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Preface

The understanding of tumor growth is closely related to the basic knowledge of homeostasis, which maintains among several other functions the balance between cell growth and cell death. The first part of the book deals with homeostasis mainly at organismal level, which one could also summarize in one word: health. The loss of growth balance by the overproduction of certain cell types is known as tumor formation, dealt with in the second major part of the book. The last part describes different tumor models, among them the renal-capsule – parathymic lymph node metastatic model.

That the long "War on Cancer" declared by president Nixon in 1971 is not over is indicated by the fact that despite all efforts, the overwhelming majority (>90%) of cancer deaths is still caused by metastasis. Another disappointing fact is that in spite of the many "magic bullets and devices" that have been discovered, none of them delivered a devastating blow to cancer. To fight more efficiently against cancer, the book follows the spread of cancer cells from chemical carcinogenesis of liver and kidney tumors to their metastasis in thoracic lymph nodes. It is expected that the revealing of the primarily lymphatic spread of tumor cells can then be mimicked and eradicated by therapeutic agents.

To follow the path of tumor spread *in vivo*, several animal models have been invented, but only a few could be used to study metastasis. The novelty of the metastatic model described in the book lies in the observation that in the malignant tumors of large abdominal parenchymal organs (liver and kidney), cancer cells enter the interstitial fluid due to inefficient angiogenesis. Tumor cells exit the primary tumor through its disruptions and enter the abdominal cavity. Tumor cells traverse through the diaphragm and are collected by the thoracic lymphatic vessels and accumulate as secondary tumors in the parathymical lymph nodes. As liver and kidney are among the major sites of tumor formation, knowledge of tumor spread from the abdominal to the thoracic cavity is of general importance.

The book can be easy used even by those who want to take only a glimpse or read the essence of tumor formation. This can be done by browsing the content

or by reading only the summary of a chapter or its specific section. The list of abbreviations is kept to the necessary minimum. Unnecessary memorization of abbreviations is avoided by giving their expansions.

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May, 2013

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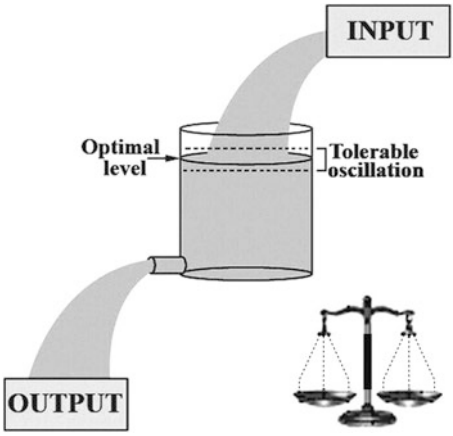
Homeostasis



Abstract Homeostasis refers to the constancy of the inner environment that secures the stability of equilibrium processes of the organism. It is also related to the relative stability of the chemical and physical environment of cells and organisms. The chemical requirements of maintaining the external requirements of homeostasis are represented by water, food, oxygen, while the physical conditions include temperature and atmospheric pressure. The organism is in homeostatic equilibrium when its cells do not suffer in any kind of shortage and can exert their activities without limitation. The dynamic equilibrium of the body (see the scheme above) is maintained by the participation of all organs helping to secure the stability of the body liquids. Stress conditions, e.g. heat, pain, hypoxia may cause the instability of the inner environment and endanger the homeostasis of the organism. Self regulatory systems have been evolved and serve to weaken the oscillations of the internal changes. Due to this involuntary regulation the ideal value ("set point") is approached and the stability of the inner environment restored. These regulatory systems act primarily by negative feedback mechanisms and with much lower frequency by positive feed-forward reactions.

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Keywords Inner environment • Dilute solutions • Osmosis • Optimal systems • Metabolism • Energy production • Biological membranes • Transport processes • Water spaces • Regulated parameters • Cellular homeostasis • Tissue homeostasis • Organs and organ systems • Dimensions of health • Control processes • Levels of bioregulation • Major regulatory systems • Immune system • Hormonal regulation • Nervous system • Regulatory mechanisms • Receptors • Reflexes • Human homeostasis • Body water • Sodium homeostasis • pH homeostasis • Regulation of body temperature • Energy storage • Energy balance • Blood composition • Regeneration • Panic syndromes

Contents

1.1 Chemical Basis of Biological Regulation

Biological balance refers to the fundamental characteristics of dynamic equilibrium that exist in living things. Homeostasis is a cornerstone of physiology; it maintains the internal environment within tolerable levels to continue the healthy functions of the organism. The disturbance of homeostasis results in disease or death. In this book when not specified, biological balance will refer to the homeostasis of the human organism. Particular attention will be paid to the relative stability of the internal environment represented by the interstitial fluid as potential mediator of tumor cell spread.

The chemical reactions in cells and organisms follow the fundamental laws of chemistry and laid down in chemical equations. A chemical equation describes the

chemical reaction and gives the chemical formulas of reactants (starting materials, substrates) on the left-hand side and products (resulting substances) on the right-hand side of the equation. The basic chemical laws include:

1. The law of conservation of mass established by Antoine Lavoisier in 1789: In every chemical transformation an equal quantity of matter exists before and after the reaction. Chemical reactions are balanced meaning that the law of conservation of mass is obeyed.

> Apart from becoming the founder of stoichiometry Lavoisier was beheaded in 1794 as a traitor during the French Revolution.

2. The law of definite proportions was recognized by Joseph Proust in 1799: In any chemical compound, the proportions by mass of the elements that compose it are constant, independent of the origin of the compound or its mode of preparation.
3. The law of multiple proportions (John Dalton 1808): When two elements form compounds, the masses of one that combine with a fixed mass of the other are in the ratio of (small) integers to each other.
4. Law of combining volumes: When two gases are allowed to react, such that the gases are at the same temperature and pressure, the volumes of each gas consumed will be in the ratio of small integers (Joseph Gay-Lussac 1809).
5. Avogadro's hypothesis (Amedeo Avogadro 1811): Equal volumes of different gases (at the same temperature and pressure) contain equal numbers of particles.
6. The law of dilute solutions established by Francois-Marie Raoult in 1882 states: The vapor pressure of an ideal solution is directly dependent on the vapor pressure of each chemical component and the mole fraction of the component present in the solution. Dilute solutions will be discussed in relation to osmosis.

Major processes that are involved in the chemical regulation of homeostasis in mammals are:

- Osmoregulation – the regulation of water and mineral household by the kidney. Water stressed animals are either osmoconformers or osmoregulators. Osmoconformers follow closely the osmolarity of their aqueous environment (e.g. ocean). Osmoregulators try to maintain blood osmolarity at a constant level, regardless of their environment.
- Excretion – the removal of metabolic waste products (e.g. urea, carbon dioxide) by excretory organs (kidneys, lungs).
- Regulation of body temperature – heat production during metabolism and release primarily by the skin.
- Carbohydrate metabolism – regulation of blood glucose (hormonal regulation)

The term homeostasis has come to use in several fields such as "ecological homeostasis", "risk homeostasis" to compensate safety measure, "stress homeostasis" to generate artificial stress, etc.

1.2 Definition of Homeostasis, Allostasis

1.2.1 Homeostasis

The term homeostasis (from Greek homoios, μοιος = similar, and stasis, στάσις = normal state) meaning regulated stability of the inner environment of an organism was coined by Walter B. Cannon in his book: *The Wisdom of the Body* and represented a modern concept of self regulation (Cannon 1926, 1939). Since then homeostasis became one of the central organizing principles in modern biology. The expression of the stable inner environment (*milieu intérior*) was defined earlier in 1865 by Claude Bernard (Bernard 1957). The inner environment as a physico-chemical puffer, and medium of intracellular communication, secures the transport of food molecules and degradation products.

Homeostasis should neither be confused with hemostasis nor with hemodynamics. Hemostasis means two combined and orchestrated physiological processes for the maintenance of vascular integrity. The first process causes the bleeding to stop by the accumulation of platelets at the site of the injured vessel. In the second process integrins interact at the surface of platelets with endothelial cells and fibrinogen generating a platelet plug covered with a fibrin meshwork. The fibrin network supports and stabilizes the blood clot while the blood is circulating inside the vessels without coagulation. Hemodynamics, another distinct term deals with the dynamics of blood circulation.

With the specialization of body cells tissues developed their own microenvironment to stabilize the inner environment. The mobile medium of the microenvironment provides optimal conditions for their macromolecular (DNA, RNA, protein) function of tissues and organs. Cannon summarized the general features of homeostasis as:

- *Constancy* of an open system such as the body, supported by mechanisms to maintain its steady state.
- *Steady state* conditions require that any change will automatically meet factors that resist change.
- *Homeostatic regulation* consists of several cooperating mechanisms acting simultaneously or successively.

A further feature of homeostasis is that the body reserves energy as ATP, creatine phosphate, glycogen, fat and protein. The storage of energy in organisms is also secured by nutrients characterized by the time required to deplete reserves ranging from few hours (amino acids), up to days (carbohydrates, sodium, water), weeks (fats, thiamin, ascorbic acid, niacin, riboflavin) and years (vitamin A, iron in men, iodine, calcium). Self-regulation has been described not only in a wide variety of living systems including embryonal development, tumor biology, self-regulation of populations and ecosystems. It also contributed to the development of cybernetics,

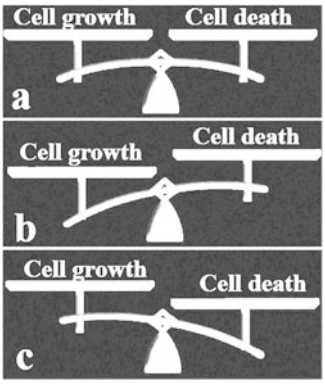

Fig. 1.1 Balance between cell growth and cell death in the organism. (**a**) Balanced cell growth and cell death is characteristic to the healthy organism. (**b**) Overwhelming cell growth with the exception of embryogenesis, in the childhood and during reconvalescence is leading to cancer. (**c**) Upsetting the balance by insufficient cell growth, increased cell death may cause apoptosis or necrosis

its application in mathematics as negative feedback, in mechanics, planning of machines and engines. It was recognized already by Cannon that the generalization of theories from one field to another may conceal the danger of applying theories too broadly. Nevertheless, self-regulation has spread from physiology to other fields gaining sense in economic and political systems after recognizing that regulation provides balanced stability.

Without intruding into other fields and drawing analogies among different self-regulatory systems this chapter is focusing on biological homeostasis and is further restricted to the balance between cell growth and cell death (Fig. 1.1a). When cell growth prevails and balance is upset in favor of increased or unlimited cell growth, cancer or metastatic growth is likely to ensue (Fig. 1.1b). The opposite tendency, namely lowered cell growth by higher rate of cell death is characteristic to apoptosis or necrosis (Fig. 1.1c).

In biological sense homeostasis is the question of life and death, balanced to keep the organism in healthy condition. As already referred to, there are several aspects of maintaining homeostasis. The balanced health of the organism is secured by three major interconnected systems:

– Nervous system
– Hormonal regulation primarily by the hypothalamic-pituitary-adrenal axis
– Immune system

These regulatory systems (hormonal, nervous, immune) will be discussed in another subchapter.

