; the restriction of viral replication within macrophages (intrinsic resistance) and the capacity of macrophages to secrete antiviral substances (e.g., IFN-$\alpha$and IFN-β) that control viral infections in other cells (extrinsic resistance). Macrophages are strategically located at various portals of entry (especially the lung, e.g., alveolar macrophages) and are in close contact with circulating blood entering the different organs. Neutrophils are a major component of leukocyte infiltrate into tissue at early stages (<24 hours) of viral infection. Neutrophils, like alveolar macrophages, express receptors for antibody (FcR) and complement and can phagocytose virions opsonized with Ab, complement, or both. This phagocytotic activity is enhanced by tumor necrosis factor (TNF)-α. In the presence of antiviral antibody, complement, or both, neutrophils can also lyse other cells infected with virus. NK cells represent an early host response to viral infection. These cells are large lymphocytes harboring cytoplasmic granules that contain a group of antimicrobial peptides including serine proteases and the membrane pore-forming molecule perforin that are cytotoxic for virus-infected cells.

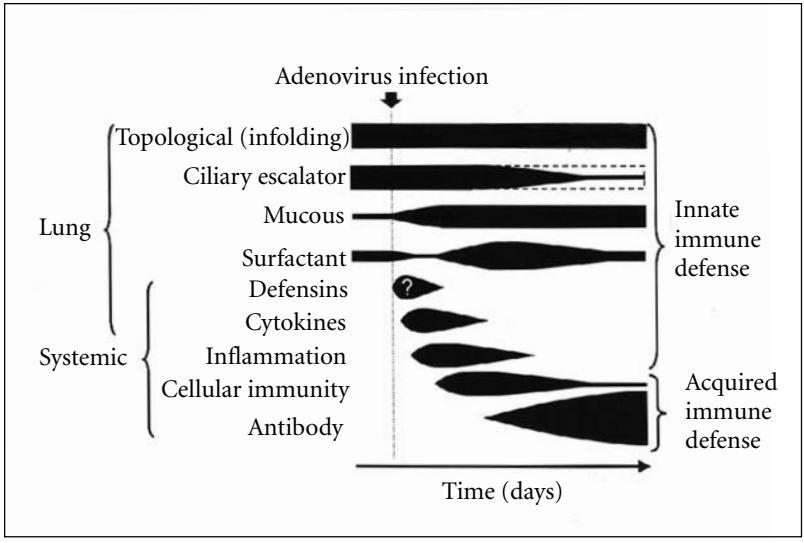

Fig. 27.1. Stepwise utilization or activation of lung defense mechanisms. The lung is protected by a variety of innate and adaptive immune responses of both lung and systemic origin. Some of these (e.g., topological invagination, mucous production, ciliary clearance and surfactant expression) are constitutive or at least active prior to the time of infection (denoted by the vertical arrow and dotted line). In contrast, others (inflammation, cellular immunity and production of antigen-specific antibodies) are activated after infection. Cytokine expression is complex, and some are constitutively active while others appear to be rapidly induced in response to infection. Defensins are expressed by neutrophils and airway epithelial cells; however, no role has been defined in adenovirus infection. See text for further details.

Soluble Factors of Natural Immunity

Natural antibodies recognize and bind to particular chemical structures including proteins, lipids and carbohydrates and may be produced in the absence or presence of a microbial infection or some other noninfectious agent.[8] In contrast to high affinity antibodies of the adaptive immune system, natural antibodies are of fixed specificity because their genes are expressed as encoded in the germ line without gene rearrangements. Examples of natural antibodies include those that recognize the blood group antigens and those that recognize species-specific cell surface carbohydrate structures. Natural antibodies to murine cell surface carbohydrates present on murine retroviral vector help mediate the rapid lysis of retroviral vectors exposed to human serum. Complement (C) can also neutralize viruses by various mechanisms, including coating of virion with protein, agglutination of virions resulting in a net loss in infectivity, opsonization of virions for degradation by C3 receptor-bearing phagocytes, and lysis of enveloped virions.[8] All three complement activation pathways (classical, alternative and collectin, see below) can be activated by viruses and can lead to opsonization, lysis of targets, chemotaxis of neutrophils and degranulation of mast cells. For example, retroviruses from mice, rats, birds, and cats are lysed by complement in human serum. Collectins are a group of collagenous, C type lectins bearing structural and functional homology with C1q and include conglutinin, mannose-binding protein (MBP) and the surfactant proteins A (SP-A) and D (SP-D).[8] Collectins bind to carbohydrate

structures on the surfaces of mammalian cells, bacteria, and presumably viruses, and some (e.g., MBP) can substitute for C1q in the activation of complement cascade. Surfactant, initially thought to be primarily involved in maintaining alveolar structural integrity and patency, also confers a protective role to the lung. For example, mice deficient in SP-A are more susceptible to some bacterial infections. Defensins are a family of proteins with bacteriocidal properties secreted from neutrophils and epithelial cells including those of the airway. A series of other proteins provide antimicrobial activity against bacteria and includes lysozyme, elastase, cathepsin G, phospholipase A2, lactoferrin and transferrin. At present, the potential role of these molecules in viral infection of the lung is not clear. A number of cytokines are associated with nonspecific host responses and mediate inflammation (e.g., Interleukin (IL)-1α, L-1β, IL-6, transforming growth factor (TGF-β), TNF-α, chemotaxis (e.g., IL-8, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1, MIP-2, TGF-α) or posses direct antiviral activity (e.g., IFN-α, IFN-β, IFN-γ) (Table 27.1). For example, mice deficient in MIP-1αfailed to mount a strong inflammatory response to coxsackie virus or influenza virus infection and delayed clearance of the virus.[9] Additional cytokines mediate activation of the immune system (e.g., IL-2, IL-12) or suppression of the immune system (e.g., IL-10, TGF-β). Many of these cytokines have multiple functions in viral and other infections and some also play a role in other biological processes such as wound healing.

Interaction of Innate and Adaptive Immune Responses

The in vivo connections between innate and adaptive immune responses are multiple, overlapping, practically inseparable and complex. Penetration of initial innate defenses results in the sequential activation of less physical and more inflammatory defenses that are more hostile for both the organism and the host, i.e., cough and mucociliary clearance vs. inflammatory cell infiltration and pneumonia (Fig. 27.1). Concurrent activation of innate and adaptive immune responses leads to production of cytotoxic lymphocytes and antiviral antibodies that recognize specific epitopes and inhibit subsequent infection (i.e., neutralizing antibodies). Expression of the network of cytokines and other soluble mediators of inflammation provides numerous links between the two systems. For example, C3d protein of complement also can modulate the antibody response through binding to cluster differentiation (CD)21 on the plasma membrane of B cells. In addition, IFN-γ and IL-12 comprise an autocrine positive feedback loop between macrophages and NK or $\gamma\delta$T cells. IFN-γ and IL-12 also modulate adaptive immunity by inducing the differentiation of T_h1 cells which subsequently modulate a variety of both adaptive and innate immune functions.

Innate Immunity to Adenovirus-Mediated In Vivo Gene Transfer

This section reviews the current evidence regarding interactions of adenovirus and the components of innate immunity. For convenience, the data will be discussed in the order of the mechanisms outlined in Figure 27.1. The physical barriers probably afford some protection from adenovirus infection of the lung, although little direct evidence is available. Defensins are expressed in the lung and have been implicated in protection against retroviral infection;[8] however, no role has yet been established for protection against adenovirus infection or in Ad-mediated gene transfer. Surfactant protein gene expression is altered and surfactant homeostasis is disrupted by Ad administration to the lung.[10] Despite information suggesting a defensive role for SP-A in bacterial infection of the lung, no similar role has yet been proven for adenovirus infection of the lung. However, it is interesting that SP-A binds to the surface of various other viruses including influenza and herpes. Since virulence factors and host defenses have coevolved, natural selection for absence of adenovirus receptor

Table 27.1. Cytokines of innate immunity

Class	Molecule	Major Functions
Cytokines/Chemokines	IL-1α, IL-1β	Inflammation, Fever
	IL-2	NK activation IFNγ induction
	IL-6	Inflammation, Fever
	IL-8	Chemotaxis
	IL-10	Immune Suppression
	IL-12	NK cell activation IFNg secretion
	MCP-1	Chemotaxis
	MIP-1α, MIP-1β	Chemotaxis
	MIP-2	Chemotaxis
	TGFβ	Immune suppression
	TNFα	Inflammation Chemotaxis Apoptosis Macrophage activation Neutrophil activation
Interferons	IFNα, IFNβ	Antiviral NK cell activation MHC upregulation
	IFNγ	Antiviral Macrophage activation MHC upregulation

molecules required for infection of host cells at necessary locations in the airway could be considered an evolutionary contribution to innate defense against adenovirus infection.

Molecular Mediators of Inflammation

Expression of pro-inflammatory cytokines, chemoattractive chemokines and activation of various cytokine signal transduction pathways mediate many aspects of Ad-induced inflammation. Multiple in vivo studies have demonstrated elevated levels of cytokine expression in response to pulmonary administration of wild type adenovirus or Ad. For example, pulmonary administration of wild type adenovirus to mice resulted in enhanced

expression of proinflammatory cytokines TNF-α, IL-1 and IL-6 in lung from 1 to 7 days after infection.[11] In one study, IL-6 but not TNF-α, expression was also elevated as early as 6 hours post infection. In non-human primates, bronchoscopic delivery of 10^{10} infectious units of an Ad expressing a β-galactosidase transgene increased IL-8 and IL-1β expression levels in lung from 3 to 28 days post-infection.[12] In mice, pulmonary administration of Ad caused a rapid expression of IL-6, MIP-1α, and MIP-2 by 6 hours post-infection.[6] Early expression of these latter two chemokines is consistent with the observed pattern of early accumulation of neutrophils for which they are chemoattractive. IL-1β, IFN-γ and MCP-1 were also increased in lung tissue, but not before 24 hours post-infection, thus excluding their direct role in the initiation of the inflammatory cascade. Similar results were obtained in one Phase I human clinical trial; IL-6 levels were increased in serum and bronchoalveolar lavage of individuals receiving Ad-mediated transfer of the cystic fibrosis transmembrane regulator (CFTR) cDNA.[13]

In vitro studies wherein Ad or wild type adenovirus was administered in vitro to lung-derived airway epithelial cells or macrophages yielded conflicting results regarding stimulation of cytokine expression. Ad administration induced IL-8 gene expression in A549 cells[14] consistent with the in vivo data in non-human primates.[12] However, in contrast to this in vitro observation and to in vivo data in several different animal models and humans receiving Ad, Noah et al showed that the exposure of airways to adenovirus or Ad did not induce inflammatory cytokines (IL-1β, IL-6 and IL-8) in airway epithelial cells and alveolar macrophages in vitro.[15] The reasons for the differences of observations in these studies are not clear. In HeLa cells, infection by either wild type adenovirus or Ad resulted in rapid activation of Raf-1, a transient increase in the tyrosine phosphorylation and activation of $p42^{mapk}$. This was followed by IL-8secretion and both MAPK activation and IL-8 production were inhibited by forskolin, a potent inhibitor of Raf-1, suggesting that adenovirus-induced Raf-1/MAPK actively may contribute to IL-8 production. Notwithstanding the conflicting in vitro data regarding Ad-induced cytokine production, the available in vivo data from a variety of sourcessuggests that adenovirus or Ad administration to the lung results in a rapid release of a variety of cytokine mediators of inflammation even if the cellular source is not known.

Inflammatory responses to Ad may also include activation of adhesion molecule expression, that enhance cellular infiltration. Infection of A549 cells with Ad induces intercellular adhesion molecule (ICAM)-1 (a ligand for inflammatory cell adhesion molecule CD11b/CD18) expression and increase CD18-dependent adhesion of activated neutrophils.[17]

Cellular Inflammation

Pulmonary administration of wild type, replication competent adenovirus to mice resulted in pneumonia at doses of 10^{10} infectious units per animal.[11] Histopathologically, the pneumonic infiltrate occurred in two phases. The first phase was apparent on day 1-2, peaked at day 3-4 but was still present on day 5 and consisting of septal and intra-alveolar infiltration by monocytes/macrophages and lymphocytes and lymphocytes. The second, overlapping phase consisted of very prominent lymphocytic and perivascular and peribronchial infiltration which were maximal on days 5-7. A similar pattern of adenovirus pneumonia was observed in the cotton rat, commonly used to evaluate human adenovirus infection.

Pulmonary administration of recombinant, replication-deficient, E1-, E3-deleted adenovirus vector to mice and a number of other animal models demonstrated similar cellular inflammatory responses, which were most frequently observed to be predominantly lymphocytic[3-6] (reviewed in ref. 1). However, it should be noted that most of these studies evaluated histopathology at late times after infection. Thus, one of the problems with

interpreting these studies regarding innate immunity is that at such late times after infection, innate responses cannot be interpreted separately from concurrent adaptive responses. Notwithstanding this confounding issue, the similarity of histopathologic responses between wild type adenovirus and Ad is interesting, in spite of the fact that the key transcriptional regulator genes (e.g., the E1 region) have been eliminated in Ad. Cellular inflammation occurred in cotton rats as early as 24 hours after pulmonary Ad infection.[5] In this study, neutrophils were noted in the inflammatory infiltrate, and within the airway, epithelium itself as early as 24 hours after infection. Incomplete virions (viral capsids composed of proteins but absent intact functional viral genomes) and UV-inactivated Ad induced pulmonary inflammation six days after infection which consisted predominantly of lymphocytes.[18] Thus, the Ad capsid is itself able to induce inflammatory responses in the absence of any viral gene expression. In this study, adaptive immune responses were also noted, thus making difficult the interpretation of innate responses distinct from adaptive responses.

In an effort to focus attention on innate responses and eliminate concerns regarding adaptive cellular immune responses, inflammation was evaluated at very early times from 6 hours to 3 days after pulmonary Ad administration to normal and athymic (nude) mice (which lack functional cellular immunity).[6] Histopathological evidence of cellular inflammation was evident by 6 hours after Ad administration and cytological evaluation of whole lung lavage cells showed a predominance of neutrophils and macrophages, with very little lymphocyte accumulation. Importantly, identical results were observed in both athymic and normal animals. Interestingly, use of dexamethasone to block inflammation resulted in a statistically significantly increased level of gene transfer expression which was not attributable to an effect on transcription of the transgene. These observations demonstrate that nonspecific inflammation is an important determinant of the efficiency of pulmonary gene transfer in vivo.

Alveolar macrophages play an important role in response to intravenous and pulmonary Ad administration. Following intravenous in vivo administration of Ad to the liver, more than 90% of vector DNA present at early time points (10 min) was eliminated within 24 hours by macrophage degradation. This elimination of Ad DNA was independent of the type of transgene and was similar in normal and immunodeficient (athymic) mice, suggesting an innate immune mechanism and excluding an adaptive cellular immune etiology.[19] Prior depletion of macrophages by administration of dichloromethylene biphosphate-laden liposomes blocked the rapid loss of Ad DNA from the lungs of infected animals.[20] These results demonstrated that alveolar macrophages played an important role in elimination of adenovirus vector from lung.

The role of NK cells in the response to adenovirus or Ad administration is less clear. Lymphocytes seen at early times following pulmonary Ad administration include NK cells; however, prior depletion of NK cells did not alter the level of adenovirus-mediated gene transfer (Otake and Trapnell, unpublished observations). Still, NK cells may also play a role in Ad-induced inflammation. Pretreatment of mice with dexamethasone is known to cause lysis of lymphocytes and NK, cells and pretreatment of mice significantly reduced Ad-induced lung histopathology. NK cells are also an important source of IFN-γ. Dexamethasone decreased Ad-induced IFN-γ expression, suggesting that, during adenovirus infection, NK cell expression of IFN-γ may amplify inflammation.

Role of Adenovirus Genes in Modification of Host Responses

Adenovirus expresses a cassette of genes that modulate the host immune system in its attempt to survive (see chapter 26 for more details). For example, the adenovirus E3 region gp19 kDa protein suppresses the expression of MHC class I antigens on the cell surface,

thereby reducing CTL from attacking infected cells.[21] Also, the adenovirus E3 region 14.7 kDa protein inhibits cytolysis by TNF.[22] In homozygous (+/+) SPC/E3 14.7k transgenic mice, the lung inflammation was reduced and the transgene expression was increased.[23] These viral mechanisms represent virulence factors that ensure viral replication.

Conclusion

Despite a large and increasing body of knowledge regarding the adaptive responses to wild type adenovirus and to Ad administration, relatively less is known about role of the diverse array of innate defenses. It is clear that Ad induces a dose-dependent, nonspecific inflammatory response that impacts adenovirus-infected and noninfected cells directly. Although data regarding adenovirus are lacking, it is likely that some of these innate responses modulate the subsequent adaptive immune responses. Importantly, data regarding some aspects of innate immune defense (e.g., inflammation) have been difficult to separate from adaptive innate responses. Future studies should focus on the innate immune responses to adenovirus infection and Ad-mediated gene transfer.

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CHAPTER 28

Host Immune Responses to Recombinant Adenoviral Vectors

Johanne M. Kaplan

Recombinant adenoviruses (Ad) are versatile gene delivery vectors capable of infecting a broad range of cell types without a requirement for cell division. They are being developed for a variety of clinical applications such as correction of inherited disorders, cancer gene therapy and treatment of cardiovascular disease. However, results from animal studies suggest that the clinical usefulness of Ad vectors may be limited by their immunogenicity. Neutralizing antibodies elicited by input virus particles interfere with vector readministration which is expected to be necessary for applications such as chronic treatment of genetic disorders. In addition, the induction of cytotoxic T lymphocytes (CTLs) directed against adenoviral proteins and, more importantly, immunogenic transgene products expressed by transfected cells typically results in limited persistence of transgene expression. To address these issues, several approaches are being developed to inhibit or circumvent host humoral and cellular immune responses and improve the therapeutic potential of Ad vectors.

Humoral Immunity

Characterization of the Humoral Response to Adenovirus Vectors

Studies conducted in several animal models have shown that administration of Ad vector via different routes leads to the development of virus-specific antibodies. The response is elicited by capsid proteins and can be induced by live as well as UV-inactivated particles.[1] Western blot analysis indicates that the antibodies are directed primarily against the viral structural proteins hexon, penton and fiber.[2,3] The magnitude of the antibody response is dependent on the dose of virus, and repeated delivery of large doses of vector leads to increasing levels of antibody which plateau after 3-4 administrations.[4-7] The IgG isotype dominates the response in serum, while both IgG and IgA are elicited in the lung.[1,5,6] The IgG and IgA isotypes both possess neutralizing activity[6] and several lines of evidence indicate that they can interfere with readministration of vector. For example, an inverse correlation was noted between levels of serum neutralizing antibodies and ability to readminister vector to the lung.[4,7] In contrast, effective readministration of high doses of vector was achievable in immunodeficient animals such as class II-deficient mice which are incapable of mounting a humoral response.[1] Finally, passive transfer of immune serum into the venous circulation or the lungs of naive mice was shown to preclude gene transfer following intravenous or intratracheal delivery of Ad vector, respectively.[1] Taken together, these findings clearly demonstrate that neutralizing antibodies present a significant barrier against repeated vector delivery.

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Circumvention of the Humoral Response Through Vector-Based Approaches

Several strategies are being investigated to circumvent interference by neutralizing antibodies through manipulation of the vector or modulation of the host immune response. At the level of the vector, it has been shown that sequential administration of Ad vectors with different serotypes represents an effective approach to evade neutralizing antibodies which are known to be specific for serotypic determinants.[8,9] This type of approach, however, is not entirely practical, as it would require the production and clinical testing of a series of Ad vectors.

In another instance, overexpression of the Ad E3 region, a genomic modification which does not alter viral coat proteins, was found to inhibit the development of Ad-specific antibodies and allow for a second administration of vector.[10] The mechanism by which E3 proteins may influence the humoral response is unclear and it remains to be determined whether this phenomenon will extend to species other than the Gunn rat which was used in this study.

Finally, a simple reduction in the effective dose of virus, which can be accomplished by improving transduction efficiency, would be expected to minimize stimulation of antibody production and improve readministration of vector. However, it is unlikely that repetitive delivery of even small doses of vector will prevent antibody titers from ultimately reaching prohibitive levels.

Suppression of the Host Humoral Response

Modulation of the host humoral response will likely be necessary to allow for multiple vector delivery, and several groups have investigated the use of immunosuppressive agents. Promising results have been reported showing that transient immunosuppression with various synthetic drugs or biological agents can prevent or reduce the humoral response to Ad vector and improve levels of gene transfer to several organs upon readministration (Table 28.1). However, with few exceptions,[43,44,50] these studies were limited to a single readministration of vector and were conducted in naïve animals not previously exposed to adenovirus. Such conditions poorly reflect the expected clinical setting since, for example, treatment of chronic conditions such as cystic fibrosis is expected to require repeated administration of vector over the lifetime of an individual. In addition, most prospective gene therapy recipients are likely to have encountered wild type Ad and, in this preimmune state, may not be as susceptible to immune downregulation, since activation requirements tend to be reduced in primed lymphocyte populations. Clearly, more stringent studies in primed animals receiving multiple doses of vector need to be conducted to assess the clinical potential of immunomodulatory agents more realistically.

The induction of tolerance to Ad vectors has also been investigated as an approach to selectively prevent immune responses against the vector without affecting the general immunity of the host. Injection of Ad vector into neonates appeared to induce tolerance, as the animals failed to develop Ad-specific antibodies.[9,11] However, a single readministration of vector induced the development of neutralizing antibodies, thus limiting the usefulness of this approach. A more promising strategy was described by Ilan et al, who reported that central tolerance to Ad vector could be achieved in adult rats by repeated low dose feeding of vector protein extracts or by intrathymic injection of transduced hepatocytes, vector or protein extracts thereof.[12,13] Tolerized rats failed to develop Ad-neutralizing antibodies and repeated intravenous delivery was possible. However, the ability to induce tolerance in primed hosts remains to be determined. In addition, the potential for inducing susceptibility to wild type (wt) Ad infection in individuals tolerized against Ad vector, as well as the impact of a wt infection on the maintenance of tolerance to vector are issues that need to be addressed.

Table 28.1. Modulation of host immune responses to adenovirus vectors

Drug/ Agent	Regimen	Decreased Ab*/ Improved readministration	Decreased CTL or improved persistence	Organ	Ref.
FK506	transient or chronic	+/+	+	muscle	41,42
Deoxyspergualin	transient	+/+	+	lung, liver	43-45
Cyclophosphamide	single high dose	+/+	not measured	liver	43
	transient	+/+	+	lung, liver	46
CTLA4-Ig	transient	+/-	+	liver	47
	transient	-/not measured	+	muscle	48
CTLA4-Ig + anti-CD40 ligand	transient	+/+	+	liver	49
Anti-CD40 ligand	transient	+/+	+	lung, liver	50,51
Anti-CD4	transient	+/+	+	muscle	48
				lung, liver	52,53
IL-12	transient	+/+	not measured	lung	54
Interferon-γ	transient	+/+	not measured	lung	54

*Ab - antibody response

Humoral Response to Immunogenic Transgene Products

Another aspect of the humoral response to Ad vectors that is being increasingly recognized relates to the development of antibodies against the transgene product. Expression of immunogenic proteins by Ad vector, whether secreted (e.g., factor IX, AAT) or expressed intracellularly (e.g., β-galactosidase), can clearly lead to the induction of an antibody response.[3,5,14] In a clinical setting, antibody responses against therapeutic human proteins may occur in patients with null mutations, who may recognize the gene product as non-self, or in the case of proteins with allotypic variants.

Cellular Immunity

$CD4^+$ T-Cell Response to Adenovirus Vectors

The administration of Ad vectors to various organs typically leads to local inflammation characterized by the infiltration of $CD4^+$ and $CD8^+$ T lymphocytes. The activation of Ad-specific $CD4^+$ T cells has been measured in vitro using proliferation and cytokine release assays.[1,6,15] $CD4^+$ T lymphocytes of the Th1 subset appear to dominate the response, as indicated by the release of large amounts of IL-2 and interferon-γ upon stimulation with Ad antigen, with comparatively low levels of the Th2-type cytokine IL-4 being produced.[1,15] Cytokines released by activated Th2 cells promote the development of a humoral response, while Th1-type cytokines are involved primarily in the cellular arm of the immune response. Interferon-γ in particular, can promote the activation and expansion of CTLs and has been reported to be essential for sensitizing transduced hepatocytes for lysis by vector-specific CTLs through upregulation of MHC class I.[16]

Characterization of the Cytotoxic T-Lymphocyte Response to Adenovirus Vectors

The induction of a CTL response by recombinant Ad vectors is well documented. Vector-specific CTLs have been detected in the spleen of mice treated with an intravenous injection of Ad vector as well as in the draining lymph nodes, spleen and bronchoalveolar lavage of mice and monkeys following delivery of Ad vector to the lung.[6,15,17-19] Studies conducted in the mouse indicate that the CTL response to Ad vector is directed against both Ad proteins and immunogenic transgene products encoded by the vector.

With regard to the CTL response against Ad proteins, the E1 region of wt Ad has been shown to encode immunodominant CTL epitopes that are recognized in the context of the 3 major mouse haplotypes ($H\text{-}2^b$, $H\text{-}2^d$, $H\text{-}2^k$). Even though the E1 region is deleted in recombinant Ad vectors, the same mouse strains were able to develop Ad-specific CTL responses of varying strengths.[18] This finding indicates that immunorecessive epitopes outside of E1 can become available for recognition in E1-deleted vectors and that the magnitude of the response and ability to recognize immunorecessive epitopes is dependent on MHC haplotype.[18,20] The non-E1-encoded determinants recognized by vector-specific CTLs appear to reside primarily in late viral proteins[21] and the E2A DNA-binding protein (Kaplan et al, unpublished), which are still expressed at low levels by E1-deleted vectors.

Comparatively little is known about the specificity of human CTLs against Ad. The observed lysis of target cells infected with E1-deleted wt Ad suggests that human CTL epitopes are present outside of the E1 region and may therefore be expressed in recombinant vectors.[22,23] Contradictory results have been obtained regarding recognition of input capsid proteins by human CTLs.[22,23] In any case, it is likely that individuals with different MHC types will recognize different sets of antigenic determinants.

Impact of Cytotoxic T Lymphocytes on Persistence of Transgene Expression

Transgene expression by Ad vectors in vivo has been found to be transient in many systems, and several lines of evidence suggest that CTLs elicited by Ad vectors are involved in limiting persistence of expression. Improved duration of expression has been reported in animals lacking effector CTLs such as nude,[17,18,24] SCID,[24,25] Rag-2-deficient[1,15] and β2-microglobulin knockout mice,[1,15,18] as well as in animals with compromised CTL activity such as knockout mice lacking expression of interferon-γ or perforin.[16] In addition, adoptive transfer of immune $CD8^+$ T cells into Rag-2-deficient or nude mice stably expressing a reporter gene was found to result in loss of expression.[1,15,18] Adoptive transfer of CTLs directed against either viral proteins or the transgene product were able to terminate expression. However, the relative contribution of these CTL populations to loss of expression in intact animals remains controversial. There is evidence to suggest that, in some situations where the host is tolerant to the transgene product, CTLs specific for Ad antigens are sufficient to eliminate vector-transduced cells. For example, loss of transgene expression was observed following administration of a β-Gal-encoding Ad vector to ROSA mice transgenic for β-Gal[26,27] or after delivery of an Ad/OTC (ornithine transcarbamylase) vector to the lung of mice expressing the same enzyme in the liver.[26] On the other hand, several instances of long term expression of non-immunogenic transgene products in immunocompetent animals have also been reported. For example, persistent transgene expression from first generation Ad vectors has been observed in the following systems: human AAT in the liver of C57BL/6 or AAT transgenic mice;[14,19,25] human factor IX in the liver of C57BL/6 mice;[24] mouse erythropoietin in the muscle of several mouse strains;[28] very low density lipoprotein (VLDL) receptor in the liver of LDL receptor knockout mice and C57BL/6 mice;[2] human CFTR in the lung of several mouse strains (Fig. 28.1) (Scaria A, St. George JA, Jiang J et al. Adenovirus-mediated presistent cystic fibrosis transmembrane conductance regulator expression in mouse airway epitheliums. J Virol 1998; 72:7302-7309). In every case, persistence of expression correlated with non-immunogenicity of the transgene product at the cellular and/or humoral level, and the documented presence of a robust CTL response against Ad proteins had no apparent effect on longevity of expression (Fig. 28.1).[2,19,24] In contrast, expression of immunogenic gene products such as β-Gal is invariably transient and is accompanied by the development of CTLs against the protein and loss of vector DNA.[14,18,24] The development of antibodies against secreted gene products such as AAT and factor IX was also found to interfere with persistence of the protein in serum.[14,24] Taken together, these findings suggest that, in many situations, host immune responses to immunogenic transgene products rather than adenoviral proteins represent the primary determinant of longevity of expression.