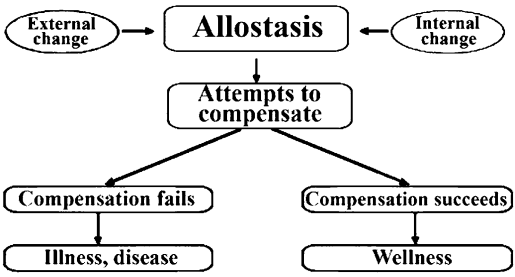

Fig. 1.2 Allostasis caused by external or internal changes. Allostasis may be compensated and maintain health or can fail and lead to illness or disease

1.2.2 Allostasis

Systems planned for *standard conditions* are functional only under stable conditions. Systems designed for optimal function work only under *optimal conditions*. Animal organisms are designed in such a manner that they can be used for a longer period of time and under changing conditions i.e. the principle of optimization has to be given up by over securing. When the sources are scarce, the system set to optimum can be destructed. Allostasis defined by Mc Ewen means altered state, the disintegration of physiological functions and can lead the loss of health (Fig. 1.2).

1.2.3 Homeostasis Versus Allostasis

When we speak about human homeostasis we normally think of the fine tuning of a single set point, e.g. blood pH, blood glucose or blood oxygen level. However, a multitude of highly complex interactions are needed to maintain balance or return systems within their normal, functional range. Allostasis in Greek means "remaining stable by being variable" (Sterling and Eyer 1988). During this hazardous stability serious pathophysiological changes may occur that need cumulative energy input and expenditure (Wingfield 2003). Allostasis is adaptation by providing a more dynamic balance. The holistic view of allostasis regards health not simply the lack of illness or disease, but a state of complex physical, mental, emotional, environmental, sociocultural and spiritual well being. However, Cannon who also took this holistic view suggested that the unity of functional cooperation among different organs provides sufficient stability for the organism without special means to maintain homeostasis. This controversy indicates that the concept of allostasis is not more than renaming the original concept of homeostasis. The concept of allostasis has largely occurred as a result of misunderstandings and misapprehensions concerning the concept of homeostasis (Day 2005). Allostasis has been characterized by:

- altered inner function,
- strong tendency for compensatory mechanisms to join in,
- separation and disintegration of integrated functions,
- extreme functions maintained for a longer period of time.

The altered function is indicating the derangement of homeostasis that can be realized as bad feeling (e.g. strong hunger) and may provoke extreme behavior. Such extrahomeostatic processes are expected to work under different mechanisms that do not serve the benefit of homeostasis. As an example, in allostasis the homeostatic processes of nutrition, namely:

- metabolic pathways,
- emptying and filling up stores,
- visceral reflexes,

are opposed by extrahomeostatic preferences such as:

- nutritional selection,
- searching for food, instincts,
- learning, vision, anticipation.

Allostasis is a critical point in homeostasis where the organism mobilizes reserves that will maintain the homeostatic balance or turn it to a pathological process.

1.3 Aqueous Internal Environment

Organisms consisting of cells are dilute aqueous systems with a water content ranging between ∼40 to 90 % and an average of 70 %. The water content of the 2 months old embryo is over 95 %, a new newborn baby's body contains more than 70 %, an adult person 60–65 %. The water content of men is higher than those of women due to the fact that men have more muscle that binds more water (∼70 %) than the higher proportion of hydrophobic fat tissue in women with a mere 10 % water content. Aging gradually reduces the water content, above age 70 the water content may sink below 50 %. Obesity can also decrease the percentage of water to as low as 45 % (Guyton 1991).

In a human body containing 40 l of fluid, the body water consists of the following compartments (Hansen and Koeppen 2002):

Intracellular fluid	∼ 25 l (62.5 % close to the 2/3 rule of thumb)
Extracellular fluid	∼ 14 l (35 % close to the 1/3 rule of thumb)
Plasma	∼ 3 l (20 % of extracellular fluid)
Interstitial fluid	∼ 11 l (80 % of extracellular fluid)
Transcellular fluid	∼1 l (2.5 %)

The specialized transcellular fluid (volume often ignored) consists of the gastrointestinal, cerebrospinal, peritoneal, ocular fluid in the eye, endolymph of the inner ear, bladder and synovial fluid of the joints.

Table 1.1 Osmotic values of inner environment represented by blood plasmas of vertebrates

Vertebrates	mOsm	Osmoregulation to maintain ionic homeostasis
Terrestrial vertebrates		
Mammals	295	Kidneys, nasal passages, lungs reduce water loss
Birds	317	Kidneys, salt-glands, intestines, internalization of respiratory surfaces, uric acid excretion (uricotelism)
Reptiles	286	Kidneys, glands, tongue, intestine, uric acid excretion
Lizards	307	Kidneys, specialized nasal salt glands
Snakes	300	Kidneys, specialized sublingual salt glands
Turtles	287	Kidneys, specialized orbital salt glands
Amphibians	160–240	Kidneys, gills, swim bladder, skin, intestine
Marine vertebrates		
Fish		
Teleosts (bonny fish)	60–80	Kidney, specialized chloride cells in gills
[a]African lungfish (*Protopterus aethiopicus*)		
Preaestivation	234	Kidney and gill ion exchange (urea: 4.2 mM)
Aestivation (13 month)	650	Ion exchange inoperative (urea: 203 mM)
Cartilaginous		
[b]Sharks	1,094	Rectal gland absorbs extra salt from the blood and passes it into the intestine to be excreted
Marine mammals	310–360	Water and electrolyte balance secured by renal function
Seawater	1,094	Multifactorial global regulation

Modified with permission (Banfalvi 1991a)
[a]African lungfish osmolarity (DeLaney et al. 1977)
[b]Bonnethead shark osmolarity (Harms et al. 2002)

When discussing osmolytes one cannot escape the thought that living cells consuming and producing substances in dilute aqueous milieu, resemble the ultimate dilute solution, the primordial ocean. Living things still retain in their ionic make-up certain characteristics of the ancient ocean. Indeed the blood plasma is regarded as a relic of the Paleozoic sea. In this context, the ocean as the oldest and most important electrolyte system is discussed in relation with osmolyte systems of biological origin. The idea that the uniform osmolarity of blood and land vertebrates reflects the osmolarity of an ancient stage, namely the concentration of the primordial ocean at the time of migration to land, is not new (Smith 1943, 1953). As the osmotic concentration of the present day ocean (1.09 Osm) is more than three times higher than that of the blood of land vertebrates (0.3 Osm) (Table 1.1), it was concluded that the salinity of oceans is gradually increasing (Banfalvi 1991a).

In contrast to unicells, most cells of a metazoan are not in direct contact with the external milieu. The motility of animals allows them to seek out optimal environment and to sacrifice certain biosynthetic capabilities. The loss of versatility and the requirement for essential elements is compensated in many cases by predation. In addition, the cells of a mammal's body maintain a higher

temperature than that of single cells and their output of waste products and heat are correspondingly greater. Many of the metazoan cells became highly specialized and intolerant to more than trivial alterations in the composition or temperature of their dilute aqueous environment. This extracellular fluid environment is Claude Bernard's *milieu interior* (Bernard 1878) which represents the internal environment for metazoan cells. The extracellular fluid consists of all the body fluids outside the cells including the interstitial fluid, lymph, blood plasma, and specialized fluids. The extracellular fluid constitutes the internal environment of the body. Extracellular fluids that are involved in the circulation constitute the interstitial fluid moving towards and forming the lymph (after intertitial fluid is collected) and the blood plasma. The lymph in man returns to blood through the major lymph vessel (*ductus thoracicus*). The constant dynamic properties of the extracellular fluid provide the basis upon which the fundamental metabolic processes go on. Higher animals have developed organs such as gills, lungs, cloacae, rectal and nasal glands, bladder and kidneys in which special mechanisms, such as respiration, digestion, absorption and excretion evolved to regulate the composition of the extracellular fluid in order to maintain homeostasis. These mechanisms depend on the circulation of blood, which stirs extracellular fluid preventing local alterations in composition or temperature.

1.3.1 Osmosis

1.3.1.1 Extrahomeostatic Dilute Solutions

The sea is the oldest dilute solution, with a much higher osmolarity than the isosmotic concentration of land vertebrates. This does not mean that the ocean concentration would be extrahomeostatic to marine life. The vapor pressure of dilute solutions is a fundamental property of vapor-liquid equilibrium, which has been exploited in several processes (distillation, absorption, stripping, flash separation, etc.). Looking at the criteria of dilute solutions it becomes evident that all their criteria apply to the ocean. In dilute aqueous solutions the molar excess of solvent over solute is more than 100 (<0.555 Osm). In dilute solutions there are practically no interactions between the molecules of the solute. Although, the osmolality of the ocean (1.09 Osm) is two times higher, the solute-solute interactions are still negligible and with some reservation, the ocean is still regarded as a dilute solution.

The birthplace of life was most probably the ocean. Oparin and Haldane proposed that life on Earth developed under reducing conditions and by the synthesis of increasingly complex organic polymers which became living systems (Oparin 1954; Haldane 1928). The transition from aquatic to terrestrial mode of life is thought to have occurred during the Devonian Era some 420 million years ago. Most of the orders of mammals, including flying bats and aquatic whales, evolved from insectivorous ancestors during the Paleocene-Eocene period not more than 15 million years ago. It is generally accepted that at the beginning of life, the intracellular body liquid and the extracellular concentration of ocean were identical.

The osmotic concentration of seawater is now more than three times higher than the isotonic concentration of land vertebrates (Table 1.1). Osmolality or osmotic concentration is the ratio of amount of different solutes in a given weight of water (solute osmoles/water in kg). As the uniform osmolarity of blood and body fluids representing the inner environment in land vertebrates is a reflection of osmolarity of seawater at the time of their evolutionary emergence from the marine environment, the stability of inner environment of land vertebrates may be an indication of a long-term concentration process of salt in the sea over the past ~420 million years.

Osmosis is a special kind of diffusion through the cell membrane. This movement is occurring passively without the input of energy. Passive transport will be discussed with the membrane systems.

Marine organisms with the notable exception of hypotonic marine mammals tend to be isotonic in relation to the seawater. Marine teleosts (bony fish) are among the most abundant vertebrates on the planet, and like those of higher vertebrates, are hypotonic to seawater. Bony fish lose water to the environment. Lungfish who have survived the movement to land without significant physiological and anatomical changes are characterized as the first land vertebrates, have attracted the attention of those who were concerned with the evolutionary transition to terrestrial life. Before aestivation the plasma osmolality of the African lungfish is lower than that of terrestrial vertebrates and similar to that of amphibians (Table 1.1). During the aestivation period lungfish dehydration constitutes an increasing fraction of total plasma osmolarity beside the elevated urea concentration (DeLaney et al. 1977).

In contrast to bony fish, cartilaginous chondrichthyans represent 20-times less species, but these ancient species were much more abundant, according to the fossil records. The body fluids of cartilaginous fish embrace an astonishing variety of forms and lifestyles, with blood plasma approximately isosmotic to the seawater. The blood chemistry reference values have been determined only for a few species of the elasmobranchs (sharks, skates and rays) (Harms et al. 2002). Sharks retain urea in their blood and gain water from the seawater through their gills and the lining of their guts. The serum sodium of the shark is higher (~260–290 mOsm) than that of man, (135–145 mOsm), but with associated anions only half the external osmotic pressure of the ocean (Epstein 1979). The hypertonicity of the sea is exactly counterbalanced with urea, which circulates in high concentration in the blood and permeates all cells. The excess water is excreted as dilute urine.

Amphibians living under both terrestrial and aqueous environmental conditions lowered to some extent their osmotic concentration. Birds, reptiles, and other terrestrial vertebrates are often subjects to limited water availability. Terrestrial vertebrates conserve water to maintain isotonic concentration. The preservation of stable isotonic concentration critical for macromolecular (DNA, RNA, protein) function is a reasonable explanation why terrestrial vertebrates maintained their nearly identical osmotic concentration.

The isotony in the blood of land vertebrates corresponds to ∼0.3 Osm, hypotony to <0.3 Osm and hypertony to >0.3 Osm. Blood cells shrink in hypertonic solution and expand in hypotonic solution. In solutions with concentration under the osmotic tolerance blood cells disrupt and cause hemolysis. Isotonic solutions can be administered parenterally by intravenous (*i.v.*), intracardial (*i.c.*), intramuscular (*i.m.*), intraperitoneal (*i.p.*), retroperitoneal (*r.p.*) or subcutaneous (*s.c.*) injection. Isotonic solutions (0.3 Osm) contain $6 \times 10^{23} \times 0.3 = 1.8 \times 10^{23}$ particles dissolved in 1 kg of distilled water. Physiological saline is a 0.15 M NaCl solution, but due to the dissociation of NaCl to Na^+ and Cl^- ions its osmotic concentration will be 0.3 Osm. Glucose does not dissociate, its O.3 M solution used for parenteral administration contains as many dissolved particles as saline corresponding to 0.3 Osm.

1.3.1.2 Global Aspects of Ocean as Dilute Solutions

These aspects are summarized briefly under the following bullet points:

- The solutes of the sea as the largest dilute solution are non-volatile. The sea contains primarily non-volatile salts. An exception is iodine at the surface of the seas (0.05 ppm ∼ 60 μg/L). Iodine-containing rain (∼2–8 μg per liter) returns iodine to the Earth. Farther from the ocean the iodine content of the rain diminishes and the greater the chance of the iodine deficiency in the human population will be.
- The solvent evaporates. Evaporation close to distilled water quality takes place on the surface of sea.
- The applicability of Charles' law to the ocean is confirmed by the fact that with the increase of the temperature the volume of the ocean expands. Charles' law was established in the 1780s and described a direct relationship between the temperature and volume of a gas.
- More water molecules break away at higher temperature (without boiling), resulting generally in increased vapor pressure over sea and precipitate formation.
- Evaporation takes place in a closed system. The amount of water on Earth together with the water vapor of the atmosphere remains constant.
- Equilibrium will be reached when the number of water particles leaving water surfaces is balanced by precipitation. Evaporation and precipitation above the sea are balanced. Global equilibrium cannot be reached, only local saturation (precipitation) occurs.

By accepting the ocean as a dilute solution of global proportion, the laws of dilute solutions apply and contribute to the understanding of climatic advances, retreats and biological consequences. Salinity changes of the sea over geological ages provide evidence for a dynamic osmolyte system against a persisting general geochemical balance. Fluctuations in sea salinity explain wet and dry climatic

periods and are among the major driving forces of biological evolution. The recent thermal expansion of sea volume, snowpack reduction, melting of sea ice and ice sheets, are the reflection of a short-term dilution period, the oscillation of which is temporarily outweighing the long-term salt accumulation of ocean.

1.4 Optimal Systems

Optimal control theory is a mathematical optimization method to approach the best control under circumstances. Consequently, optimal control deals with finding a control law for a given system such that a certain optimality criterion is achieved (Pontryagin et al. 1962; Athans and Falb 1966). Similarly to bioinformation, the basic concepts of optimization come from mathematical optimization. The key elements of mathematical optimization problems are:

(a) *decision variables,* those which can be varied during the search of the best solution,
(b) *objective function,* the performance index which quantifies the quality of a solution defined by a set of decision variables, and which can be maximized or minimized, and
(c) *constraints,* requirements that must be met, usually expressed as equalities and inequalities.

Decision variables can be continuous (represented by real numbers), resulting in *continuous optimization* problems, or discrete integer numbers, resulting in integer optimization, also called *combinatorial optimization*. In many instances, there is a mix of continuous and integer decision variables (Banga 2005). Optimization theory not only explains current adaptations of biological systems, but also helps to predict new designs that may yet evolve (Alexander 1982; Sutherland 2005). Optimization means to find the best solution (compromise) among several conflicting demands subject to defined requirements (constraints). In evolutionary context of increasing demand the homeostasis of living organisms needs constant development to maintain the fitness to the environment it is co-evolving with.

Red Queen Hypothesis This theory tries to explain two controversial evolutionary tendencies: the constant extinction of species caused by the co-evolution among competing species and the reproduction of individuals (Van Valen 1973; Bell 1982). The expression comes from Lewis Carroll's "*Through the Looking-Glass*" where Alice and the Red Queen were constantly running, without going anywhere.

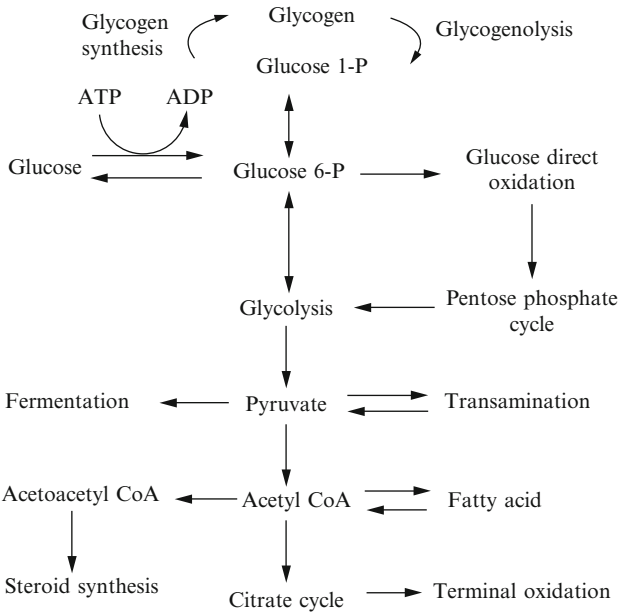

Fig. 1.3 Major pathways connected with glucose metabolism. Glucose 6-phosphate (glucose 6-P) is the center of the major pathways: glycolysis, glyconeogenesis, glycogen synthesis, glycogenolysis, glucose direct oxidation. Among several additional connections, the pentose phosphate cycle, fermentation, transamination alanin, ketone body formation (acetoacetate, hydroxybutyrate, acetone), cholesterol biosynthesis, fatty acid synthesis and their β-oxidation, the citrate cycle and terminal oxidation are also indicated

Organisms have to keep improving homeostasis ranging from behavior and ecology to speciation, macroevolution, human language etc. to survive, in conformity with the so called "Red Queen Effect" (Nowak and Sigmund 2004). The Red Queen Effect postulates, that organisms constantly adapt, evolve and proliferate to reproduce themselves and to survive while challenged by the changing environment.

1.4.1 Optimization of Glucose Metabolism

As an example for the optimization the glucose metabolism of organisms is given in Fig. 1.3. For optimization consider the cross roads connected directly to different pathways including glycolysis, gluconeogenesis, glycogen synthesis, glycogenolysis, pentose phosphate pathway and back to glucose from glucose-6-phosphate catalyzed by glucose-6 phosphatase and further connections with other pathways. The question is how to regulate and synchronize these processes to work efficiently by minimizing consumption and optimizing glucose metabolism with the smallest, but unavoidable ancillary constraints. The simplest solution is

reducing or increasing certain functions by systematically choosing input values (set points) from within a small set and computing the value of the function. Indeed, the regulation of an overall flux along a metabolic pathway takes place only at one or perhaps two key reactions catalyzed by their so called regulatory enzymes. The physicochemical factors of the reactions, primarily the substrate concentrations are of primary importance to control the overall metabolic rate, while those factors that are held constant (e.g. temperature and pH in warm-blooded vertebrates) have little regulatory significance. Notable exception is the pH in the gastrointestinal tract that affects digestion.

Body Optimization The goal of optimization of body functions is not just stay healthy, but as healthy as possible and not just fit, but as fit as possible. The signs that the body will not last forever is physical sickness and the warnings of doctor about health. Body optimization *via* caloric restriction is also known to optimize for longevity by calorie cut by about 1/3 of normal levels. During caloric restriction those extensive exercises that require high caloric intake and increase the chance of injury are also avoided. Practitioners also caution that caloric restrictions can lead to extremely lean figures for their height, far from the aesthetic standard deemed "ideal". Caffeine and smoking appears to boost metabolism, but can lead to physical dependence. Chain smoking, the practice of lighting a new cigarette immediately after the previous one is a common form of addiction causing significant harm.

The economics of glucose metabolism is exemplified by its relationship with lipid metabolism. The conversion of glucose to fat occurs regularly under conditions of optimal nutritional intake. To the contrary fat (triacylglycerol), with the exception of glycerol, cannot be converted back to glucose due to the irreversible decarboxylation of pyruvate to acetyl-CoA. As certain tissues (nervous system) and cells (erythrocytes) are more dependent on continual glucose supply than others, a minimal constant supply of glucose level is necessary to maintain the citrate cycle. The direct oxidation of glucose (pentose phosphate cycle), the fetal nutrition and the synthesis of lactose in milk also require glucose.

Biological systems, particularly organisms are so complex that due to the multitude of interplay among the large number of components, until recently it seemed to be impossible to establish system biology. Recent technological development allowed a shift from the reductionist to the holistic view of organisms. Reductionism argued that the processes of biology can be explained by chemistry and the laws of chemistry. The idea of holism is that natural systems, including organisms should be viewed as wholes, not as collections of parts. The complexity theory comprises both the holistic view and specific subparts towards the understanding of the body as a complex adaptive system upon interaction with the external environment in conformity with the internal requirements.

1.5 Basic Properties of Living Systems

The Living System Theory of James Grier Miller is a general theory on how all living systems work, maintain themselves, develop and change (Hammond and Wilby 2006). The living organism is distinguished from the inanimate world as the exclusive system that assimilates both the mass and energy of its environment into its living substance. Consequently, the same chemical and physical rules apply, the mass-energy laws are the same in the inanimate, plant, animal and human realms. The bioelements (CHNOPS group) can be combined into innumerable compounds including informational macromolecules (DNA, RNA, protein) that are present only in living organisms, but not in inanimate nature. In the mnemonic CHNOPS group C stands for carbon, H for hydrogen, N for nitrogen, O for oxygen, P for phosphorus and S for sulfur. These six light elements make up nearly 98 % of the human body. The electronic configuration of light elements including 20 elements with atomic numbers from 1 to 20 is similar, containing exclusively *s* and *p* electrons in their covalent bond formation but no *d* or *f* electrons that make elements more reactive. The first 20 elements of the periodic table up to calcium with a similar electronic configuration are regarded light, with a density less than 3 g/cm^3. The transition elements start with the 21st element scandium (Sc, $1s^2 2s^2 2p^6 3s^2 3p^6 3s^2 d^1$). The microelements consisting of 38 transition elements with incomplete *d* subshells belong to the heavy metals (Banfalvi 2011).

Although, the chemical laws apply equally to non-living and living systems, the selection of elements based on their electronic structure shows that significant selection took place to reduce and to control the biological reactions in cells and organisms. This selection contributed to the adaptation of the entire organism to its fitness to the environment and its survival through the inherited genetic makeup. In spite of the selection of light elements and limited biological application of heavy metals mainly for enzymatic catalysis, the chemical composition of animals is complex, interacting with environmental factors and operating by taking in carbon based food as energy source. The primary source of macromolecular information is DNA coding for functional proteins (enzymes, structural proteins, regulatory proteins, signal molecules, defensive immune molecules). Lipids constitute the membrane barrier, and serve as storage of energy. Carbohydrates are the primary energy suppliers for all organisms and provide faster and more energy supply than lipids and proteins.