Modification of Adenovirus Vectors to Circumvent the Cytotoxic T-Lymphocyte Response

Even though the role of Ad-specific CTLs in limiting persistence of expression may not be as significant as believed initially, several investigators have designed second and third generation Ad vectors from which additional open reading frames have been deleted to minimize viral gene expression and consequent stimulation of Ad-specific CTLs. Incorporation of a temperature sensitive version of E2A or complete deletion of the E2A region from Ad vectors was found to prevent expression of E2A and reduce late viral gene expression, but conflicting results were obtained regarding the impact on longevity of expression.[29-31] Differences in the results obtained may be due to variations in the vector backbone and model systems used by different investigators.

Partial or complete deletion of the E4 region from Ad vectors also resulted in decreased expression of E2A DNA-binding protein and late viral proteins.[29,32-34] However, in this

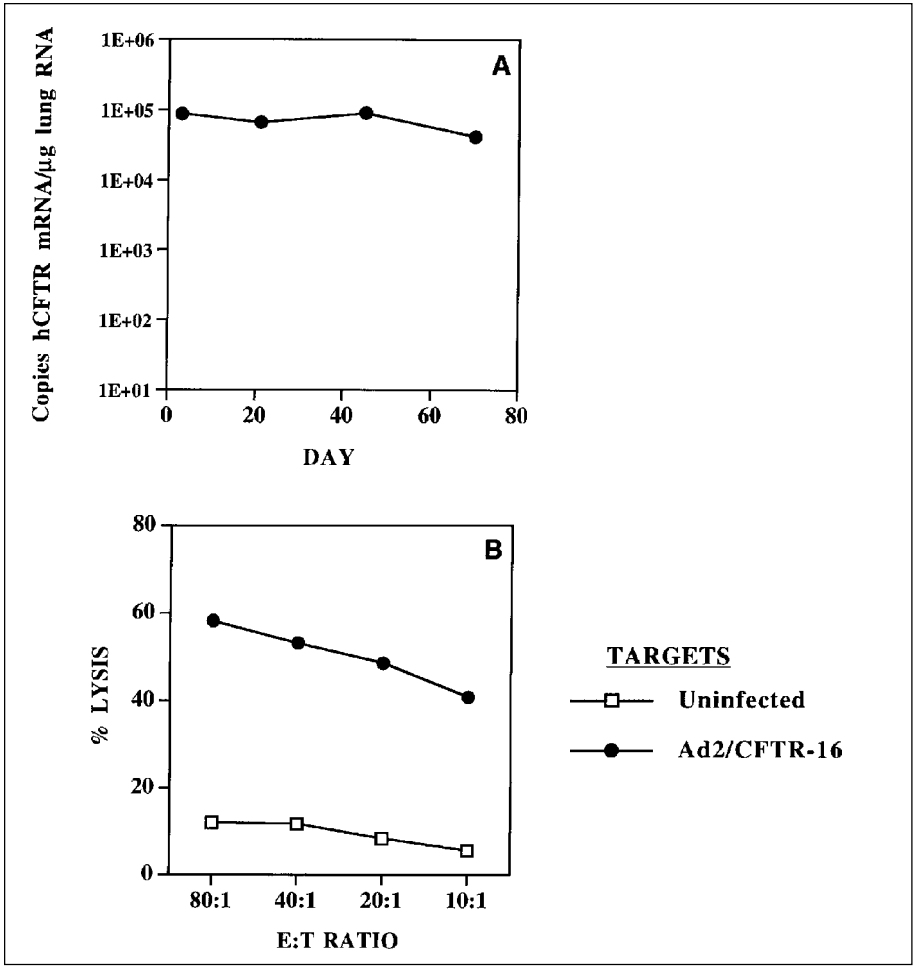

Fig. 28.1. Persistent expression of CFTR in the presence of vector-specific CTLs.(A) BALB/c mice were instilled intranasally with 2 x 10^9 i.u. of a CFTR-encoding Ad vector (Ad2/CFTR-16). Levels of CFTR mRNA expressed in the lung at different time points were measured by quantitative RT-PCR. Lung samples from 4 mice were pooled and values shown are the average of duplicate RT-PCR assays. Similar results were obtained in BALB/c (shown here), C57BL/6 and C3H mice.(B) Spleens were collected from BALB/c mice 21 days after instillation of 2 x 10^9 i.u. Ad2/CFTR-16. Pooled spleen cells were restimulated in vitro with Ad2/CFTR-16-infected syngeneic fibroblasts and tested for cytolytic activity against uninfected or Ad2/CFTR-16-infected fibroblasts. Results shown are the mean percentage lysis from triplicate wells at various effector:target (E:T) ratios. Results from Scaria A, St George JA, Jiang C, Kaplan JM, Wadsworth SC, Gregory RJ. Adenovirus mediated persistent CFTR expression in mouse airway epithelium. J Virol 1998; 72:7302-7309.

instance, assessment of the effect on persistence of non-immunogenic transgene expression has been complicated by the fact that several of the viral gene promoters used to control expression (e.g., CMV, RSV) are dependent on E4 proteins to maintain their activity.[18,32,34] Evaluation of viral DNA persistence as an alternative measurement has provided conflicting results.[29,32] Again, differences in vector structure and animal models may have contributed to discrepancies between different groups.

Finally, high capacity Ad vectors lacking all viral coding sequences have also been produced. One such vector encoding the complete human AAT locus was compared to a first generation E1-deleted Ad/AAT vector in C57BL/6 mice, a strain in which human AAT is non-immunogenic. Expression from the first generation vector in the liver slowly declined to 10% of peak levels over a 10 month period, while expression from the high capacity vector remained stable.[35] The gradual loss of expression from the first generation vector was also observed in immunodeficient Rag-1$^-$ C57BL/6 mice and appeared to be due to liver toxicities which were not observed with the high capacity vector. A reduction in toxicity has also been observed with E2A and E4-deleted vectors.[31,32] Therefore, even though long-term expression can be achieved with first generation vectors expressing nonimmunogenic transgene products, vectors with additional deletions offer the potential advantage of reduced toxicity and decreased probability for emergence of replication-competent Ad.

Another strategy in the area of vector modification is inclusion of immunomodulatory molecules in the vector genome to prevent or evade host immune responses. For example, expression of viral IL-10 from an Ad vector was found to prevent CTL priming,[36] while inclusion of CTLA4Ig inhibited the development of humoral responses against the vector and the secreted transgene product.[37] Similarly, overexpression of the Ad E3 region under the control of a CMV promoter resulted in decreased CTL responses and improved persistence of expression, presumably due to the ability of the E3 gp19K protein to interfere with MHC class I antigen presentation.[10,38,39] Not all approaches were equally successful and, contrary to expectations, the use of an Ad vector encoding Fas ligand to provide protection against lysis by Fas$^+$ effector cells proved ineffective.[40] Clearly, this type of approach represents a promising avenue of research that will require selection of the appropriate immunomodulatory molecules and treatment regimens to achieve the desired effect.

Immunomodulation of the Cellular Immune Response

Various immunosuppressive treatments have also been tested to inhibit cellular immune responses against Ad vector and prolong longevity of expression (Table 28.1). Improved persistence of expression was achieved in many cases, but the majority of the studies were conducted with Ad vectors encoding immunogenic transgene products. As described above, suppression of cell-mediated immunity may not be required in situations where the transgene product is nonimmunogenic. Nevertheless, it is possible that, in a clinical setting, CTL responses against Ad and/or the transgene may become limiting so that immunological intervention becomes necessary. For example, expression of a therapeutic protein with allotypic variants or expression of a protein in an individual with a null mutation may provoke a CTL response.

The induction of tolerance against Ad vector and the encoded transgene product has also been investigated as a possible strategy to control cell-mediated immunity. Approaches such as administration of Ad vector to neonates,[9,11] intrathymic inoculation of vector antigen[12] and oral tolerization[13] were all successful in permitting long-term expression and allowing for renewed expression upon a second administration of vector. However, as mentioned above in the context of the humoral response, several concerns remain to be addressed regarding the feasibility and safety of tolerance induction.

Conclusion

Host immune responses against Ad vectors still present a barrier to the development of successful Ad-based gene therapies. However, significant advances have been made with regard to characterization of the nature and impact of immune responses elicited by Ad vectors, as well as approaches to circumvent such responses. For example, it is now apparent that the presence of Ad-specific CTLs does not necessarily correlate with loss of expression and that long term expression of nonimmunogenic transgene products can be achieved in many systems without any immunological intervention. Repeated delivery of Ad vector, which will be required for many but not all applications, remains problematic. However, promising approaches are being developed to inhibit the development of Ad-specific antibodies and/or allow the vector to evade neutralization. Finally, it must also be kept in mind that, while the inherent immunogenicity of Ad vectors may present a problem for many gene therapy applications, the induction of immune reactivity against immunogenic transgene products is highly beneficial for purposes of immunization against pathogens or tumor antigens.

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Chapter 29

AdCFTR for Cystic Fibrosis

Samuel C. Wadsworth

Advantages of Ad Vectors for Cystic Fibrosis (CF) Gene Therapy

Cystic fibrosis (CF) is a recessive, monogenic, lethal disorder caused by defects in a protein termed the cystic fibrosis transmembrane conductance regulator (CFTR) .[1,2] Approximately 60,000 individuals are affected worldwide. CFTR is an ion channel that functions to allow the secretion of chloride from within cells. While epithelia throughout the body are affected by mutations in CFTR , the predominant cause of morbidity and mortality in CF patients is lung disease. As there is no known way to restore airway cells to the lung following ex vivo manipulation, treatment of airway disease in vivo is required.

Thus the rationale for CF gene therapy is clear: Introduce a copy of the normal CFTR cDNA (because the gene is very large[1]) into airway cells within the lung to restore CFTR activity. The number of CFTR molecules per airway cell is low,[3] suggesting that even a modest level of vector-directed gene expression would suffice. Moreover, it has been estimated that restoration of CFTR activity to 3 to 6% of airway cells would be sufficient to restore the chloride and fluid secretion properties of the airway epithelium.[4] The rationale for attempting CF gene therapy with Ad vectors also seems clear: well defined molecular biology, ability to infect nondividing cells, potential for large-scale growth at high titer, and ability to infect cells within the lung. However, since the target cells within the lung are not permanent, repeated administration will be required for Ad-based CF gene therapy, increasing the likelihood of the generation of neutralizing antibodies and the attendant potential to limit greatly the efficacy of long term treatment.

CF Gene Transfer Clinical Studies with Ad Vectors

Ten clinical studies designed to explore the utility of Ad vectors encoding CFTR (AdCFTR) for CF gene therapy have been proposed, nine in the US[5-12] and one in France.[13] All studies were carried out with CF patients. Results from only five of the proposed studies have been published.

Properties of AdCFTR Vectors

Each of the proposed studies has employed Ad2 or Ad5 serotype-based vectors with the CFTR cDNA replacing the E1 region; some investigators have employed vectors with an intact E3 region[6,10,14-17] while all others have employed E3-deleted vectors; vectors with partial E4 deletions have been employed in three studies.[14,15,17] A variety of promotors has been employed to direct CFTR cDNA expression: viral promoters, Ad E1A,[6,16] Ad MLP,[9,13,18,19] CMV IE,[12] RSV LTR;[9] cellular promoters, e.g., PGK;[10,14,15] and a hybrid promoter with the CMV IE enhancer linked to the minimal chicken β-actin promoter.[7,8]

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Clinical Strategies

Nasal Administration

The first AdCFTR clinical studies were proposed in 1992 when the safety of administration of Ad vector to the lung was untested. With safety as the primary consideration, and the knowledge that the chloride secretion defect was also manifested in the nasal epithelium of CF patients, an early clinical strategy was to administer AdCFTR vector to the nasal cavity.[6,11] The nasal cavity is also readily accessible for measurement of electrophysiological changes across the epithelium after AdCFTR gene transfer. Three of the ten CF gene therapy protocols involved administration to the nasal cavity (or maxillary sinus) alone,[6,8,10] with one study being an escalating, repeat dosing protocol;[10] another three involved administration to the nasal cavity followed by lung administration.[9,11,13]

Lung Administration

Of the seven lung administration protocols, two studies proposed aerosol administration to the entire lung.[13,15]

Safety Parameters

Because each of the clinical studies proposed to date is classified as a phase I trial, safety is the primary issue under test. A broad array of safety-related patient responses have been measured, ranging from the nonspecific general examination, to specific assays for lung function such as FEV1, chest X-ray or CT scan, to vector-specific responses such as alterations in cytokine release and antibodies specific for Ad.

Measures of Efficacy

Considering the early stage of CF gene therapy, there is no expectation of clinical benefit to patients at this time. Thus, each of the various groups has used a variety of assays to measure efficacy at the molecular and/or cellular levels; PCR for vector DNA, RT-PCR for vector CFTR mRNA expression, immunohistochemistry for CFTR protein expression, transepithelial electrical potential difference measurement (V_t) for improvement in chloride secretion.

Results from Clinical Studies

Nasal Administration

The first published data from an AdCFTR clinical study was from a single-dose, nasal administration protocol.[16] Three individual patients were treated with vector doses of 2×10^6, 2×10^7, or 5×10^7 infectious units. The vector was applied to a limited region of the nasal epithelium with the aid of a plastic applicator to reduce vector dilution by spreading. V_t measurements were recorded before and after vector administration.

A striking improvement in nasal V_t was observed in each of the treated patients for up to four weeks, a result consistent with gene transfer of normal CFTR and subsequent expression of the protein within the nasal respiratory epithelium. Moreover, vector-specific CFTR mRNA was detected by RT-PCR, providing evidence of vector gene transfer and expression at the molecular level. Patients experienced local discomfort following vector administration, likely due to "damage" produced by the applicator used to deliver the vector to the nasal epithelium. Given the increased understanding available today of issues limiting gene transfer to the respiratory epithelium,[20] it can be hypothesized that the administration-associated damage also allowed greater efficiency of gene transfer.