Specific functions of multicellular organisms are carried out by four basic groups of cells forming tissues, namely the epithelial, nervous, muscle and connective tissues. Characteristic functions are performed by different tissues working together and forming an organ, such as the heart pumping the blood, or the skin protecting the organism from the environment. The cooperation of organs brings about organ systems, responsible for reproduction, digestion, absorption, respiration, excretion, etc. Animals, including mammals consist of organ systems, the coordinated functions of which secure stability. Homeostasis is manifested throughout the life span of species ranging from as short as 1 day to thousands of years (e.g. fungi). Aging and its relationship to cancer will be discussed in Chap. 2.

The basic characteristics of living systems include:

- organization and maintaining organization
- regulation
- metabolism
- reproduction: passing the genetic information to the next generation, the production of descendants resembling ancestors
- responsiveness
- growth: cell division
- development: series of morphological changes from fertilized egg to sexual maturity
- adaptation to external and internal changes
- evolution

Unicellular (fungus, microorganism) and multicellular (animal, plant) organisms as their name suggests are organized, capable to maintain this organization for a longer period of time, take up food as energy source, brake down these molecules and utilize them in multistep catabolic and anabolic processes. Living organisms respond to stimuli, grow, develop, reproduce themselves and maintain their homeostasis. As far as organization is concerned viruses are not regarded as living organisms. They evolved in their hosts in co-evolution with their host cells. Without host cells viruses could not exist and evolve. The living unit is the cell. Different prokaryotic and eukaryotic cells are shown in Fig. 1.4. The size of cells varies from the small microbes (μm) to large egg cells (cm). The diameter of an average mammalian cell is about 30 μm. The largest existing cell is the ostrich egg with a diameter of 16.3 cm (1.5 kg). The eggs of dinosaurs were much larger. That size matters during the spread of cancer will be discussed in Chap. 3.

Of the four major tissue types (epithelial, connective, nervous, muscle) (Fig. 1.5) epithelia are by far the most prolific and cancerogenic cells. Epithelial cells cover flat surfaces and line the cavities in the body held together more tightly than other cells by tight junctions, desmosomes and hemidesmosomes. Taxa are distinguished groups of organisms ranked in hierarchical order from domains to species.

As an example the classification of the human is given:

Domain	Eukaryota	All life form with eukaryotic cells including plants and animals
Kingdom	Animalia	Mobile multicellular organisms requiring preformed organic food
Phylum	Cordata	Embryonic skeletal rod (notochord), dorsal nerve cord, gills
Subphylum	Vertebrata	Vertebral column enclosing spinal cord, skull enclosing brain
Class	Mammalia	Female nourishes young on milk, warm blooded
Order	Primate	Descendants of tree living mammals, flattened face and fingers, poor sense of smell
Family	Hominidae	Bipedal locomotion, binocular vision forward, specialized hands
Genus	*Homo* (man)	Large brain, long childhood, ability to speak, keen vision
Species	*Homo sapiens* (wise man)	Reduced body hair, high forehead, large brain, shortened face, thumb opposition, nails rather than claws, increased metabolic rate in pregnancy and higher brain complexity relative to other hominidae

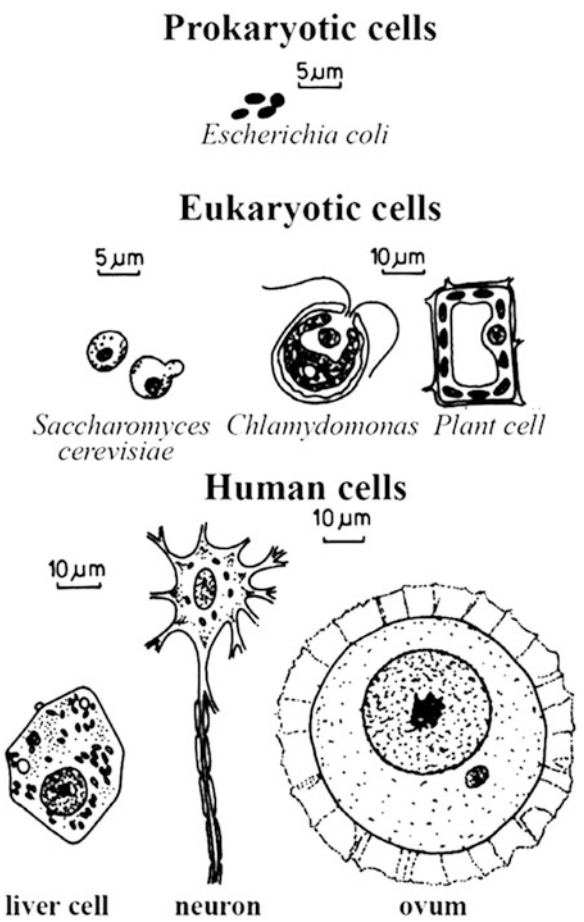

Fig. 1.4 Size and shape of prokaryotic and eukaryotic cells. Prokaryotic cells are the smallest ones. The size of eukaryotic cells varies within a relatively broad range from µm to cm (With permission Banfalvi 2009, p. 5)

1.6 Metabolism and Energy Production of Living Systems

1.6.1 General Rules of Bioenergetics

Bioenergetics is a biochemical term that is concerned with the energy production and consumption by breaking chemical bonds within living things. From bioenergetic point of view there are two types of chemical reactions. Thermodynamically favorable *exergonic reactions* are spontaneous and release energy. To the contrary *endergonic*, anabolic reactions consume energy. Endergonic reactions have positive ΔG values, meaning that energy is required to break their bonds. Bioenergetics is characterized by basic laws:

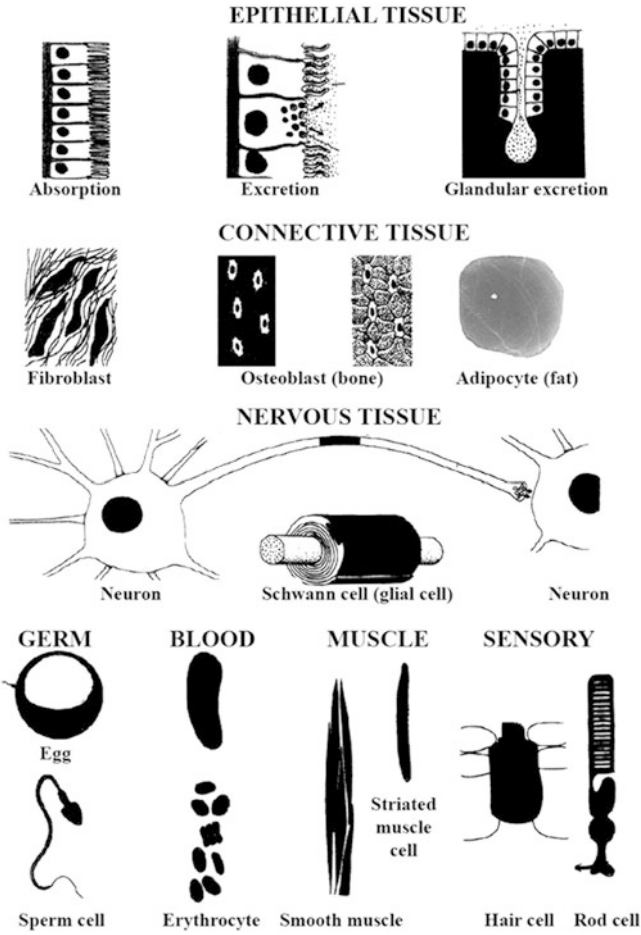

Fig. 1.5 Major tissue types of the human body (With permission Banfalvi 2009, p. 6)

- Energy conservation: energy is not lost, but can be converted into another form.
- In isolated systems the randomness increases (rule of entropy changes).
- The system cannot by cooled down to absolute zero (0 K = −273.15°C) temperature.

Biological forms of energy are:

- chemical energy (ATP) consumed in 3 major fields shown in Fig. 1.6,
- heat energy (e.g. homeo- and heterothermic animals),
- electric energy, difference between the inner and outer membrane of mitochondria (1.1 V), or the action potential (from −70 mV to +40 mV = 110 mV),
- light energy (e.g. the luciferase enzyme of firefly),
- kinetic energy (flagellar, ciliar, ameboid, muscular movement)

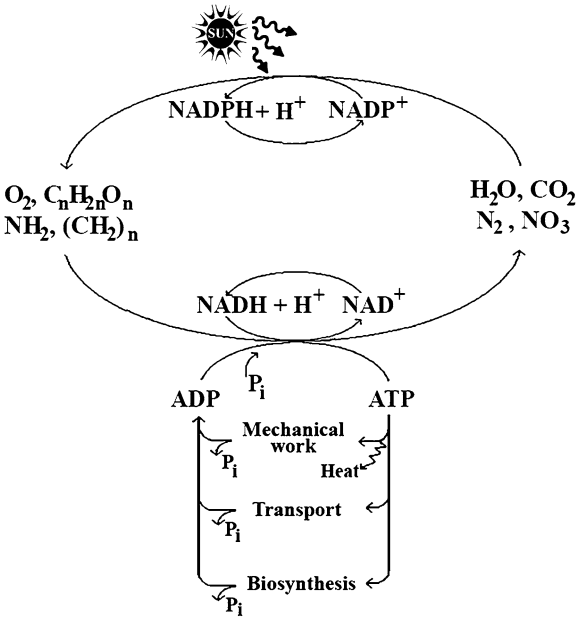

Fig. 1.6 Sun as the recent ultimate source of biological energy. The light energy of sun is converted to the chemical energy of ATP through photosynthesis by the conversion of an organic compounds to organic ones, primarily to carbohydrates ($C_nH_{2n}O_n$). The three major ATP consuming processes of animals are: mechanical (muscle) work, active membrane transport and biosynthetic processes

The energy of food can be utilized only in multistep degradation processes. The energy of direct burning of a sugar cannot be utilized, while its multistep oxidation by activated carrier molecules lowers the activation energy that can be overcome by body temperature. Biological energy conservation is based on Hess's law stating that during the complete course of a multistep process the same amount of energy is released as in a single step (Hess 1840).

1.6.2 Energy Content of Organic Compounds

The energy released or consumed during a chemical reaction is given as Gibbs' free energy change (ΔG). The free energy (ΔG) gained or lost in a reaction can be calculated:

$$\Delta G = \Delta H - T\Delta S$$

where H is the heat content, ΔH the change in heat content, T the temperature in Kelvin, S the entropy or the measure of disorder of the reaction and ΔS the entropy change. The energy of oxidation and reduction in redox reactions can also be expressed as the potential difference expressed in volts:

$$\Delta G = -nF \times \Delta E$$

where n represents the number of charges, F is the Faraday constant (96,500 C), and E the potential difference in volt (V). Standard redox potential is measured under standard conditions (298 K, 1 atm and 1 M concentration of the reactants).

To estimate the heat content (oxidation energy) of a compound a simple equation was applied to hydrocarbons (Kharash 1929), which however could not be applied to biological compounds containing functional groups (hydroxyl, amino, keto, aldehyde, etc). The Kharash equation has been extended to metabolites containing a great variety of functional groups, double bonds and aromatic rings. The combustion energies of different types of organic compounds were calculated and compared with those of calorimetrically measured values. Estimated values were within the range of error of calorimetric measurements with an average deviation of 0.4 %. By means of the modified Kharash equation the heat content of nutrients and metabolic intermediates could be calculated for organic compounds even if calorimetric values were not available. These estimated values were used to construct energy maps of metabolic cycles (Banfalvi 1999).

One of the basic properties of life is the maintenance of the highest organization against the general tendency of disorder (entropy), organized systems have to fight against constantly. The basis of highly organized structural and functional order is the potential of living organisms to obtain energy from their environment through different chemical processes regulated by genes. During evolution cells maintained their energy and apart from a few exceptions their genetic independence. Genes are involved in the regulation of cell growth, division and differentiation. The logic and strategy of gene control in prokaryotes is to allow the cells to adjust to nutritional changes of their environment and to optimize major functions, namely growth and division. Correspondingly, the elements of gene control in prokaryotes are centered on transcriptional regulation. In unicellular yeasts the major focus of regulation is also placed on cell growth and division with more attention being paid to differentiation. In multicellular organisms more genes are involved in differentiation than in growth and division. All cells get their food molecules primarily through diffusion and these energy rich molecules are then broken down to produce ATP. Similarly, the cellular information in the nucleus of the eukaryotic cells is maintained from generation to generation with few exceptions of cells that have lost their nuclei (e.g. red blood cells of most mammalians). The maternal genetic information is stored in mitochondria, responsible for the oxidative energy production of ATP.

Originally cells in the ancient ocean were using the chemical energy of the “warm broth” and could have developed the metabolic network conforming the “evolution backward” theory (Horowitz 1945):

Essential organic compound	O
Beginning of evolution:	$D \rightarrow O$
After the exhaustion of D:	$C \rightarrow D \rightarrow O$
After exhausting C:	$B \rightarrow C \rightarrow D \rightarrow O$
Photosynthesis upon exhaustion of B	$A \rightarrow B \rightarrow C \rightarrow D \rightarrow O$

The essential organic compound (O) was present at the beginning of evolution in huge quantities. After it was consumed other high energy containing compounds joined in (D followed by C, followed by B) to produce organic food molecules. As an alternative solution the energy might have been provided at least partially, and periodically from the internal heat of the Earth. The major energy producers of this alternative system could have been the bacteria and archeae by utilizing hydrogen sulfide, hydrogen, methane and other highly reduced compounds as evidenced by the still existing Mid-Ocean Ridge system, other seafloor volcanic centers and anaerobic lake sediments.

The energy crisis culminated when the organic energy substances were exhausted in the primordial ocean. The prebiotic chemistry became driven by the solar energy. The energy crisis was solved by the utilization of the unlimited light energy of the Sun by photosynthesis to generate organic molecules from inorganic (A) substances (CO_2, H_2O, N_2, NO_3) (Fig. 1.6). ATP is generated in plant cells by photophosphorylation, in animal cells by substrate level phosphorylation (e.g. in glycolysis, citrate cycle) and in their mitochondria during terminal oxidation coupled to the oxidative phosphorylation of ADP to ATP.

The construction of energy maps and linking them together can serve as a first thermodynamic check to predict alternative routes to be taken when metabolic roads are blocked at certain points. Even if such a thermodynamic transition is feasible, there is a second enzymological aspect to be considered, namely the activation energy which determines the speed of an energetically favorable and enzymatically catalyzed transition. It will be interesting to compare the energy maps of linear, spiral, branched pathways and metabolic wheels to see how they are linked together, what kind of metabolic transitions can be predicted, and which are the key junctions serving as branch points in different species. Ultimately, the tracing of metabolic shift will bring us closer to the understanding of metabolic identity which is unique and characteristic to the metabolism of individuals.

1.6.3 Energy Production

The oxidation of hydrogen to water is among the most efficient ways to drive the synthesis of ATP, the universal energy currency in living organisms. The complete oxidation of one molecule of glucose to CO_2 and water under oxidative conditions can produce 38 molecules of ATP in the following reactions:

Biochemical processes	ATP yield per glucose
Glycolysis: glucose to pyruvate	
Activation using ATP energy:	
Hexokinase, glucokinase reaction	−1
Phosphofructokinase reaction	−1
Substrate level phosphorylation:	
Phosphoglycerate kinase reaction	+2

(continued)

(continued)

Biochemical processes	ATP yield per glucose
Conversion of pyruvate to acetyl-CoA (in mitochondria):	
Substrate level phosphorylation:	
Pyruvate kinase reaction	+2
Formation of 2 NAD in glyceraldehyde 3-phosphate dehydrogenase reaction	
Citrate cycle:	
Substrate level phosphorylation:	
SuccinylCoA → Succinate (GTP production)	+2
Terminal (mitochondrial) oxidation and coupled oxidative phosphorylation	
2 NADH (formed in the glycolysis, each yielding only 2 ATP due to the cost of the glycerol phosphate shuttle)	+4
2 NADH (generated in oxidative decarboxylation of pyruvate)	+6
6 NADH (generated in the citrate cycle: isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, malate dehydrogenase reactions)	+18
2 $FADH_2$ (generated in the citrate cycle: succinate → malate reaction)	+4
Net ATP yield (glycolysis + citrate cycle + terminal oxidation)	38 ATP

In earlier calculations each NADH was estimated to yield 3 ATP, and $FADH_2$ worth 2 ATP. Other calculations estimate that each $FADH_2$ generates 1.5 ATP and NADH is yielding 2.5 ATP when transported through the mitochondrial membrane by the malate-aspartate shuttle. NADH is yielding only 1.5 ATP when transported by the glycerol phosphate shuttle (Hinkle et al. 1991). As there is no complete agreement on this matter and the P/O ratios obtained with isolated rat liver mitochondria consistently exceeded 2.5 with NAD-linked substrates and 1.5 with succinate (Lee et al. 1996) we have used earlier calculations.

When pyruvate is converted to lactic acid or ethanol under reductive conditions in the process known as fermentation, the reduced $NADH + H^+$ is oxidized back to NAD. Due to the lack of oxidative pathways in the absence of molecular oxygen (O_2), the citrate cycle and terminal oxidation are nonfunctional and the net ATP gain of glycolysis will be only 2 ATP *versus* the 38 ATP obtained after the terminal oxidation in mitochondria. These data clearly show the advantage of oxidative energy production and the importance of mitochondria as the major energy power stations of cells. The energy obtained in terminal oxidation can be expressed both in calories and in electrovolts (Fig. 1.7).

Most energy is gained from highly reduced fatty acids that are degraded to C2 carbon units (acetyl-CoA) by β-oxidation in a spiral pathway. As an example the schematic view of β-oxidation of stearinic acid is shown in Fig. 1.8a. In each spiral cycle acetyl-CoA, reduced $NADH + H^+$ and reduced $FADH_2$ are obtained. The calculation of ATP energy during the complete oxidation of stearinic acid is given in Fig. 1.8b.

Amino acids and carbohydrates are already oxidized to some extent, consequently they contain less (∼ 50 %) energy than fatty acids. Krebs' citric-acid cycle is the oven where the C2 carbon billets of active acetate (acetyl-CoA) are burned

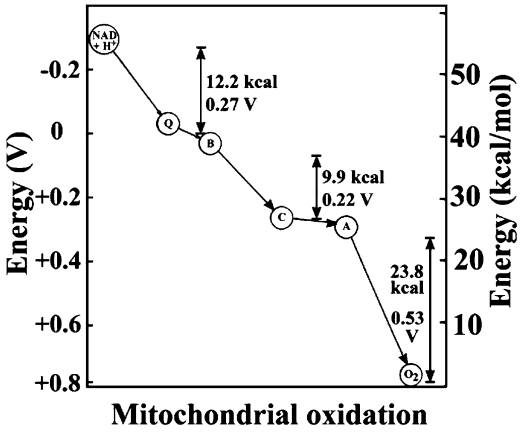

Fig. 1.7 Mitochondrial production of Gibbs' free energy. Free energy is expressed in volts and in kcal/mol. For the conversion of these energies the equation $\Delta G = -nF \times \Delta E$ can be used. In the equation n represents the number of charges, F is the Faraday constant (96,500 C), and is E the potential difference in volts (V). 0.1 V corresponds to 4.5 kcal/mol. Noteworthy to mention that there are only three steps in this cascade where the released energy exceeds the 7.3 kcal/mol threshold sufficient to produce ATP. In the last step where water is formed in the cytochrome A reaction catalyzed by cytochrome oxidase the energy gain would be enough to produce three ATPs, but in one step only one ATP can be obtained. Abbreviations: *Q* coenzyme Q, *B* cytochrome B, *C* cytochrome C, *A* cytochrome oxidase

a

$C_{16} \rightarrow$ Acetyl-CoA
$C_{14} \rightarrow$ Acetyl-CoA
$C_{12} \rightarrow$ Acetyl-CoA
$C_{10} \rightarrow$ Acetyl-CoA
$C_8 \rightarrow$ Acetyl-CoA
$C_6 \rightarrow$ Acetyl-CoA
$C_4 \rightarrow$ Acetyl-CoA
C_2 Acetyl- CoA

b

$$C_{17}H_{35}COOH + 9\ CoA + 8\ FAD + 8\ NAD^+ + 8\ H_2O =$$

$$= 9\ AcetylCoA + 8\ FADH_2 + 8\ NADH + 8\ H^+$$

ATP production:

9 AcetylCoA	9 x 12 =	108 ATP
8 FADH	8 x 2 =	16 ATP
8 NADH + H^+	8 x 3 =	24 ATP
		148 ATP

Fig. 1.8 Spiral pathway and energy production during the β-oxidation of stearic acid. (**a**) AcetylCoA is released in each cycle. (**b**) The net energy production of 148 ATPs comes from the complete oxidation of 9 acetylCoAs and the terminal oxidation of coenzymes (8 NADH + H^+ and 8 $FADH_2$)

to carbon dioxide. While several aspects of the citrate cycle have been reviewed (Kay and Weitzman 1987) less attention has been paid to the origin of the increased reducing power (3 NADH + H^+, $FADH_2$) funneled into the respiratory chain from the citric acid cycle. The production of reducing equivalents, 8 [H] in each round of the cycle gives the impression that energy is derived exclusively from energy rich electrons. This concept is misleading in most biochemistry textbooks while the other factor of respiration, namely the oxidation of carbon atoms of acetate has been

Table 1.2 Values of $\Delta G^{o'}$ for some important chemical reactions

Reaction		$\Delta G^{o'}$ (kcal/mol)
Hydrolysis of anhydrides:		
Acetic anhydride:	$CH_3\text{-}\overset{O}{\overset{\|\|}{C}}\text{-O-}\overset{O}{\overset{\|\|}{C}}\text{-}CH_3 + H_2O \rightarrow 2CH_3COOH$	−21.8
Pyrophosphate	$Ppi + H_2O \rightarrow 2Pi$	−8.0
ATP	$ATP + H_2O \rightarrow ADP + Pi$	−7.3
Oxidation:		
Stearic acid	$C_{17}H_{35}COOH + 27O_2 \rightarrow 18CO_2 + 18H_2O$	−2,628[a]
Stearic acid (estimated combustion energy)		−2,655[b]
Glucose	$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$	−686
Photosynthesis:		
Glucose synthesis:	$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6$	+686
Esterification (phosphorylation):		
	Glucose + Pi → Glucose 6-phosphate + H_2O	−1.7
Ester hydrolysis (dephosphorylation)		
	Glucose 6-phosphate + H_2O → Glucose + Pi	+1.7
Substrate level phosphorylation using high energy bonds (>7.3 kcal/mol)		
	Phosphoenolpyruvate + ADP → Pyruvate + ATP	−14.8
	1,3-phosphoglycerate + ADP → 3-phosphoglycerate + ATP	−11.8
	Creatine phosphate + ADP → Creatine + ATP	−10.3

Source: Lehninger (1975)
Pi phosphate
[a]Oxidation energy of stearic acid from Lehninger (1975)
[b]Combustion energy estimated value (Banfalvi 1999)

neglected. Based on energetic considerations, it is pointed out, that the metabolic energy of oxidation of carbon to carbon dioxide is converted to the reducing power of [H] and conserved in the citrate cycle summarized as:

$$CH_3COO^- + H^+ + 2H_2O \rightarrow 2CO_2 + 8\,[H] \qquad \Delta G^{o'} - 61\ \text{kJ/mol}$$

$\Delta G^{o'}$ of the reaction is the free energy change (ΔG) under standard conditions.