Using similar measurements of chloride secretion, the findings of AdCFTR gene transfer to the nasal epithelium and subsequent expression of CFTR have been confirmed. Crystal et al[18] reported evidence of gene transfer and expression in the nasal epithelium as determined by immunohistochemical and RT-PCR assays, and the same group subsequently reported electrophysiological evidence of CFTR expression.[19] Bellon et al[13] provided evidence of CFTR gene transfer and expression by RT-PCR and immunohistochemical assays. In a repeat dosing protocol to the nasal epithelium, Zabner et al[17] reported only modest improvement in chloride secretion in the middle of their dosing scheme, and no correction at the highest vector dose. The vector used in this study has the weakest gene promoter (PGK) of all vectors tested in clinical studies to date. The authors speculated that repeated vector dosing may have stimulated a neutralizing antibody response

Not all studies of nasal administration of AdCFTR have resulted in detection of improved chloride secretion. Knowles et al[21] carried out a double-blind, vehicle-controlled study in which AdCFTR was administered to the nasal cavity in logarithmically increasing doses from 2 x 10^7 to 2 x 10^{10} infectious units. These authors reported that none of the electrophysiological parameters that were measured indicated improved CFTR function. However, a proportion of the treated patients had evidence of AdCFTR gene transfer by RT-PCR. One explanation for the discrepancy between this study and those summarized above is that Knowles et al applied the AdCFTR vector to the nasal passage in a relatively large volume, 2 ml, compared to an administration volume of between <0.1 ml to 0.4 ml in the other studies. The larger volume would have the effect of reducing the multiplicity of infection and thus could result in a lower efficiency of gene transfer. Studies in mouse model systems have shown that the most efficient AdCFTR gene transfer occurs when care is taken to increase the contact of vector with the nasal epithelium.[22,23]

Lung Administration

Results from two lung administration studies have been published, one employing bronchoscopic administration and another employing aerosol administration. Crystal et al[18] reported evidence of CFTR gene transfer and expression in one patient in bronchiolar epithelial cells after bronchoscopic administration of 2 x 10^6 infectious units of AdCFTR. These results were not consistent between the three patients in the study that were tested. Three patients in this study received a vector dose of either 2 x10^6 (one patient) or 2 x 10^7 (two patients) infectious units, and there were no or minor adverse reactions to the vector in these patients. Nor were there significant or consistent increases in the neutralizing antibody response. However, one patient that received a vector dose of 2 x 10^9 infectious units had a systemic as well as a local response following vector administration. Responses included headache, fatigue, and fever, altered lung function, altered chest X-ray and CT, and a transient rise in serum IL-6 levels. These reactions were treated clinically and the patient recovered without exhibiting chronic sequelae. In three out of four patients in this study, including the patient with the adverse reaction, vector was delivered in a volume of 20 ml. This procedure is likely to force the vector solution, and purulent mucus present in the CF lung, beyond the airways and into the alveolar spaces. There has been speculation that this phenomenon may have contributed to the patient's adverse response, especially in light of the absence of reports of similar patient responses in other lung trials, even those with higher vector doses (David Meeker, personal communication).

Bellon et al[13] delivered AdCFTR to the lung by aerosol, a method likely to be the eventual preferred mode of administration to CF patients. Six patients were treated, with two patients each receiving vector doses of 1 x 10^7, 1 x 10^8, or 5.4 x 10^8 infectious units, and cells were recovered from patients' lungs by bronchial brushing at several intervals after administration. There was evidence of vector mRNA expression at 14-15 days after treatment in one patient

treated with the lowest vector dose. Evidence of CFTR protein expression was obtained in one patient in each of the two higher vector dosing groups up to 7-8 days post-administration. Approximately 7-11% of cells recovered by brushing were positive for CFTR by immunohistochemistry. No significant changes in the Ad-specific immune status were observed in the treated patients, nor were inflammatory responses to vector administration noted.

Conclusion

To date few adverse reactions to AdCFTR administration to the nose or lung have been reported, indicating that AdCFTR vectors are generally rather safe, at least at the doses tested so far and by the routes of administration used. Moreover, the anticipated robust secondary antibody response in treated patients has not been observed. However, caution in interpretation of these findings is advised since the experience with repeated vector administration is minimal.

What has been demonstrated clearly by the published reports is that it is possible to achieve AdCFTR gene transfer and expression of the normal CFTR protein in cells within CF patients. Correction of chloride secretion, expression of vector-encoded CFTR mRNA, and expression of CFTR protein have all been demonstrated. We should not be surprised by these findings; RT-PCR for example, is a very sensitive technique. Likewise, we should not be convinced by these findings that a therapeutic level or duration of CFTR expression has been achieved; it is challenging to quantify the levels of vector-derived CFTR mRNA by RT-PCR, and expression in patients for longer than a few days has not been demonstrated routinely. We should not be convinced that an adequate proportion of cells within the airways can be transfected routinely, even though a measurable percentage of vector-positive cells are present within some bronchial samples. It is estimated that gene correction in between 3 and 6% of cells within the respiratory epithelium will be required, and although the results are far from conclusive, dramatic improvements in the efficiency of AdCFTR gene transfer probably will be required to achieve this level of gene transfer throughout the airways. Nonetheless, we should be encouraged by the published findings.

A successful CF gene therapy treatment will require a combination of persistent vector-directed CFTR gene expression, effective repeat dosing, low toxicity, and gene delivery to a substantial proportion of cells lining the lower airways. Researchers in the field are focusing on the remaining limiting issues; progress has been made at the basic research level in many of these areas and advancement in Ad vector design is accelerating. To quote from a review of gene therapy written in September of 1995, "...the promise of gene therapy is intact. There is good reason to be optimistic about the ultimate success of this treatment."[24]

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Chapter 30

Ad-p53 Clinical Trial in Patients with Squamous Cell Carcinoma of the Head and Neck

Gary L. Clayman, Douglas K. Frank, and Patricia A. Bruso

Our laboratory has been involved in the investigation of wild type p53 gene transfer for the selective induction of apoptosis in human upper aerodigestive tract squamous cell carcinoma of the head and neck (SCCHN). Transient overexpression of the wild type p53 gene in various malignancies has been explored as a potential therapeutic intervention strategy.[1-6] This strategy is based on the role that wild type p53 plays as a tumor suppressor gene and inducer of cell-cycle arrest and apoptosis.[7-11]

SCCHN is a devastating disease. The overall poor survival rate for these tumors has not changed over the last several decades with current standard treatment modalities (radiation, surgery, chemotherapy).[12,13] After undergoing standard therapy (including radiotherapy), the advanced stage and recurrent SCCHN patient has a poor prognosis, and treatment often has major effects upon cosmesis and function. The principal cause of death in SCCHN is local/regional recurrence.[14,15] Clearly, new treatment strategies need to be developed and investigated. Given these issues, the study of novel molecular intervention therapies involving genes such as wild type p53 seemed appropriate. The fact that local/regionally recurrent SCCHN is readily accessible, even in the most advanced cases, enhanced its candidacy for investigation as a target for wild type p53 molecular intervention.

The recombinant adenovirus vector, Ad-p53, has been used as the gene delivery tool in all of our preclinical and clinical studies to date regarding wild type p53 gene transfer to SCCHN. This vector contains the CMV promoter, and wild type p53 cDNA in a minigene cassette inserted into the E1-deleted region of human adenovirus type 5.[16] Details regarding the preparation of recombinant adenovirus can be found in the publication by Zhang et al.[16] The tropism of adenovirus for tissues of the upper aerodigestive tract has made it an attractive gene delivery vehicle for our purposes. This point has been confirmed by the fact that adenovirus transduction efficiency of SCCHN cell lines is particularly high.[3] It should be noted that the genetic material introduced into mammalian cells via Ad-p53 remains episomal (not integrated into the DNA) and is overexpressed. Gene expression is transient, as the episomal DNA is not passed on to successive cell generations.

Introduction of the wild type p53 gene via Ad-p53 into SCCHN cell lines and established tumor nodules in nude mice suppressed in vitro and in vivo tumor growth, respectively, in our preclinical laboratory investigations.[3] Suppression of cell growth was demonstrated to occur through physiologic cell death (apoptosis),[2] and the apoptotic process occurred in malignant cells regardless of their p53 status. The induced apoptotic process was selective

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for malignant cells.[1] Furthermore, introduction of Ad-p53 into sites of microscopically implanted SCCHN cells in nude mice prevented the establishment of tumors.[1] This finding was of particular potential translational relevance because of the high incidence of local/regional failure in this disease,[14,15] presumably secondary to microscopic residual disease following initial standard therapy. These extensive preclinical laboratory investigations laid the groundwork for the current adenovirus-mediated wild type p53 clinical human gene therapy trial in the Department of Head and Neck Surgery at the University of Texas M. D. Anderson Cancer Center for patients with advanced local/regionally recurrent head and neck squamous cell carcinoma of the upper aerodigestive tract that has failed other standard therapeutic modalities, including radiation therapy.

Review of Current Research

The first phase of the Ad-p53 clinical trial at the University of Texas M. D. Anderson Cancer Center has been completed.[17] An international phase II trial has been initiated. The 33 patients in phase I were entered into one of two treatment arms for the purposes of determining toxicity of Ad-p53 gene therapy. The first arm consisted of inoperable, incurable patients (nonresectable arm, n=18). The second arm consisted of patients who were deemed operable for the purpose of debulking, but were otherwise considered incurable (resectable arm, n=15). Overall, 48% of patients were determined to be mutant at the p53 locus as determined by direct sequencing analysis.

The patients in the unresectable treatment arm had Ad-p53 injected directly into the tumor three times per week (every other day) for two weeks. After a two week rest period, this cycle was repeated until disease progression for up to seven treatment cycles. The patients in the resectable treatment arm had Ad-p53 injected directly into the tumor preoperatively in six doses over two weeks. Twenty-four hours after their last preoperative dose, these surgical patients had their operation, during which they had Ad-p53 delivered as a single dose to the surgical bed. Seventy-two hours following surgery, resectable treatment arm patients received a retrograde administration of Ad-p53 through drains which had been placed intraoperatively.

Our phase I work was carried out in a dose escalation fashion. Three to six patients were assigned to each Ad-p53 dose level. The administered doses of Ad-p53 were escalated in log increments from 10^6-10^9 particle forming units (pfu) per dose, and in one-half log increments from 10^9-10^{11} pfu.

As stated, Ad-p53 was always injected directly into tumors. This was accomplished either through direct visualization or through manual palpation (injection transcutaneous in these cases). Ad-p53 delivery was always done under sterile conditions, in a respiratory and body secretion isolation hospital room, under reverse isolation procedures for the medical staff.

All patients tolerated Ad-p53 gene therapy. There were neither serious dose-related effects or dose-limiting toxicities associated with this treatment modality. Treatment-related adverse events included mild flu-like symptoms (fever, headache, sinus congestion, mild sore throat) as well as mild erythema around the injection site. Flu-like symptoms would resolve within two days of treatment. Flu-like symptoms and injection site erythema tended to be associated with higher Ad-p53 treatment doses ($\geq 10^9$ pfu). The most frequently noted adverse event was pain at the time of Ad-p53 injection. This was felt to be due to injection of 4°C Ad-p53 solution. Recent stability studies have established that Ad-p53 is stable at room temperature and we anticipate decreasing the pain at injection sites with this warming.

Patients with Ad-p53 delivered at doses $\geq 10^7$ pfu tended to develop antibody responses against the type 5 adenovirus used as the gene delivery vector in this study. Despite this finding, expression of the p53 transgene could be detected in biopsy samples from a high-dose treated

patient 67 days after the first administration of Ad-p53, suggesting that a humoral antibody response against adenovirus does not prevent Adp53 tumor gene delivery and subsequent expression of p53 in the local/regional tumor environment.

The biodistribution of Ad-p53 after administration was determined for various body fluids in the phase I patients. Patient blood, urine, and upper aerodigestive tract secretions were assayed for the presence of Ad-p53 by a cytopathic effect assay (CPE) and Ad-p53-specific polymerase chain reaction (PCR)-based assay. Ad-p53 was detected in all patients in blood and urine at higher vector doses ($\geq 10^{10}$ pfu). No Ad-p53 was detected in blood 24 hours after a treatment. At high doses, vector could be detected in the urine of patients throughout treatment. The presence of vector in the urine ultimately disappeared within 3-17 days after the last Ad-p53 administration. As with blood and urine, Ad-p53 could also be detected in the sputum of patients after treatment at high doses (10^{11} pfu), and would be present throughout a cycle. Ad-p53 would usually be cleared from the sputum within a week. Despite the relatively constant appearance of the adenovirus vector in various body fluids, when administered at high dose, there were no serious systemic toxicities as noted above. Blood and urine from the health care workers with the most direct Ad-p53 and patient contact during the clinical trial remained free of any detectable vector.

Although the purpose of the phase I study of Ad-p53 gene therapy in head and neck cancer patients was primarily designed to determine patient safety, tolerance, treatment-related toxicity, and vector biodistribution, some interesting observations were made regarding potential antitumor activity of this novel treatment modality. The median survival of patients in the nonresectable treatment arm was not significantly different from other phase I and phase II studies of advanced, recurrent SCCHN. However, the median survival of patients in the resectable treatment arm was 13.6 months, with 2 patients remaining disease free at 21.5 months median follow up, despite the predicted incurable nature of their recurrent neoplasm. This finding was quite encouraging and seems to support the continued investigation of Ad-p53 gene transfer in SCCHN.

Conclusion

Our success of in vitro and in vivo adenovirus-mediated wild type p53 gene transfer in SCCHN[1-3] (selective induction of apoptosis in transduced cells) led to a phase I clinical trial in patients with advanced local/regionally recurrent disease that had failed standard treatment, including radiotherapy. Patients with such disease have a median survival of four to eight months, with few meaningful treatment options and no known effective means of prolonging survival.

Ad-p53 intratumoral injections, in a dose escalation fashion, to patients in both arms of the phase I clinical trial were safe and well tolerated. There were no dose-limiting local or systemic toxicities, despite wide vector distribution in various body fluids when administered at high doses. Additionally, wild type p53 gene transduction seems to occur despite the development of a humoral anti-adenovirus type 5 antibody response.