Standard conditions: [products] = 1 M and [reactants] = 1 M (M = moles/l)

$$\Delta G = \Delta G^0 + RT\ \ln [\text{products}]\,/[\text{reactants}]\,,$$

where: R = universal gas constant, T = temperature in ^{0}K, when T = 25 °C, RT = 0.59 kcal/mol.

Negative values refer to exergonic (spontaneous or energy producing), positive signs indicate endergonic (nonspontaneous, energy consuming) reactions. Values of $\Delta G^{o'}$ for some important chemical reactions are given in Table 1.2. The energy values of calories can be easily converted to joules, by multiplying them with 4.18 and *vice versa* dividing joules by 4.18 to obtain calories.

The net chemical energy gain of the citrate cycle is only one GTP produced when succinyl-CoA releases the high energy of its thioester bond and succinate is formed. This energy represents only 7 % of the total combustion energy of acetate. Two hydrations take place in the citrate cycle: one in the succinyl-CoA → succinate and the other one in the fumarate → malate reaction. The idea of saving metabolic energy in the form of reductive power led to the conclusion that in the citrate cycle the two major energetic factors of respiration are coupled. The energy of oxidation of carbon atoms of fuel acetate to CO_2 is converted to the second one, i.e. to reducing equivalents (Banfalvi 1991b). Similar oxidative – reductive energy coupling *via* hydration is taking place in the oxidation of fatty acids leading to an increase and conservation of reductive power (Banfalvi 1992a). Hydrolytic oxidations are also involved in the metabolism of amino acids (Banfalvi 1992b).

A general conclusion can be drawn from these reactions: the hydrolytic oxidation of the carbon skeleton of fuel molecules in the citrate cycle, β-oxidation and α-keto-acids is a recurring motif seen throughout the extraction of oxidative energy derived from fats, polysaccharides and proteins, testifying the fundamental importance of hydrolytic oxidation in all forms of animal life. The importance of hydrolytic oxidations is shown by the fact that hydration is followed by or coupled to dehydrogenation indicated by the presence of coenzymes carrying reducing equivalents. The reductive way of life is maintained by each cell, in fact the metabolism of basic food molecules (carbohydrates, lipids, proteins) starts with anaerobic reactions including hydrolytic oxidations. With respect to evolution hydrolytic oxidations belong to the oldest reactions, anaerobic decarboxylation *via* hydrolytic oxidations contributed to the appearance of carbon dioxide in the primordial ocean and atmosphere serving as a basic compound for the photosynthesis that in turn led to the production of molecular oxygen. The presence of oxygen meant an important advantage in energy and material economy, but also hided hazardous disadvantages related to the toxic effects of oxygen (radiation damage, inflammation, autoimmune diseases, reactive oxygen species, poisons, carcinogens, ischemia, aging, etc.) during the evolution of terrestrial animals.

We have sufficient data to calculate the efficiency of the 38 ATPs produced during the complete oxidation of glucose in glycolysis, citrate cycle and terminal oxidation:

Combustion energy of glucose:	−686 kcal/mol	(100 %)
Energy of 38 ATPs (38 × 7.3 kcal):	−277.4 kcal	(40.3 %)

The efficiency of complete biological oxidation of stearic acid can also be calculated from its combustion energy, ATP production during β-oxidation and from the energy of acetyl-CoA molecules:

Estimated combustion energy of stearic acid:	−2,655 kcal/mol	(100 %)
Energy of 148 ATPs (148 × 7.3):	−1, 080.4 kcal	(40.7 %)

These calculations show that oxidation energy of organic food molecules is supporting the biological energy (ATP) need of the organism quite effectively. The efficiency (∼40 %) of carbohydrate (glucose) and lipid (stearic acid) utilization is significantly higher than those of mechanical engines planned and produced by men. The biological energy saving significantly contributes to homeostasis. In mammals that maintain constant body temperature ∼75 % of the energy intake is released as heat and only 25 % is utilized for the physical activity and basal metabolism (transport, biosynthesis).

Eating highly nutritious food is increasing the risk of the toxic effects of oxygen. Food consumed in smaller quantity is extending the healthy years of life. Calorie restriction to a level 20–40 % less than the *ad libitum* diet intake delays the aging process, the reproductive senescence, immunologic decline, reduces the frequency of neoplastic diseases, extends the period of life span and substantially reduces the risk of virtually all the diseases (Weindruch and Walford 1988).

1.7 Contribution of Biological Membranes to Homeostasis

1.7.1 Permeability

Living animal cells similarly to bacteria, fungi, insects, fish and birds consist of individuals that maintained their flocking behavior. Flocking is the collective motion of a large number of self-propelled entities and a collective behavior arising from simple rules followed by individuals without the involvement of central coordination. Living eukaryotic cells came into being by separating them from their external environment by plasma membranes. Intracellular membranes of eukaryotic cells isolate compartments, among them the nucleus, endoplasmic reticulum, Golgi complex, lysosomes, mitochondria, and chloroplasts. It was recognized that the cellular membrane of each eukaryotic cell is composed of a lipid bilayer (Gorter and Grendel 1925; Hendler 1971), where the hydrophobic non-polar fatty acid tails are sandwiched between the outer and inner hydrophilic polar heads of phospholipids. The fluid mosaic model proposed the dynamic properties of membranes, where both the lipid bilayers and the embedded proteins are arranged in a tightly locked, water excluding mosaic (Singer and Nicolson 1972). The lipid composition of the plasma membranes of mammalian cells consists of amphypatic phospholipids (phosphatidate, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol), sphingomyelin, glycolipids (cerebrosides, gangliosides), glycoproteins and cholesterol. Carbohydrate containing glycoproteins are particularly abundant in membranes of eukaryotic cells, but absent in prokaryotic cell membranes. Transport processes of the cellular membrane belong to the most important cellular regulations. The two basic types of transport processes are the energy requiring active transport and passive transport processes along ionic gradients.

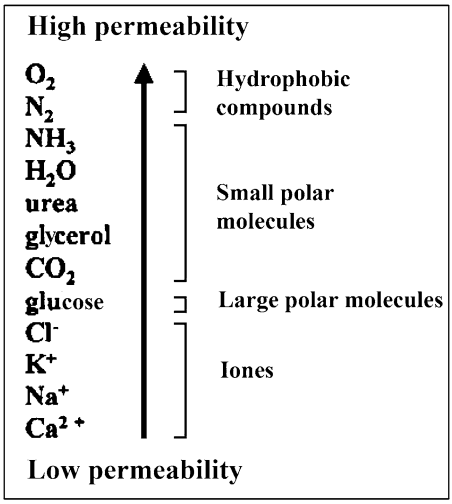

Fig. 1.9 Relative permeability of plasma membrane

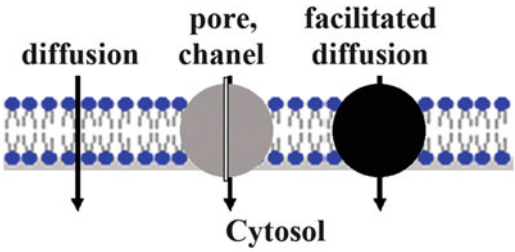

Fig. 1.10 Passive transport by diffusion, transporters and facilitated diffusion

Major functions of biological membranes are: (a) to separate cells from the external environment, (b) to form organelles inside the cell, (c) to be selectively permeable to different substances, (d) to provide biological connections and (e) to conserve energy. The selective permeability of the lipid bilayer (Fig. 1.9) shows how the relative permeability of the lipid bilayer decreases (from top to bottom):

1.7.2 Membrane Transport Processes

The movement across the plasma membrane of the cell may take place without energy consumption. Passive transport takes place by simple diffusion, transporters or facilitated diffusion driven by the concentration gradient (Fig. 1.10).

1.7.2.1 Simple Diffusion

When soluble sugar is dissolved in tea, initially the particles are sedimented and are all near to each other at the bottom of the teacup. The sugar molecules will move around randomly in the liquid and become distributed uniformly. The process where dissolved particles become randomly distributed is called diffusion. The concept of

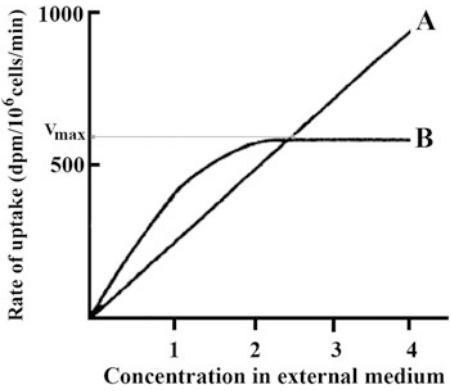

Fig. 1.11 Kinetics of simple and facilitated diffusions. Simple diffusion is dependent exclusively on the concentration of the diffusible substance taken up by the cells (*A*). Facilitated diffusion is also driven by the concentration gradient but reaches maximal value (V_{max}) determined by the number of transporter protein molecules (*B*). Disintegration per minute (*dpm*) refers to the radioactive substance used in the measurement

diffusion is used in physics, chemistry, biology, sociology, economics, finance, etc., but here only the chemical meaning will be considered.

Simple diffusion is the chemical process whereby a chemical substance passes through a membrane without the aid of an intermediary integral membrane protein. The substance has to penetrate the hydrophobic core of the phospholipid bilayer of the membrane. The diffusion force that drives the substance from one side of the membrane to the other one is dependent exclusively on the concentration of the substance. Natural diffusible compounds that cross freely the biological membranes are oxygen, water, carbon dioxide, nitric oxide.

1.7.2.2 Facilitated Diffusion

During facilitated diffusion the movement of molecules through the cell membrane is also concentration-dependent but aided by transport proteins embedded in the cell membrane. The difference in the kinetics between the simple diffusion of substance A and the facilitated diffusion of substance B is shown in Fig. 1.11.

The saturation curve of facilitated diffusion resembles the kinetics of enzymatic catalysis. Although, the saturation curve of facilitated diffusion can be characterized by V_{max} but has no saturation constant that would belong to $V_{max/2}$ and would represent that concentration of the diffusible molecule at which the speed of the diffusion would be half of the maximum. The uptake of substrate transported into the cell by facilitated diffusion and measured as a function of the external concentration, does not usually show the rectangular hyperbolic function characteristic to the Michaelis-Menten enzyme kinetics. Rather, a seemingly biphasic curve is observed, consisting of Michaelis-Menten kinetics at low concentrations and no further uptake at higher concentrations (ter Kuile and Cook 1994).