The observations made with regard to antitumor activity among resectable treatment arm patients is encouraging as we proceed with the international phase II evaluation of this novel molecular therapy. It has been found that patients with molecular evidence of residual disease (as determined by polymerase chain reaction-based assay detection of p53 mutation) at tumor margins recur and die with a higher incidence.[18] Thus, as our phase I results suggest, Ad-p53 gene transfer may find an application as an adjuvant modality in surgical wound beds to treat residual disease that may not yet bear any of the histologic characteristics of malignancy. Ad-p53 gene transfer may also be advantageous in combination with radiotherapy or chemotherapy. Enhanced therapeutic effects have been seen in several preclinical combination treatment models.[5,19,20] Finally, in the future, Ad-p53 gene transfer may also

be investigated in premalignant head and neck lesions, which have been associated with p53 mutation.[21]

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CHAPTER 31

Adenoviral Vectors for Liver Cancer—Clinical Trials

Ragai R. Mitry, Catherine E. Sarraf, Nagy A. Habib

Malignant tumors of the liver are among the commonest cancers in the world, with an estimated annual global incidence of 1,000,000 cases per year.[1] This includes primary tumors such as hepatocellular carcinoma (HCC) and metastases in the liver from various primary sites, for example the colon-colorectal liver metastases (CLM). In most cases, especially with primary liver tumors, the prognosis is very poor and those with advanced disease are unlikely to survive more than three months.[2] Moreover, many cases of HCC are beyond radical resection when detected, and treatment is rarely beneficial.[3] Gene-based therapies are being developed for many diseases, including cancer, and these will involve augmentation of immunotherapeutic and/or chemotherapeutic approaches.[4]

Gene Therapy for Liver Cancers

Gene therapy offers the potential of developing innovative treatments for both inherited monogenic diseases like cystic fibrosis and adenosine deaminase deficiency, and also for polygenic disorders such as cancer, cardiovascular and infectious diseases.[5,6] Methods of modern molecular genetics have been developed to allow stable transfer and expression of foreign DNA sequences in human somatic cells, making human gene therapy possible.[7] Some of the many preclinical studies (see Table 31.1) and the few clinical studies on gene therapy for liver tumors are mentioned in the following subsections (see Table 31.2).

Preclinical Studies

The use of tumor suppressor genes for the treatment of liver malignancies has been/is being studied both in vitro and in vivo. These studies involve the use of non-viral and also viral techniques, and many make use of adenoviruses as vectors. For example, adenovirus-mediated reintroduction of the wild type *p53* (wt *p53*) gene into liver and colorectal cancer cell lines leads to inhibition of proliferation of these cells that originally had mutant *p53*, or lacked wt *p53* expression.[8-10]

The use of adenoviruses encoding cytokines and suicide genes for the treatment of liver malignant primary and metastatic tumors seems to be a promising approach. For example, Kanai and colleagues (1997),[11] showed that intratumoral injection of a replication-deficient adenovirus encoding the *Escherichia coli cytosine deaminase* (*CD*) gene under the control of the α-fetoprotein (AFP) promoter/enhancer, and subsequent injection of 5-fluorocytosine (5-FC) resulted in regression of established subcutaneous HCC xenografts in mice. Also, in vivo, adenovirus-mediated *thymidine kinase* (*tk*) gene transfer and expression in the presence of ganciclovir (GCV) resulted in a substantial reduction in liver metastases

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Table 31.1. Preclinical studies in gene therapy for liver cancers

Cells/Tissue type	Gene(s) encoded by adenovirus used	Type of study carried out in vitro	in vivo	Reference
Normal liver and HCC	LDL receptor gene	+	+	19
HCC	CD (in presence of 5-FU)	+	+	11
HCC and colorectal cancer	p53	+	+	9
HCC	p53 (in presence of paclitaxel)	+	-	10
Colorectal cancer cells	p53	+	+	20
HCC	TK (in presence of GCV)	+	+	15,21
HCC and CLM	TK (in presence of GCV)	-	+	13
Metastic colon carcinoma	TK + IL-12	-	+	12
Hepatic metastases of lung cancer	TK + IL-2	-	+	14

+ = yes; - = no

of colon[12-13] and lung carcinomas,[14] and also inhibited HCC tumor growth in mouse models.[13,15]

Recent studies also showed that ribozymes, for example, can be used to inhibit telomerase activity in HCC cell extracts.[16] Telomerase activity is believed to be important for the continuous proliferation that occurs in malignant tumor cells; therefore, it may be beneficial to use ribozymes as cancer therapeutic agents.

Table 31.2. Clinical studies in gene therapy for liver cancer

Tumor/Cancer type	Gene(s) encoded by adenovirus used	Reference
CLM	CD (in presence of 5-FU)	18
HCC	*p53*	Habib and colleagues (work in progress)
HCC and CLM	*p53*	22

Targeting Primary and Secondary Liver Tumors

Some tumor cells possess antigens which are specific enough to be immunolabeled when confirming tumor type during diagnosis. Adenoviruses used in gene therapy could be modified to express the gene(s) of interest under the control of specific promoter(s). These promoters could be activated by such tumor specific molecules. For example, AFP can be used as a marker for HCC, while carcinoembryonic antigen (CEA), more generally associated with colonic carcinomas, can be used to demonstrate that a liver lesion might be a CLM. Thus, the construction of an E1-deleted type 5 adenovirus vector to express, for example, a tk gene under the control of an AFP promoter, would result in a vector preferentially specific to HCC,[11] and the *tk* gene would then be expressed in liver tumor cells. Hallenbeck and colleagues[17] were able to modify promoters to produce adenoviral vectors specifically designed to replicate in particular cancers, such as the modified version of AFP promoter, which increased the efficacy and specificity of the vector injected in HCC xenografts in animal models. Their results indicated that no more than 1% of tumor cells need to be transduced in order to prevent tumor growth.

Clinical Studies

To date, few gene therapy clinical trials/protocols have been adopted for liver malignancies. Crystal et al[18] published a clinical protocol for treatment of patients with CLM which combines the use of an adenovirus containing *E.coli* CD gene and 5-FC. Horowitz et al[9] started a phase I, dose escalation clinical trial to evaluate the safety and gene expression following hepatic artery administration of a recombinant adenovirus encoding human *p53,* in patients with liver tumors. The results showed there were no dose limiting-toxicities, and the only adverse effects were fever and flu-like syndrome, which they believed were in response to the injected adenovirus alone.[18]

In 1998, Horowitz and colleagues presented the results of a phase I pilot investigation at the 89th Annual Meeting of the American Association for Cancer Research, New Orleans, LA. The program included 62 patients with CLM, ovarian carcinoma, melanoma, head and neck, and non-small cell lung cancer. Each patient received a single administration of a replication-deficient, E1-deleted adenovirus containing human wt *p53* gene. Prior to adenovirus administration, all patients showed presence of antibodies to adenovirus. Dose levels evaluated ranged from 7.5×10^9 to 7.5×10^{12} viral particles per dose; the route of

Table 31.3. p53 Transgene expression detection

Tissue/cells	Route of adenovirus administration	Detection at partical level (pfu)
Normal liver	intrahepatic artery	2.5×10^{10}
Tumor tissue	intrahepatic artery intratumoral	7.5×10^{11} 7.5×10^{9}
Tumor cells	intraperitoneal	7.5×10^{10}

administration was intrahepatic, interperitoneal or intratumoral. They measured the transgene expression using RT-PCR and internal mimic confirmation, and found that it was first detected in normal liver, then in the tumor (see Table 31.3); thus, they confirmed that *p53* transgene expression followed intrahepatic, intraperitoneal and intratumoral administration. They established that the presence of endemic baseline antibodies to adenoviruses did not preclude transgene expression, and their results suggested that such gene expression appears to be a function of viral particle dose delivered.[24]

Previously published results of a phase I pilot study involving the direct injection of wt *p53* plasmid DNA showed that partial/total regression of HCC tumors is possible.[25] The cohort included five HCC patients with primary lesions, three of which, after treatment, showed objective response with reduction of tumor volume on CT scan measurements; also, there was a significant fall in serum AFP level. There was no mortality nor morbidity due to the injections, and only minor complications were observed, such as transient fever, hypotension, or hypertension. These side effects lasted no more than two hours and reverted spontaneously to normal without further intervention. One of these patients showed total regression of a 14 cm tumor Fig. 31.1) with a drop in AFP from 1900 IU/l to 6 IU/l, and the other two demonstrated a decrease in tumor volume of 75% and 90%. These results encouraged Habib and colleagues to start a phase I trial to evaluate the safety and beneficial effects of the use of E1-deleted type 5 adenovirus encoding human p53 under the control of CMV/IE promoters, for treatment of HCC. Treatment was initiated by a single direct intratumoral injection of 1×10^8 pfu of Ad-*p53*. Injections were repeated once a month for a period of three months. Target tumor size was determined prior to the first injection by using CT scan measurements of tumor diameters; changes in tumor volume were calculated and compared to results obtained prior to the first injection. No side effects or toxicity have been observed following the Ad-*p53* injections so far. Liver biopsies were analyzed for viral accumulation in tumor and non-tumor tissue by transmission electron microscopy (Fig. 31.2) and are being analyzed for wt *p53* gene transfer and expression. Analysis also will address efficacy of gene transfer and expression using the Ad-*p53* vector, as various routes of administration and dose levels will be tested.

Conclusion

It can be predicted that the technology for injecting adenoviruses encoding appropriate 'killer' or antiproliferation genes into a peripheral vein will be perfected for the treatment of liver malignancies. Accurate delivery exclusively to tumor cells will ensure the survival of normal tissue and the death of tumor cells.

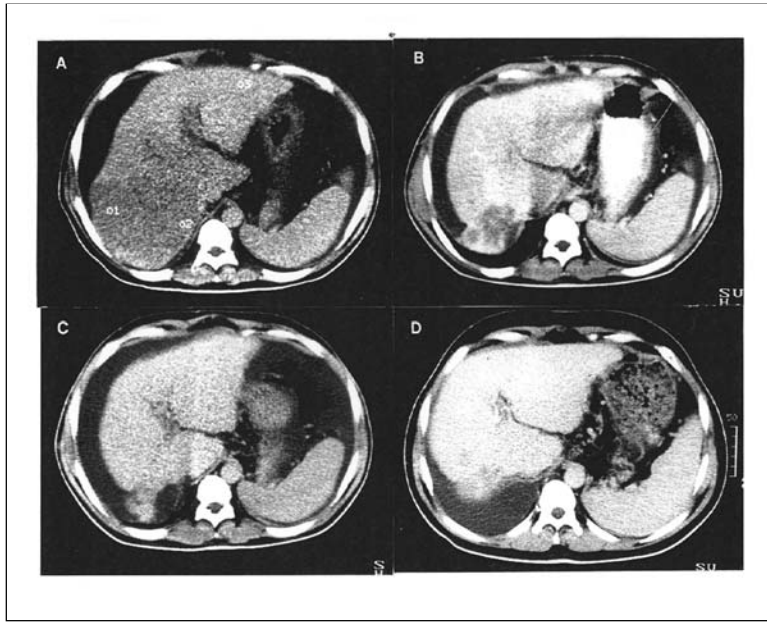

Fig. 31.1. CT scans of one of the HCC patients treated with wt p53 plasmid. (A) Unenhanced CT scan prior to therapy. A 14 cm diameter tumor is seen in the posterior position of the right lobe of liver. (B) Contrast enhanced CT scan of the same patient 3 months after two intratumoral injections of wt p53 shows a considerable reduction in size of the tumor, which now measures 5 cm in diameter (normal AFP). (C) Contrast enhanced CT scan of same patient 6 months after commencement of therapy. The tumor now measures 2 cm in diameter (normal AFP). (D) Contrast enhanced CT scan of the same patient 19 months after commencement of therapy. No tumor is seen (normal AFP).

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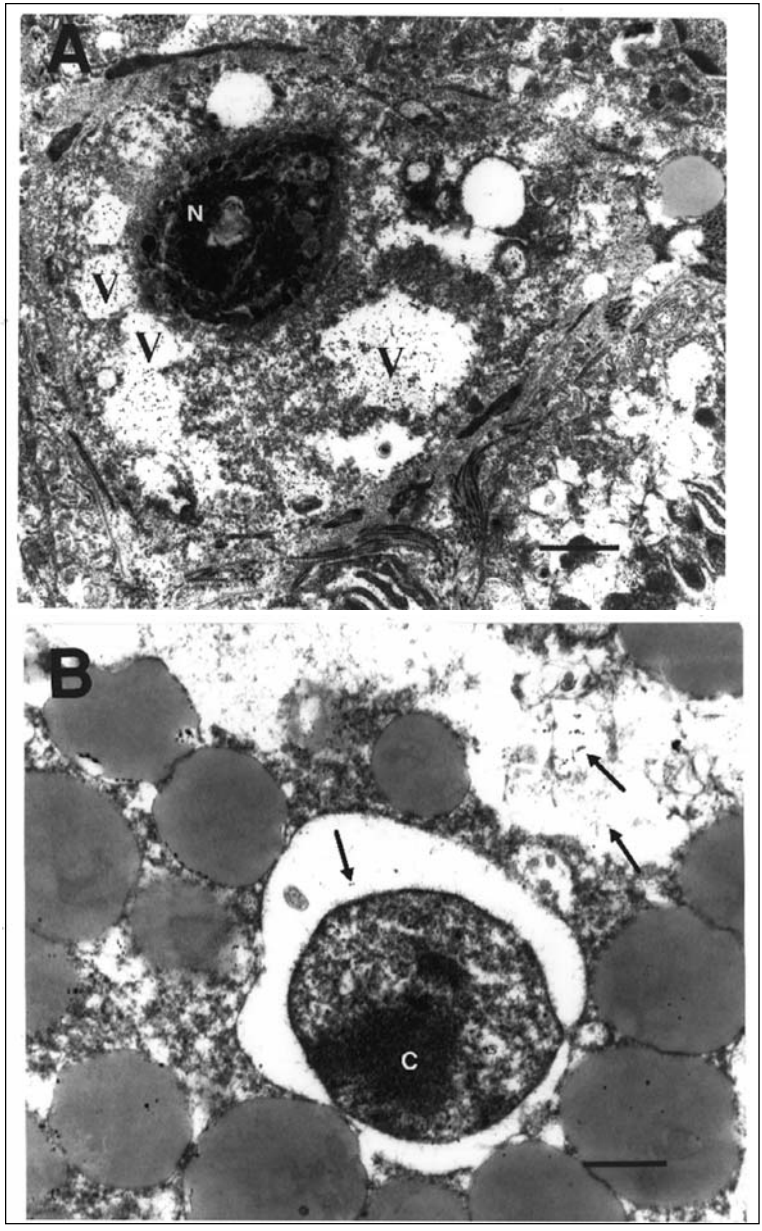

Fig. 31.2. (A) An electron micrograph of a non-neoplastic liver cell adjacent to an area of hepatocellular carcinoma in a patient who has had therapeutic application of adenovirus encoding wt p53. The cell nucleus, N, shows unequivocal early apoptotic changes, such as chromatin condensation and blebbing. The cytoplasm has yet to undergo characteristic shrinkage, but contains large vacuoles containing aggregations of virus particles, V. Bar = 1.5 μm. (B) An electron micrograph of an early apoptotic nucleus from a hepatocellular carcinoma cell. Chromatin, C, is condensed and marginated and the nuclear envelope is highly dilated. Degrading adenovirus particles are visible both in the cytoplasm and in the nuclear envelope (arrows). Bar = 0.6 μm.

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CHAPTER 32

Opportunities for p53 Tumor Suppressor Gene Therapy in Ovarian and Other Peritoneal Cancers

Loretta L. Nielsen, Mark Pegram, Beth Karlan, John Elkas, and Jo Ann Horowitz

Schering-Plough Research Institute is currently sponsoring phase I/II clinical trials of adenovirus-mediated p53 gene therapy for cancer in several countries. The drug used in these clinical trials (Ad p53; ACN53; SCH58500) consists of a replication-deficient, type 5 adenovirus vector expressing human p53 tumor suppressor gene under the control of the cytomegalovirus promoter.[1] Clinical targets for initial trials with p53 gene therapy were chosen on the basis of frequency of p53 mutation and pattern of spread for specific tumors which would make them amenable to local or regional therapy and subsequent biopsy. The incidence of p53 mutations for most tumors is dependent on stage of the disease. In general, early stage disease has a much lower incidence of p53 mutation, with more advanced and invasive cancer having a higher incidence of mutation. The natural history of ovarian cancer results in 75% of patients having cancer spread beyond the ovary and 60% beyond the pelvis at diagnosis. Neoplastic cell dissemination within the peritoneal cavity is the most common pathway for progression in patients with advanced disease. Five year survival for patients with regional disease at diagnosis is 55%. Preexisting anti-adenoviral antibodies and the need to achieve an effective concentration of Ad p53 suggest this drug will be most effective when administered regionally. Ovarian cancer limited to the abdominal cavity, with small volume disease either naturally or optimally debulked, seems ideal for regional p53 gene therapy.

Preclinical Pharmacology: Intraperitoneal Administration of p53 Adenovirus

Many studies have examined the in vivo efficacy of Ad p53 in preclinical models when adminstered intratumorally or intravenously;[2] however, published information does little to guide clinicians in the design of intraperitoneal (i.p.) dosing trials for ovarian cancer. To this end, we examined several parameters with special significance for i.p. adminstration of Ad p53. First, cell proliferation was measured in two ovarian tumor lines (SK-OV-3, OVCAR-3) and one prostate tumor line (DU-145) after in vitro treatment with Ad p53. SK-OV-3 cells are $p53^{null}$, while OVCAR-3 and DU-145 cells express mutant p53. The ED_{50} values (ciu/cell required for a 50% reduction in cell number) were approximately 30 for SK-OV-3 cells, 2 for OVCAR-3 cells, and 5 for DU-145 cells. The effects of dosing volume

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and frequency were next examined, using the SK-OV-3 tumor xenograft model in SCID mide.[3] When the effect of vehicle volume on antitumor efficacy was examined, all mice treated with Ad-p53 had reduced tumor burden compared to controls. Vehicle volumes between 0.2 and 1 ml were equally effective, and all were more effective than a vehicle volume of 0.1 ml. However, reduced efficacy was observed when a 1.5 ml vehicle volume was administered. These results are somewhat puzzling, but might be explained by considering the fact that 1.5 ml is a large volume of liquid to inject into the peritoneal cavity of a 20 gram mouse. It appears that the peritoneal tumor was not exposed to the entire dose of Ad-p53 when this volume was used. Therefore, hydrostatic pressure may have caused leakage back out of the injection site and/or into the systemic circulation prior to adenovirus transduction of peritoneal tumor cells. Volume considerations have also determined the maximum viral dose available for phase II clinical trials in human patients, given the lack of dose-limiting toxicities observed in our phase I trials (see clinical section in this chapter). Next, the effect of dosing frequency was examined in the same SK-OV-3 model. Fractionated doses of Ad p53 had greatly enhanced efficacy compared to fewer, bolus injections. By contrast, bolus and fractionated doses were equally effective in the DU-145 model. This suggests that the optimal dosing regime in mouse models will be dependent on specific characteristics associated with each tumor cell line. Multiple variables may be involved; therefore, extensive studies with many different i.p. models will be needed to isolate the critical factors. For example, i.p. DU-145 tumors grow faster than i.p. SK-OV-3 tumors in SCID mide, but SK-OV-3 tumors kill the mice faster at tumor burdens between 1 and 2 g. By contrast, DU-145 tumors can reach tumor burdens of 5-6 g before they kill their host. Tumor burden at the start of dosing is also important, if the "peeling-the-onion" theory of drug action is correct. According to this hypothesis, the particulate drug (Ad p53) has limited ability to penetrate outer cell layers in a peritoneal tumor. Therefore, each dose, no matter how concentrated, can only kill the outer tumor layers. Once those cells die and disintegrate, the next dose of drug can access another discrete layer of tumor. Preliminary studies using immunohistochemistry and Laser Scanning Cytometry to assess adenovirus transduction, transgene expression, and p53-induced apoptosis support this theory.[4] However, the biology is probably more complex than this simple model. For example, Nielsen et al[3] have shown that recombinant adenoviruses can be absorbed from the peritoneal cavity into the systemic circulation when delivered at high concentrations for several consecutive days.

Ad-p53 Gene Therapy Combined with Chemotherapy

Cancers containing nonfunctional p53 tumor suppressor protein are generally less sensitive to chemotherapy.[5] Many anticancer agents induce apoptosis via p53-dependent (cisplatin, doxorubicin, 5-fluorouracil) or p53-independent (paclitaxel) pathways.[5-7] Therefore, the introduction of wild type p53 into cells with nonfunctional p53 protein should enhance their sensitivity to most chemotherapeutic drugs. Although systemic delivery of chemotherapy is commonly employed in the treatment of metastatic disease, the results are frequently disappointing. Enhancement of its efficacy may result in more effective treatment outcomes.

Ad p53 combined with cisplatin, doxorubicin, 5-fluorouracil, methotrexate, etoposide, or paclitaxel (taxol) inhibited cell proliferation more effectively than chemotherapy alone in $p53^{null}$ SCC-9 head and neck, $p53^{null}$ SCC-15 head and neck, $p53^{null}$ SCC-25 head and neck, $p53^{null}$ SK-OV-3 ovarian, $p53^{mut}$ OVCAR-3 ovarian, $p53^{mut}$ DU-145 prostate, $p53^{mut}$ MDA-MB-468 breast and $p53^{mut}$ MDA-MB-231 breast tumor cells.[8,9] Responses were independent of the type of p53 gene mutation in the cells, and cells expressing mutant p53 protein were indistinguisable from $p53^{null}$ cells. Also, no obvious schedule dependence was observed.

In addition to the reasons mentioned above, further rationale for combining p53 gene therapy with chemotherapy in the clinical setting are:

1. Combinations of agents with different toxicological profiles can result in increased efficacy without increased overall toxicity to the patient;
2. Combinations of therapeutic agents may thwart the development of resistance to single agents;
3. Combinations of therapeutic agents may offer a solution to the problem of heterogeneous tumor cell populations with different drug sensitivity profiles;
4. Combinations of therapeutic agents allow physicians to take advantage of possible synergies between drugs, resulting in increased anticancer efficacy in patients.[10] Synergy (or antagonism) between two chemical agents is an empirical phenomenon, in which the observed effect of the combination is more (or less) than what would be predicted from the effects of each agent working alone. Although mathematical synergy cannot be directly proven in the clinic, it does predict in vivo synergy when the two therapeutics are combined. By contrast, overt antagonism warns of future clinical problems.

Sophisticated statistical modeling techniques were used to evaluate the presence of synergistic, additive, or antagonistic efficacy between Ad p53 and paclitaxel in a panel of human tumor cell lines.[8] Tumor cells with altered p53 were treated with paclitaxel 24 hours before Ad p53 or treated with both agents simultaneously. Paclitaxel had synergistic or additive efficacy in combination with Ad p53, independent of whether the cells expressed mutant p53 protein or no p53 protein at all. Most importantly, antagonism between the two drugs was never observed. Paclitaxel increased the number of cells transduced by recombinant adenovirus 3-35% in a dose-dependent manner at paclitaxel concentrations up to 12 nM. This is one possible mechanism to explain the observation of drug synergy. In other words, more tumor cells were infected with Ad p53 and exposed to high levels of wild type p53 protein when paclitaxel "sensitized" them to transduction by recombinant adenovirus. Of particular note, the concentrations of paclitaxel responsible for increased adenovirus transduction were lower than the concentrations required for microtubule condensation. Also, the rate of change in the number of cells transduced by adenovirus appeared to be independent of paclitaxel-induced cell death.

The antitumor effects of combination therapy with Ad p53 and paclitaxel were also evaluated in tumor xenograft models in vivo. It has been well documented that Ad p53 is a drug with antitumor efficacy attributable to both the p53 tumor suppressor gene and the adenovirus delivery vector.[2] The in vivo experiments were designed to mimic the clinical situation, in which efficacy of the Ad p53 drug (with or without chemotherapy) will be compared to clinical outcome with traditional chemotherapy. In this situation, it is unethical and prohibitively expensive to include study arms for an empty adenovirus vector. In the intraperitoneal SK-OV-3 model of ovarian cancer, a dose of Ad p53 which had relatively minimal antitumor effect by itself had significantly enhanced efficacy when combined with paclitaxel. Similar results were observed using a higher dose of Ad p53. Paclitaxel also enhanced the antitumor efficacy of Ad p53 in the DU-145 prostate, MDA-MB-468 breast, and MDA-MB-231 breast cancer xenograft models. In summary, adenovirus-mediated p53 gene therapy for cancer shows enhanced therapeutic benefit when combined with paclitaxel. The ability of paclitaxel to increase adenovirus transduction rates in tumor cells could explain part or all of the observed enhancement.

Greater anticancer efficacy was also demonstrated with other chemotherapy drugs in tumor xenograft models in vivo.[9] These data support the combination of p53 gene therapy with chemotherapy in clinical trials. Of particular significance, there was enhanced efficacy using the three drug combination of Ad p53, cisplatin, and paclitaxel in the SK-OV-3 ovarian

tumor model. Clinical trials combining Ad p53 with chemotherapy in liver, lung, and ovarian cancers are currently underway.

Tumor/Host Factors Predictive of Response to p53 Adenovirus

Nonfunctional p53 in and of itself may not be a sufficient predictive factor of clinical response, because other tumor and host factors may interfere with adenovirus-mediated gene delivery. To cite an analagous situation, chondroitin sulfates in malignant pleural effusions were recently shown to inhibit gene transfer by retroviral vectors.[11] Analyses of the mechanism of this effect indicated that interaction of chondroitin sulfates with retroviral vector in solution was responsible for inhibition. Further, pretreatment of pleural fluid with chondroitinases abolished the inhibitory activity. To investigate whether there are host factors which might inhibit adenoviral vectors, we analyzed the effect of malignant ascites fluid obtained from patients with ovarian carcinoma on transduction efficiency of Adβ-Gal into MDA-MB-231, $p53^{mut}$, breast carcinoma cells. The results demonstrate that a 50/50 mixture of malignant ascites and cell culture medium inhibited β-Gal transduction efficiency by 50-100% in 17 of 18 samples analyzed (Fig. 32.1). In addition, this inhibitory factor resides in the soluble, rather than the cellular, fraction of the ascites. Conditioned media from tumor cell cultures derived from the same patients were not able to block Adβ-Gal transduction, suggesting that this inhibitory factor is derived from normal tissues rather than from malignant cells. Further isolation and characterization studies are ongoing. This data strongly suggests that removal of malignant ascites from patients prior to intraperitoneal treatment with adenovirus-based gene therapies could facilitate transgene delivery to tumor cells in vivo.

We are also examining the influence of factors which may be predictive of response to Ad p53. Preliminary data suggests that the transduction efficiency of Adβ-Gal in a panel of primary ovarian tumor cell cultures is predictive of in vitro tumor cell response to Ad p53 (Fig. 32.2). For example, the primary ovarian carcinoma cell cultures CSOC 823c and 790-5,6 have mutations of p53 (determined by immunohistochemistry) and yet are not growth suppressed by Ad-p53. This lack of p53 activity correlates with low transduction efficiency by Adβ-Gal. We are currently analyzing expression levels of the common receptor for coxsackie B and adenoviruses 2 and 5 (CAR)[12] in a large panel of ovarian tumor-derived cell cultures to test the hypothesis that CAR is necessary for efficient transduction by Adβ-Gal. The predicted amino acid sequence from HeLa cell-derived CAR cDNA indicates that CAR is a 365 amino acid transmembrane protein with two extracellular immunoglobulin-like domains. Though the cellular function of CAR remains unknown, transfection and expression of CAR in CHO cells is sufficient for adenovirus-mediated gene transfer by Adβ-Gal. It is our hope that investigation of tumor cell factors, such as CAR, and host factors which influence adenovirus-based gene delivery systems will allow for optimization of patient selection and improved therapeutic potential for Ad p53.