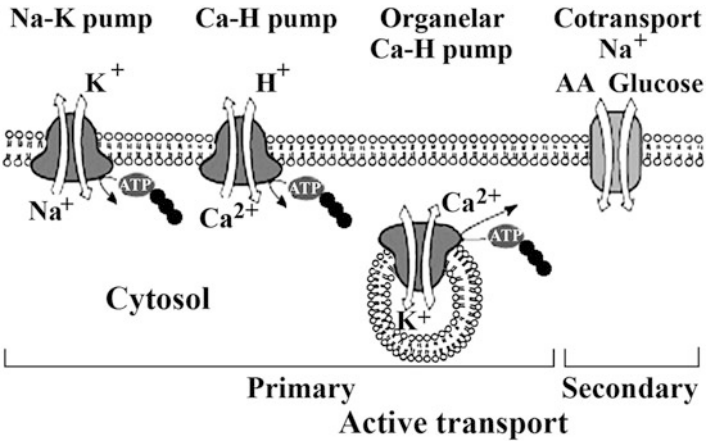

Fig. 1.12 Active transport processes across the plasma membrane. The energy for the primary active transport is provided by ATP and for the secondary active transport by the accumulated Na^+ ion concentration generated by the primary active transport of the Na^+-K^+ pump

1.7.2.3 Active Transport

1.7.2.3.1 Primary Active Transport

The primary active antiport of Na^+ and K^+ catalyzed by sodium-potassium ATPase takes place in opposite directions. The energy-powered pump utilizes the phosphorylation energy of ATP where the phosphorylated enzyme induces conformational changes allowing to pump out 3 Na^+ ions. Dephosphorylation in turn drives 2 K^+ ions inside the cell against the concentration gradient. The sodium-potassium pump is practically present in each cell and is thus of universal importance maintaining the basic ionic balance of cells. Cardiac glycosides (e.g. quabain, digoxin) that inhibit Na^+-K^+ ATPase are useful in cardiac heart disease by lowering the extracellular Na^+ concentration. This results in less effective Na^+ coupled Ca^{2+} release from the myocytes and in elevated intracellular Ca^{2+} concentration that is beneficial in the treatment of congestive heart failure and cardiac arrhythmia as it causes more frequent and greater contraction of the cardiac muscle.

The basic resting potential is maintained by the Na^+-K^+ pump. Active transport processes across the plasma membrane are summarized in Fig. 1.12. Sudden change in the resting potential (1–200 msec) may generate an action potential characteristic to nerve (1–2 msec), muscle (20 msec) and heart (200 msec) cells. Besides being a homeostatic, "housekeeping" ionic pump, Na^+-K^+ ATPase is also regarded as a computation element in the brain evidenced by its mutation causing the rapid onset of dystonic parkinsonism indicating the loss of cerebellar function (Cannon 2004).

1.7.2.3.2 Secondary Active Transport

The secondary active transport is based on the accumulation of sodium ions outside cells generated by the primary active transport. Examples of Na^+-coupled transporters are Na^+-K^+-Cl^-, Na^+-amino acid, Na^+-glucose, Na^+-fatty acid cotransporters, Na^+-H^+, Na^+-Ca^{2+} antiporters, Na^+-PO_4^{3-}, Na^+-HCO_3^-, Na^+-SO_4^{2-}, Na^+-cholate, Na^+-folate, Na^+-ascorbate and Na^+-choline symporters. Sodium-coupled secondary active symporters are responsible for the intestinal absorption of amino acids and glucose.

1.7.3 Ionophores

Ionophoric proteins can be categorized as ion channels and ion transporters. Ion channels are pore forming membrane proteins that: (a) help to establish resting membrane potential, (b) control the flow of ions across the cell membrane of secretory and epithelial cells, (c) control the action potential *via* their gating function and regulate cell volume.

1.7.3.1 Ion Channels

Voltage gated ion channels open and close in response to membrane potential (voltage-gated sodium, calcium, potassium channels, transient receptor potential channels, cAMP-, cGMP-gated channels and voltage gated proton channels).

Ligand-gated ion channels (ionotropic receptors) open channels in response to specific ligand molecules (nicotinic or acetylcholine receptor, glutamate-gated receptors, ATP-gated P2X receptors, $GABA_A$ receptor). Neurotransmitter receptors can be classified into two broad categories: ionotropic and metabotropic receptors. Ionotropic receptors form an ion channel pore. Metabotropic receptors are indirectly linked to signal transduction often *via* G proteins.

Other gatings may be activated from the inside or outside of the membrane by second messengers, work as inward-rectifier potassium channels, calcium-activated potassium channels, two-pore-domain potassium channels and as adhesion molecules may also have role in tumor suppression.

There are several diseases of ion channels that are caused by chemicals (tetrodotoxin, saxitoxin, conotoxin, lidocain, endrotoxin, etc.) and genetic mutations (Shaker gene, hyperkalemic periodic paralysis, epilepsy, ataxia, migraine, long QT syndrome, cystic fibrosis, mucolipidosis) with serious consequences for the individuals affected. These diseases are related to the ionic imbalance, but normally not related to cancer. An exception could be cystic fibrosis, where the overall risk of cancer in patients was similar to that of the general population, but an increased risk of digestive tract cancer was reported (Neglia et al. 1995).

Ion channels can be classified simply by the type of ions: chloride, potassium, sodium, calcium, proton, non-selective cation channels. These and further classifications would lead to unnecessary repetitions of channels already mentioned.

Some microorganisms also produce mobile ion carriers or channel forming ionophores. These lipid soluble ionophores increase the permeability of the bacterial membrane to certain ions. Certain ionophores have been used as antibiotics e.g. valinomycin as mobile ion carrier and gramicidin A as channel former.

1.7.3.2 Ion Transporters

Ion transporters, also called ion pumps, are transmembrane proteins that move ions across the plasma membrane against their concentration gradient, in contrast to ion channels, where ions move *via* passive transport. Ion transporters, among them the sodium-potassium pump, the sodium-calcium exchanger, sodium-glucose and other transporters have already been mentioned among the active transporters.

1.7.4 Water Spaces

Water representing the bulk of the organisms is present in an average of 55–65 % within the cells. Two third of the water is in the intracellular fluid with high potassium content and one third as extracellular fluid with high sodium content. Extracellular spaces have been mentioned as: interstitial space (18 %) between the cells, intravasal liquid (4–5 %) in the vessels, and transcellular (2–5 %) spaces. The intravascular fluid in animals is known as blood sharing 7–8 % of total body water and 25 % of the extracellular fluid. The most important difference between these two compartments is higher concentration of protein and positively charged ion in the plasma than in the interstitial fluid. The osmotic pressure is similar in the extracellular and intracellular compartments.

From evolutionary viewpoint animals belonging to coelomates have developed coelomes (body cavities) filled with a uniform extracellular fluid known as coelom liquid. The coelom, a fluid-filled body cavity that allows inner organs to shift, is lined by an epithelium derived from the mesoderm. Bilateral animals, including vertebrates, are coelomates. Organisms, with open circulation among them arthropods were the first to circulate hemolymph in their extracellular space. Fish with closed circulation started to circulate blood in their vessels. Humans have body spaces consisting of dorsal and ventral cavities. The ventral cavity that has two parts, the thoracal and abdominal cavities being by far the largest ones in volume. The abdominal cavity can be subdivided into abdominal and pelvic cavities. The

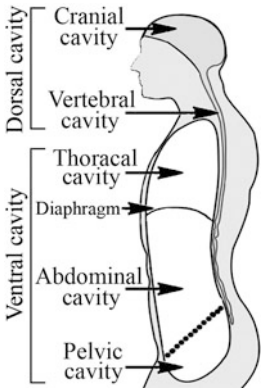

Fig. 1.13 Diagram of body cavities. Cranial cavity: brain, vertebral canal: spinal cord, thoracic cavity: lungs and heart, abdominal cavity: digestive organs (stomach, liver, pancreas, guts), spleen, kidneys, pelvic cavity: bladder, reproductive organs

much smaller dorsal cavity contains the cranial and the vertebrate (spinal) cavities (Fig. 1.13). We will return to the importance of body cavities when tumor spread will be discussed in Chap. 5.

1.8 Function of the Inner Environment and Its Regulated Parameters

Regulated parameters of the inner environment include:

- Isosmosis
- Isoionia
- Stability of pH by buffers
- Isovolemia
- Body temperature
- Food content

1.8.1 Osmotic Homeostasis

Despite of local variations the amount of body water remains stable. The kidney is representing the first line of defense to maintain osmotic homeostasis in mammals. Body water is controlled by several hormones with the arginine vasopressin (AVP) antidiuretic hormone being the key regulator (Fig. 1.14). AVP synthesized in the hypothalamus is transported to and stored in the posterior pituitary until the increased osmolarity of body fluids initiates its secretion into the blood.

Arginine vasopressin (AVP) binding to the V2 receptors in the kidney:

1. increases water permeability by inserting aquaporin channels into the membranes of the distal convoluted tubules of the kidney,

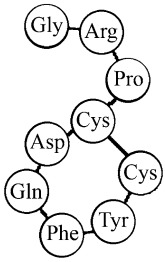

Fig. 1.14 Antidiuretic hormone. Vasopressin is a cyclic peptide consisting of nine amino acids. The three letter abbreviations of these amino acids are: gly = glycine, arg = arginine, pro = proline, cys = cysteine, tyr = tyrosine, phe = phenylalanine, gln = glutamine, asp = aspartic acid

2. water moves through these channels and decreases osmolarity,
3. increased water reabsorption by the kidney and decrease in urine flow also contribute to the osmotic homeostasis.

Loss of blood volume (hypovolemia) leading to reduced perfusion pressure and distal tubular Na^+ delivery, as well as elevated prostaglandin level can be the consequence of increased sympathetic (muscular) activity, sensed by baroreceptors causing the kidney to secret renin:

– Renin interacts with angiotensinogen generated by the liver and promotes the production of angiotensin I.
– Angiotensin I is converted to angiotensin II catalyzed by the angiotensin converting enzyme produced by the lung.
– Angiotensin II acts as strong vasoconstrictor and initiates aldosterone secretion in the adrenal cortex.
– Aldosterone increases sodium and water content and decreases its potassium and hydrogen ion content of the body.

Loss of blood volume (hypovolemia) is compensated by thirst and salt consumption. The renin-angiotensin system and AVP initiates antidiuresis and vasoconstriction. Both plasma hyposmolarity (hypovolemia or hypotension) and hyperosmolarity (hypervolemia or hypertension) interact to control AVP levels to maintain osmotic homeostasis. The most common disease related to antidiuretic hormone resulting in excessive urine production (*diabetes insipidus*) is caused either by the deficiency in secretion of AVP (*hypothalamic diabetes insipidus*) or by the inability of kidney to respond to AVP (*nephrogenic diabetes insipidus*).

1.8.1.1 Isotonic Blood Concentrations of Land Vertebrates

The concentrations of most important electrolytes in blood plasmas are summarized in Table 1.3.