Clinical Results: Intraperitoneal Administration of p53 Adenovirus

A multinational phase I clinical trial was initiated in January 1997. The original intent of this trial was to determine drug safety and transgene (p53) expression after intraperitoneal administration of Ad p53. The drug was administered in cohorts of three patients in a rising dose fashion. Patients with intraperitoneal disease amenable to biopsy or cellular cytospin, either by laparoscopy or paracentesis, were enrolled. All patients had anti-Ad antibodies prior to gene therapy, a good performance status, acceptable baseline laboratory values, and no active wild type adenovirus infection, as confirmed by ELISA. The initial dose was 7.5×10^{10} viral particles (pn) diluted in 1 liter immediately prior to intraperitoneal injection. Subsequent cohorts of three patient were enrolled at the following dose levels: 7.5×10^{11} pn,

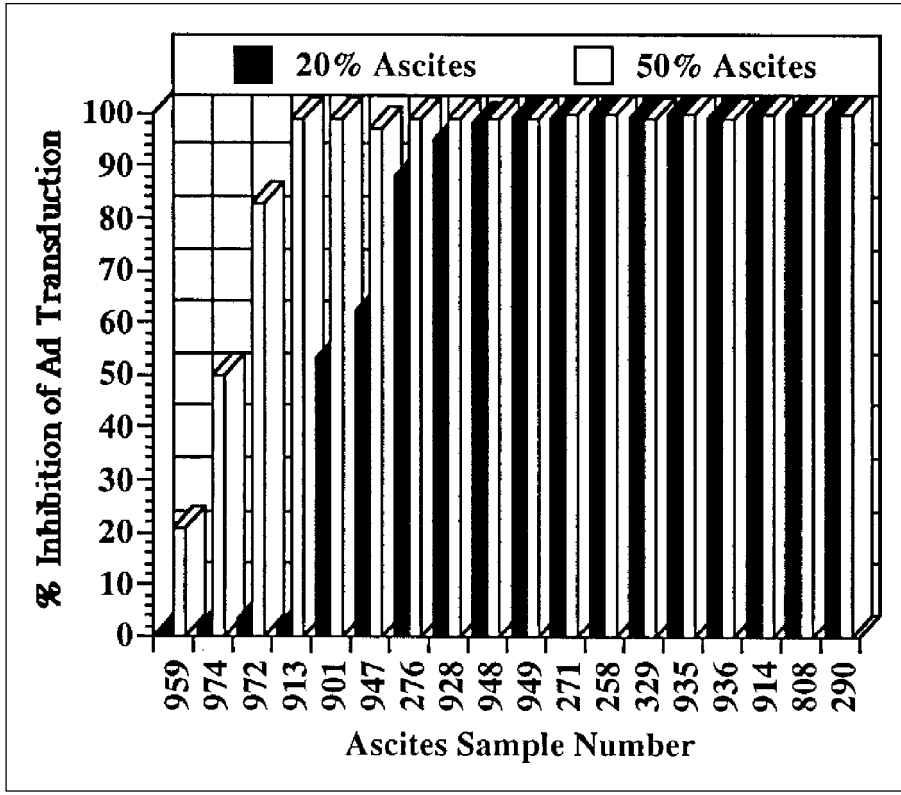

Fig.32.1. Effect of malignant ascites on Adβ-Gal transduction of $p53^{mut}$ MDA-MB-231 breast carcinoma cells. Patient biopsies had ovarian papillary serous histology. MDA-MB 231 cells were plated at 5 x 10^4 cells/well and allowed to adhere overnight. Next, 20% or 50% ascites in culture medium and 1 x 10^9 viral particles Adβ-Gal were added to each well for a final volume of 1ml. Twenty-four hours later, cells were fixed, assayed for β-galactosidase activity, and quantitated. The results shown are the means from triplicate wells. The % inhibition of Ad transduction was determined relative to MDA-MB-231 cells not exposed to ascites. These cells typically have 95-100% Ad transduction efficiency under these experimental conditions.

2.5 x 10^{12} pn, and 7.5 x 10^{12} pn given in single i.p. injections. The study goals of an acceptable safety profile and transgene expression in biopsy tissues were achieved despite the presence of anti-Ad antibodies prior to treatment. All patients had elevations in anti-Ad antibody levels subsequent to Ad p53 administration. The protocol design was then modified to allow for the addition of chemotherapy and multiple doses of Ad p53. The goals of the modification were to expand the safety profile of Ad-p53 to include multiple doses and to establish the safety profile of Ad p53 in combination with chemotherapy. The initial Ad p53 dosing level was 7.5 x 10^{12} pn daily (x2) in combination with i.p. cisplatin. An additional three patients received 2.5 x 10^{13} pn daily (x3) with i.p. cisplatin. An acceptable safety profile and transgene expression were both confirmed. Another modification was implemented in order to test intravenous carboplatin/paclitaxel instead of i.p. cisplatin while continuing to escalate the

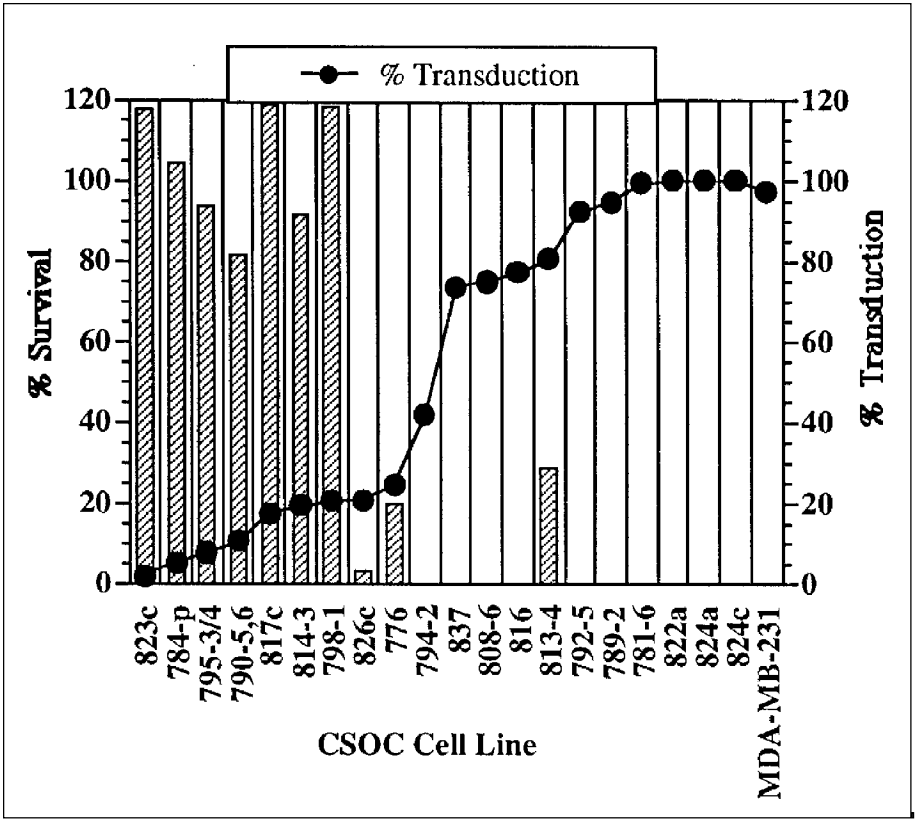

Fig. 32.2. Transduction of Cedars Sinai primary ovarian carcinoma cultures (CSOC) by Adβ-Gal and tumor cell response to Ad-p53 treatment. Cells were cultured with 1 x 10^9 viral particles/ml Adβ-Gal or Ad-p53 for three days, then assayed for β-galactosidase activity or cell proliferation. Results are relative to untreated control. The plotted values are means from triplicate wells. (The standard errors were typically 2-5%).

dose of Ad-p53. Three patients received Ad p53 at 2.5 x 10^{13} pn with i.v. carboplatin (AUC of 6) and paclitaxel (175 mg/m^2). Then fifteen patients were dosed at 7.5 x 10^{13} pn daily (x5) plus i.v. carboplatin/paclitaxel. Accrual to this last dose level continues, but the preliminary data indicates this regimen is well tolerated. Table 32.1 summarizes the dose escalation schema. Related serious adverse events included transient fever, increased liver function tests in one patient, abdominal distention, leukopenia, anemia, diarrhea, hypokalemia, dehydration, nausea and vomiting. All of these events were manageable, and other than one episode of elevation in alkaline phosphatase, did not preclude escalation to the next dose level. One patient, who received Ad-p53 and chemotherapy, experienced a cascade of events including sepsis, neutropenia, renal insufficiency, anemia, hypokalemia , nausea, vomiting, thrombocytopenia and edema. No evidence of viral shedding was seen in stool or urine. Although this study was designed to address safety and biological activity, subjective evidence for tumor response was also noted. Preliminarily, there have been three subjective reductions

Table 32.1. Preliminary results from a Phase I gene therapy clincial trial for ovarian cancer

Patient No.	No. Doses	Dose (Viral Particles)	Chemotherapy	Transgene Expression
1	1	7.5×10^{10}	None	+
2	1	7.5×10^{10}	None	-
3	1	7.5×10^{10}	None	- (x2)
13	1	7.5×10^{10}	None	-
4	1	7.5×10^{11}	None	+
5	1	7.5×10^{11}	None	+
6	1	7.5×10^{11}	None	+
7	1	7.5×10^{11}	None	NR
8	1	7.5×10^{11}	None	+ (x1)
9	1	7.5×10^{11}	None	NR
10	1	2.5×10^{12}	None	+ (x1)
11	1	2.5×10^{12}	None	+ (x1)/- (x2)
12	1	2.5×10^{12}	None	+ (x2)
14	1	7.5×10^{12}	None	-
15	1	7.5×10^{12}	None	+
16	1	7.5×10^{12}	None	-
207	2	7.5×10^{12}	IP cisplatin	BQL (C1)
212	2	7.5×10^{12}	Ip cisplatin	NR
17	3	2.5×10^{13}	Ip cisplatin	ND (C1), + (C2 and 3)
18	3	2.5×10^{13}	IV Carbo/ Taxol	Pending
19	3	2.5×10^{13}	IV Carbo/ Taxol	+ (C1 and 2)

Table 32.1. Preliminary results from a Phase 1 p53 gene therapy clinical trial for ovarian cancer; cont.

Patient No.	No. Doses	Dose (Viral) Particles)	Chemotherapy	Transgene Expression
216	3	2.5×10^{13}	IV Carbo/Taxol	+ (C1)
20	3	2.5×10^{13}	IV Cisplatin/ Taxol	- (C1),+(C2)
21	3	2.5×10^{13}	IV Carbo/ Taxol	+ (C1 and 2)
22	3	2.5×10^{13}	IV Carbo/ Taxol	+/- (C1)*
23	5	7.5×10^{13}	IV Carbo/Taxol	Pending

NR= sample degraded, no results; BQL= below quantifiable levels; ND= not done; C=dosing cycle; Patient 20 was allergic to carboplatin. *Ascites positive for transgene expression, tumor biopsy negative

in ascites, one decreased CA-125, and one short lived CT-objective decrease in tumor mass. Future development of this treatment modality remains promising.

Conclusion

Seldom does such a new and exciting therapeutic category of drug make it into the clinic. We are just starting to evaluate the extent to which p53 gene therapy can achieve clinically meaningful outcomes and add to our currently inadequate cancer treatments. Key to this discussion is the definition of appropriate clinical endpoints for gene therapy trials. Development in an unprecendented area results in reliance on endpoints used in the past to justify approval of more traditional forms of cancer therapy. Historically, improvement in overall or disease-free survival has been the clinical "gold standard". Most practitioners would acknowledge that there are other meaningful endpoints which guide them in the care of their patients. Endpoints such as improvement in quality of life and response rate may translate into an improvement in signs and symptoms of the disease. Other surrogates, such as improvement in tumor markers like CA-125, which parallels tumor burden, are significant to the patient yet more difficult to prove to regulatory authorities. In the gene therapy arena, other potentially meaningful endpoints include the ability to express the transgene, and downstream effects such as tumor cell apoptosis. It must still be determined whether clinically meaningful results and regulatory requirements could include the combination of a surrogate marker, such as CA-125, and cellular apoptosis. Survival studies are extremely long and significantly delay the introduction of new therapeutics to the patient population. There is ample evidence that the use of replacement gene therapy, either alone, or in combination with chemotherapy, translates into anticancer effects. The preliminary clinical results of transgene expression hold out hope that future applications in the clinic will result in improvement in the current response and survival rates for cancer patients.

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Abbreviations

$p53^{null}$	no p53 protein expressed
$p53^{mut}$	mutant p53 protein expressed
$p53^{wt}$	wild type p53 expressed, but not necessarily functional; ciu, cellular infectious units
moi	multiplicity of infection = ciu/cell
pn	viral particles.
AUC	mg/ml/min = "area under the curve" of the carboplatin serum concentration versus time plot. Because elimination o fcarboplatin is almost entirely dependent on renal glomerular filtration rate (=creating clearance rate in ml/min), carboplatin dosing is basedon the projected area under the curve of the pharmacokinetic plot for each individual patient based on direct measurement of their creatine clearance or an indirect estimate of renal function based on patient age, mass, and serum creatinine concentration.

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CHAPTER 33

Adenoviral Gene Therapy for Malignant Pleural Mesothelioma

Daniel H. Sterman, Larry R. Kaiser, and Steven M. Albelda

Malignant mesothelioma is a primary neoplasm of the mesothelial lining of the pleural (80%) or peritoneal cavities (19.5%). It has been linked conclusively to prior exposure to asbestos and may also be associated with certain genetic predispositions and past viral exposures, including SV40.[1] Although relatively rare, mesothelioma accounts for approximately 3000 deaths per year in the United States.

To date, standard treatment for mesothelioma (including surgical resection, radiation therapy, and chemotherapy) have not proven effective in significantly prolonging patient survival.[1,2] A number of characteristics make this tumor an attractive target for gene therapy. First is the absence of any currently effective therapy. Second is its unique accessibility in the pleural space for vector delivery, biopsy, and subsequent analysis of treatment effects. A surgical "debulking" procedure to remove gross disease, followed by gene therapy to remove residual disease, would thus be technically feasible. Third, local extension of disease, rather than distant metastases, is responsible for the morbidity and mortality associated with this neoplasm. Thus, unlike other more widespread neoplasms, small increments of improvement in local control could engender significant improvements in palliation or survival.

Based on this rationale, we have recently conducted a phase 1 clinical trial using an E1/E3-deleted replication-incompetent adenovirus carrying the herpes simplex virus thymidine kinase (HSVtk) gene aimed at treating mesothelioma.[3] This approach serves as a model for treatment of other localized malignancies such as ovarian, bladder, and brain carcinoma.

Gene Therapy Using the Herpes Simplex Thymidine Kinase Gene

One prominent approach in current experimental cancer gene therapy is the introduction of toxic or "suicide" genes into tumor cells, facilitating their destruction (molecular chemotherapy). One such "suicide" gene approach involves the transduction of a neoplasm with a cDNA encoding for an enzyme, such as the HSV*tk* gene, that would render its cells sensitive to a "benign" drug, such as ganciclovir (GCV), by converting the "prodrug" to a toxic metabolite.[4] GCV is an acyclic nucleoside that is poorly phosphorylated by mammalian cells and is thus normally relatively non-toxic. After being converted to GCV-monophosphate by HSV*tk* , however, it is rapidly converted to ganciclovir triphosphate by mammalian kinases. Ganciclovir triphosphate is a potent inhibitor of DNA polymerase and a toxic analog that competes with nucleosides for DNA replication.[5] One important feature of the HSV*tk*/GCV system is that not every cell within a tumor needs to be transduced. This so-called "bystander" effect was demonstrated in in vitro experiments and subsequently

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in experiments where complete tumor regression was noted in animals after GCV treatment when only 10-20% of the tumor cells contained the HSV*tk* gene.[6]

Early experiments with the HSV*tk* gene involved the use of retroviral vectors (i.e., ref. 7); however, our group and others have produced replication-deficient, recombinant adenoviral vectors encoding the HSV*tk* gene and shown that this vector, in combination with GCV, could eradicate tumor cells in vitro and in in vivo models of localized tumors.[8-12]

Preclinical Data: Animal and Toxicity Studies

Based on experiments showing that replication-deficient adenovirus efficiently transduced mesothelioma cells both in tissue culture and in animal models[8,9] and that infection with an adenovirus containing the HSV*tk* gene driven by the Rous sarcoma virus promoter (Ad.RSV*tk*) rendered human mesothelioma cells sensitive to doses of GCV that were 2-4 logs lower that the doses required to kill cells infected with control virus,[8] the Ad.RSV*tk* vector has been used to successfully treat established human mesothelioma tumors and human lung cancers growing within the peritoneal cavities of SCID mide.[9] Marked decreases in tumor size have also been seen in an intrapleural rat model of syngeneic mesothelioma; however, survival increases have been more modest in this system.[10] These in vitro and in vivo experimental results have been confirmed by other independent investigators.[12]

Based on this efficacy data in animals, we conducted preclinical toxicity testing for submission to the DNA Recombinant Advisory Committee of the NIH and the Federal Drug Administration (FDA). Rats and baboons were given high doses of virus intrapleurally followed by intraperitoneal administration of GCV at the same proposed dose for initial use in the clinical trial. Toxicity was limited to localized inflammation of the pleural and pericardial surfaces.[13]

Clinical Data: Results from Phase I Clinical Trial

On the strength of these animal studies, a phase I clinical trial for patients with mesothelioma began in November, 1995 at the University of Pennsylvania Medical Center in conjunction with Penn's Institute for Human Gene Therapy. The results of this trial were recently reported.[3]

The purpose of the phase I trial was to determine the maximally tolerated dose of Ad.RSV*tk* virus instilled into the pleural space, to evaluate the biological effects of therapy, and to evaluate, in preliminary fashion, any response rate.[14] Patients were eligible for this study if they had a histologically proven diagnosis of malignant pleural mesothelioma, were not candidates for resection, had an ECOG performance status of 0, 1, or 2, and had the presence of at least some residual pleural space. The protocol was designed as a dose escalation study, starting with a vector dose of 1 $x10^9$ plaque forming units (pfu) and increasing in half log intervals to the current dose level of 1 $x10^{12}$ pfu.

On day 1 of the study, patients 1-15 underwent videothoracoscopy for tissue acquisition, confirmation of diagnosis and placement of a chest tube. For patients 16-21, a chest tube was inserted at the bedside with no pretreatment biopsies taken. On day 2, the Ad.RSV*tk* viral vector, diluted in 50-100 ml normal saline, was instilled via the thoracostomy tube. Three days later (on study day 5), a repeat videothoracoscopy was performed for tumor specimen acquisition. The following morning (day 6), intravenous GCV was initiated at 5 mg per kg given over one hour twice daily for 14 days. At the completion of the 14 day GCV course, patients were discharged for outpatient follow-up. Throughout the study, the patients were carefully evaluated for evidence of toxicity, viral shedding, immune responses to the virus and radiographic evidence of tumor response.

As summarized in Table 33.1, between November 1995 and November 1997, 21 (16 male, 5 female) patients were enrolled in the study; none left the study prior to completion. The ages of the patients ranged from 37 to 74 years with a median age of 66 years. All stages and histologic subtypes of mesothelioma were represented.

Clinical toxicities of the Ad.RSV*tk*/GCV gene therapy were minimal and a maximally-tolerated dose (MTD) was not achieved. Four non-dose-limiting toxicities were commonly noted: fever, liver enzyme abnormalities, myelosuppression, and skin rash. Temperature elevations to 100-102°F within 6-12 hours of vector instillation were seen in 20 of 21 patients, but with spontaneous defervescence after 48-72 hours and without associated hemodynamic or respiratory compromise. In two of the three patients treated at the highest dose level, we noted some transient hypotension occurring within 1-4 hours of receiving vector. Thirteen of 21 patients demonstrated minor abnormalities of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyltransferase (GGT). No patients developed elevated bilirubin or prothrombin time, or clinical evidence of hepatotoxicity. Minor hematological toxicity was common, with 17 of 21 patients demonstrating a decline in hemoglobin, but only two patients required blood transfusions. There was only one episode of moderate neutropenia and one episode of lymphopenia. Both resolved spontaneously and without infectious complications. About half the patients developed a vesicular skin rash at the site of the chest tube site. The etiology of these eruptions are not known for certain but are thought to be similar to contact dermatitis.

No shedding of recombinant adenovirus was detected via an antibody fluorescent unit (AFU) assay from sterile swabs of the nares, rectum and urethra. In addition, we found no evidence of adenoviral or HSV*tk* DNA using PCR techniques in the tumor or in any of the distant organ samples obtained from seven patients at the time of autopsy.

Detectable gene transfer (Table 33.1) has been documented in 12 of 20 evaluable patients in a dose-dependent fashion by either DNA polymerase chain reaction (PCR) (Fig. 33.1A), RT-PCR, in situ hybridization (Fig. 33.1B,C), and immunohistochemistry (IHC) (Fig. 33.1D, E), the latter method utilizing a murine monoclonal antibody directed against HSV*tk*. Once the dose level of 3.2×10^{11} was achieved, all patients except one demonstrated evidence of *tk* protein on post-treatment biopsies via IHC, with positive staining of tumor cells as deep as 40-50 cell layers below the mesothelial surface (Fig. 33.1D, E).

Strong anti-adenoviral humoral and cellular immune responses have been noted. These include acute neutrophil-predominant intratumoral inflammation in the post-treatment biopsy sections, generation of high titers of anti-adenoviral neutralizing antibodies in serum and pleural fluid, significant increases in inflammatory cytokine production (TNF-α, IL-6) in pleural fluid, generation of serum antibodies against adenoviral structural proteins, and increased lymphocyte proliferative responses to adenoviral proteins.[15]

As in most phase I trials, the actual clinical effects of Ad.RSV*tk*/GCV gene therapy upon the patients' tumors has been difficult to gauge. This is made more difficult because of the heterogeneity of our patient population in terms of age, stage, histology, and vector dose. Chest radiography and CT scanning, although quite sensitive for detecting tumor progression, are poor in determining therapy-related response in mesothelioma. Given these caveats, with a median follow-up of approximately 12 months, 12 of 21 patients have died, with no fatal complications attributable to the gene therapy protocol (Table 33.1). Although no definite tumor regressions were noted, three of the 21 patients do remain clinically stable, with no evidence of tumor growth on serial chest radiographs and chest CT scans. Of those three patients without evidence of progression, all presented with early-stage mesothelioma (Stage IA/IB).

Table 33.1. Results of PENN phase 1 clinical trial using Ad.RSVtk

Meso Patient	Stage/Cell Type	Vector Dose (pfu)	Status	Survival s/p Rx (months)	Gene Transfer (PCR/IHC)
1 62/M	IA/E•	1×10^9	progressed	28	-
2 56/M	III/E	1×10^9	deceased	8	-
3 69/M	III/B	1×10^9	deceased	20	+
4 66/M	II/E	3.2×10^9	deceased	11	-
5 71/M	IA/E	3.2×10^9	stable	24	+
6 71/M	II/B	1×10^{10}	deceased	4	+
7 70/M	II/E	1×10^{10}	deceased	6	-
8 60/M	II/E	1×10^{10}	progressed	21	+
9 74/M	II/B	3.2×10^{10}	deceased	2	*
10 60/M	III/E	3.2×10^{10}	deceased	9	-
11 37/F	IV/E	1×10^{11}	deceased	16	-
12 37/M	III**	1×10^{11}	deceased	2	-
13 65/F	III/E	1×10^{11}	deceased	10	+
14 66/F	IA/E	3.2×10^{11}	progressed	18	+
15 60/M	IV/B	3.2×10^{11}	deceased	5	+
16 69/M	IB/E	3.2×10^{11}	deceased	8	+
17 70/F	IB/E	3.2×10^{11}	progressed	11	+
18 69/F	IB/E	3.2×10^{11}	progressed	11	+
19 72/M	II/E	1×10^{12}	stable	5	+
20 65/M	II/E	1×10^{12}	progressed	5	+
21 67/M	IA/S	1×10^{12}	stable	4	+

*Patient 009 was unable to have the follow-up thorascopic biopsy; **Patient 012 had a pseudomesotheliomatoid adenocarcinoma; •E-Epithelioid; B-Biphasic; S-Sarcomatoid

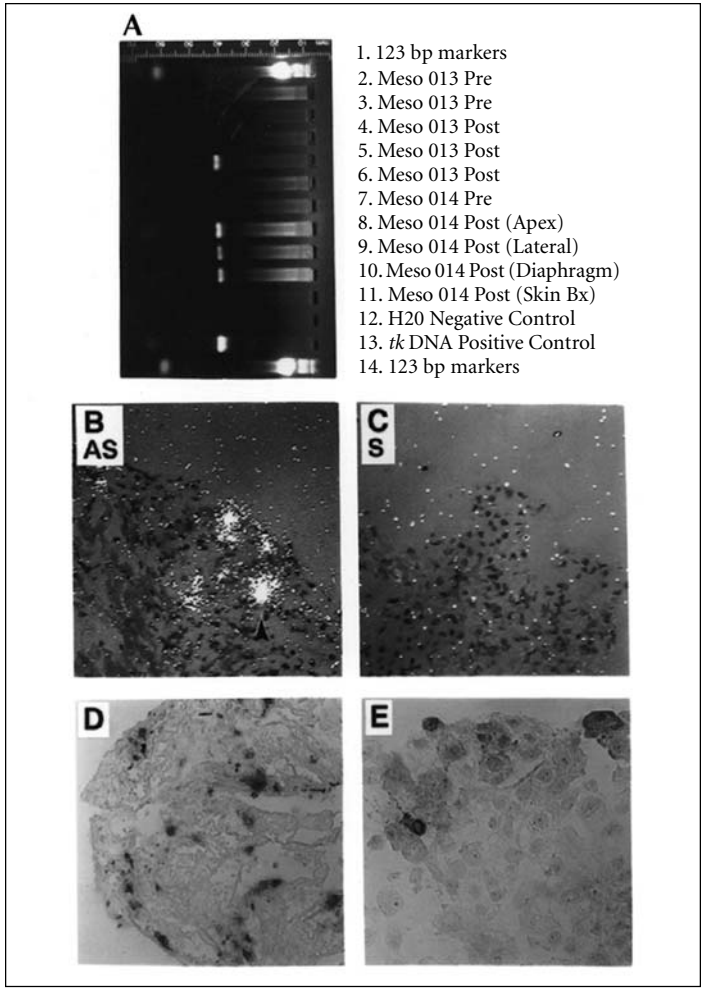

Fig.33.1. Transgene detection in the Ad.tk gene therapy trial. (A) PCR detection of HSVtk DNA from pre- and post-vector delivery pleural biopsies of Patients 13 and 14. A 536 bp fragment is detected in the positive control lane as well as the post-treatment specimens from patients 13 and 14, including three diverse intrapleural locations for patient 14. No HSV*tk* DNA was detectable by ethidium bromide gel or Southern blot from any of the pretreatment samples or a skin biopsy obtained from the chest tube site after virus instillation. (B) Photomicrographs (x200) of in situ hydridization assay performed with antisense oligonucleotide probes on post-vector biopsy specimens from Patient 13. Arrowhead demonstrates positivity for HSVtk mRNA in tumor cells 10-20 cell layers from pleural edge. (C) Similar section hybridized with HSV*tk* mRNA sense control probe. (D,E) Immunohistochemical staining of tumor biopsy from Patient 16 with the anti-HSV*tk* monoclonal antibody mixture at x100 (D) and x200 (E). Black staining denotes transgene expression. Note strong nuclear staining in some cells.

Problems and Future Approaches

The recently completed phase 1 trial has clearly demonstrated that delivery of large doses of an adenoviral vector to the pleural space is well tolerated and resulted in significant gene transfer to the surface and upper layers of tumor nodules. Our major challenge for future will be to opimize gene delivery. We plan to approach this problem in a number of ways. Since gene transfer appears to be dose related, one strategy will be to deliver higher doses of vector. To accomplish this, we plan to continue dose escalation; however, we are switching to an E1/E4-deleted "third generation" HSV*tk* -expressing vector. The main advantages of this vector will be lower production costs (due to lower levels of replication-competent adenovirus) and potentially lower hepatoxicity[16] and less immunogenicity. Preclinical in vitro, in vivo, and toxicity studies indicate that this new vector performs almost identically to the "first generation" Ad.RSV*tk* .

A second approach to optimize efficacy will involve tumor debulking and lavage of pleural space prior to gene therapy treatment to maximize intrathoracic vector to tumor cell ratios. A phase 1 trial to study the toxicity of delivering virus using this approach is planned for the near future. Future investigation might also focus on more efficient HSV*tk* mutant enzymes or drugs (such as retinoids) that might enhance bystander effects.[17,18]

One future strategy that might be particularly efficient in increasing gene delivery to mesothelioma cells could be the use of replicating viral vectors which have the capability of killing tumors by primary viral lysis and/or via delivery of therapeutic genes to cancer cells.[19] Promising viruses in this regard are tumor-selective replication-competent adenoviruses (see ref. 20 and chapter 21).

Conclusion

Cancer gene therapy is still in its infancy. Even though clinical trials of gene therapy for mesothelioma have begun, it is important to realize the preliminary nature of these studies. Although it is very unlikely that any of these early trials will result in practical therapies for advanced tumors, well-designed trials that are aimed at testing specific hypotheses and generating useful information about issues such as toxicity, gene transfer, and immune responses will be important first steps that must be taken for the advancement of cancer gene therapy. As more information is obtained about tumor immunology and biology, and as better vectors are developed, gene therapy will almost certainly play a key role in the treatment of mesothelioma in the next decade.

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