Table 1.3 Isoionic concentrations represented by the blood plasma electrolytes of land vertebrates

Vertebrates	HCO_3^-	Ca^{2+}	Cl^-	Mg^{2+}	PO_4^{3-}	SO_4^{2-}	K^+	Na^+	mOsm (total)
Mammals	27	5	107	5	3	—	4	144	295
Reptiles	15	3	114	2	1	—	6	145	286
Snakes	12	3	125	1	2	—	4	153	300
Lizards	19	3	121	1	2	—	4	157	307
Turtles	39	4	92	2	2	—	3	145	287
Birds	25	6	111	1	1	—	5	168	317
Amphibians	14–21	1–2	70–104	1–2	2–4	—	3–9	86–129	160–240
Components of seawater[a]	2	10	535	54	—	27	10	456	1,094

Values of plasma electrolyte are from the Biology Data Book (1972–1974) edited by Altman and Dittmer. Minor constituents of electrolytes have been neglected, since their total contribution to salinity is less than 10 mOsm

[a]Based on total salinity of 34.325 g/kg, or standard salinity of 19 g/kg at 20 °C and atmospheric pressure. With permission of Banfalvi (1991a)

The contribution of organic ions to osmolarity in land vertebrates is less than 10 %. Probably much more important, especially in amphibians, are non-ionic substances like urea, which is known to make significant contributions to plasma osmolarity. *Rana carnivora* has up to 0.48 M urea in its blood (Gordon et al. 1961). These organic substances may account for the anomalous electrolyte concentrations of amphibians. In spite of evolutionary divergence and different ways of osmotic regulation, terrestrial vertebrates have nearly identical ion concentrations in their blood. Exceptions are amphibians which have lower and variable ion concentrations and to some extent birds with somewhat elevated electrolyte concentrations (Table 1.3). The strikingly similar values undoubtedly indicate the biological importance of ion balance and the evolutionary stability of the inner environment of land vertebrates. The uniform osmolarity raises the question whether or not evolution selected against terrestrial vertebrates that changed their vascular ion concentration after leaving the sea. The variable ion levels in fish and amphibians may be a reflection of such a selective pressure for the early forms of tetrapods. In view of the uniform osmolarity it is reasonable to assume that osmotic concentration of vertebrate blood and body fluids (inner environment) is a relic of ancient stage, namely the osmotic concentration of the ocean (external environment) at the time of vertebrate migration to land some 400 million years ago. This interpretation of osmolarity is consistent with current thinking of paleontologists that terrestrial vertebrates arose from fish in marine waters rather than slightly saline inland seas or lakes (Pough 1979).

1.8.2 Stabilization of pH by Buffers

The regulatory mechanism of buffers prevents the pH changes of body fluids. Buffers act against acidic and alkaline effects and keep the pH of body fluids constant. The biologically important properties of water and buffers are traditionally

Table 1.4 Dissociation constants of weak acids of physiological buffers

Name	Dissociation to ions	Dissociation constant	pK
Carbonic acid	$H_2CO_3 <==> H^+ + HCO_3^-$	$4.30 \cdot 10^{-7}$	6.37
	$HCO_3 <==> H^+ + CO_3^{2-}$	$5.61 \cdot 10^{-11}$	10.25
Phosphoric acid	$H_3PO_4 <==> H^+ + H_2PO_4–$	$7.25 \cdot 10^{-3}$	2.14
	$H_2PO_4^- <==> H^+ + HPO_4^{2-}$	$6.31 \cdot 10^{-8}$	7.2
	$HPO_4^{2-} <==> H^+ + PO_4^{3-}$	$3.98 \cdot 10^{-11}$	12.40
L-histidine (amino acid)	Side chain imidazole ring	10^{-6}	6.0

Table 1.5 Preparation of phosphate buffer

Preparation of 1 M pH 7.4 buffer based on the Henderson-Hasselbalch formula:

$$\boxed{pH = pK + \log \frac{[salt]}{[acid]}}$$

$$7.4 = 7.2 + \log \frac{[Na_2HPO_4]}{[NaH_2PO_4]}$$

$7.4 = 7.2 + \log x$ $\log x = 7.4 - 7.2 = 0.2$ num $\log x = 0.2$ $x = 1.75$

$$0.2 = \log \frac{[Na_2HPO_4]}{[NaH_2PO_4]} = \frac{1.75 \text{ 1 M } Na_2HPO_4 \rightarrow \mathbf{35\ ml}}{1 \text{ 1 M } NaH_2PO_4 \rightarrow \mathbf{20\ ml}}$$

Dissolve in 50 ml distilled water:

4.96 g Na_2HPO_4 (Mw: 141.98×0.035) and 2.40 g NaH_2PO_4 (Mw: 119.98×0.20)

Add distilled water to get 55 ml 1 M pH 7.4 phosphate buffer.

This solution can also serve to calibrate your pH meter.

Note: avoid making large volumes of buffers, that will not be used.

incorporated in biological, primarily in biochemistry textbooks. The pH of buffer is calculated by using the dissociation constants of the weak acid or base and applying the Henderson-Hasselbalch formula. The dissociation and dissociation constants of acids of physiological importance are given in Table 1.4. To determine the pH and to prepare a buffer the Henderson-Hasselbalch formula applies the negative logarithm of the dissociation constant (pK) of the weak acid (Table 1.5).

In conformity with the isoionic requirements of the inner environment, the pH value of 7.4 (± 1) of intravasal and transcellular spaces (blood, cerebrospinal fluid) of animals are kept constant and under strict control. The pH values of other liquids may vary within a relatively broad spectrum as exemplified by human biological samples in Table 1.6.

1.8.3 Isovolemia

Chemical reactions in the laboratory can be pushed against their equilibrium by applying extreme conditions (temperature, pressure, catalysts). Chemical reactions in cells take place under constant pH, isoionic, isotonic, isothermal and isovolumic conditions. As already pointed out the physiological range allows some oscillation and deviation from set points. Due to their water permeability cells behave as

Table 1.6 pH values of human biological samples

Sample	pH
Bile	6.2–8.5
Blood plasma	7.4 (7.3–7.5)
Cell (average)	7.0
Cerebrospinal fluid	7.3–7.5
Colon mucus	7.9–8.0
Duodenal content	4.8–8.2
Liver cell	6.9
Milk	7.1 (6.6–7.6)
Pancreatic fluid	8.0
Saliva	6.8 (6.5–7.5)
Stomach	1.4 (1.0–3.0)
Tear	7.2
Urine	6.0 (4.8–8.4)

Values in alphabetical order have been taken from Handbook of Chemistry and Physics (Weast and Astle 1982–1983)

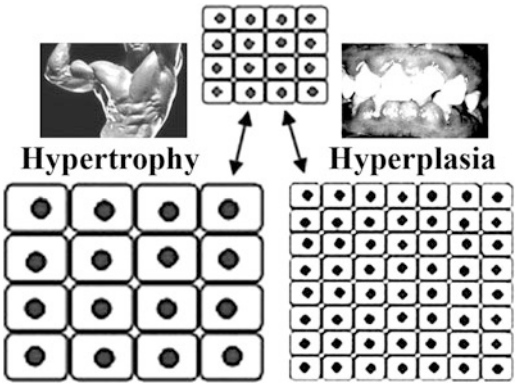

Fig. 1.15 Hypertrophy and hyperplasia. Hypertrophy with increased cell volume is demonstrated by body building (*upper left photo*). Hyperplasia with increased cells number is seen as gingiva cell proliferation (*upper right photo*)

osmometers. The regulation of cell volume within physiological values takes place through the uptake or removal of osmotically active particles. Hypertrophic cells increase their volume and capacity to cope with functional requirements. This does not simply mean the expansion of cells, but also the active synthesis of cellular components. Hypertrophy i.e. the volumetric expansion of cells is not to be confused with hyperplasia, the elevated cell number upon increased functional requirements. The reversible processes of hypertrophy and hyperplasia are demonstrated in Fig. 1.15. Both hypertrophy and hyperplasia may have physiological and pathological deviations.

Examples of physiological hypertrophy are:

– increased functional requirements: sport, body building (up to a certain limit)
– hormonal effects (estrogen, prolactin): hypertrophy of uterus during pregnancy and breast during lactation

Pathological hypertrophies of heart will be detailed under “Hypertrophied heart” in Chap. 3.

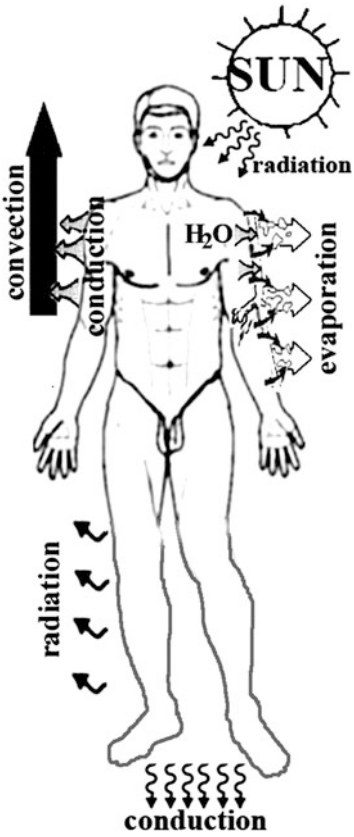

Fig. 1.16 External heat transfer mechanisms: radiation, convection, conduction, evapotranspiration. The typical skin temperature is 34 °C compared to the body core temperature of 37 °C

1.8.4 Body Temperature

The heat transfer normally takes place from a higher temperature object to a lower temperature one and changes the internal energy of both systems in accordance with the first law of thermodynamics. Beside body cooling there are other well known heat transfer examples such as, home heating, heat pump, heat engines, refrigerator, greenhouse effect, global energy, etc. With respect to body temperature animals can be divided into two major groups: endothermic and exothermic ones. Birds and mammals among them humans are warm-blooded (endothermic). All other animals are cold-blooded (exothermic).

Mechanisms of heat transfer through the skin such as radiation, conduction, convection and evaporation are shown in Fig. 1.16. Radiation is the emission of electromagnetic waves that carry energy away from the emitting body. For ordinary body temperatures the radiation is in the infrared region of the electromagnetic spectrum. Radiation is the most important heat transfer mechanism at temperatures near the ordinary room temperature.

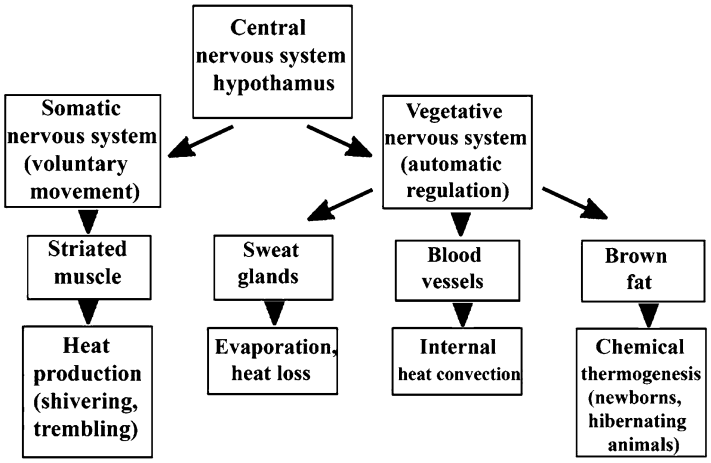

Fig. 1.17 Heat regulation by the nervous system

Conduction is the heat transfer that takes place by means of molecular agitation within the body without any motion of the body itself.

Convection is the heat transfer by mass motion of a fluid of air or water when the warm fluid is caused to move away from the body surface carrying heat energy with it. Convection occurs because the warm vapor on the surface is less dense than cold water and rises, causing convection currents.

Evapotranspiration takes place when part of the sweat evaporates and cools the liquid remaining on the surface, as it extracts the heat from the liquid and makes phase change to gaseous state. This is the most effective cooling mechanism of the human body, to give off heat energy when the ambient temperature is higher than the body temperature in order to approach thermal equilibrium. Sweating begins at a skin temperature of 37 °C and increases as the skin temperature rises, while the heat production under these conditions remains almost unchanged. Under 37 °C skin temperature various regulatory response reactions take place (see: Regulation of body temperature). Core temperature is the temperature of an organism at which it is meant to operate. It tends to refer to the temperature of organs and parts of the body that are well insulated, as opposed to the skin and other surface areas, with fluctuating temperatures.

1.8.4.1 Heat Regulation

As noted the heat production and heat loss are higher in warm blooded mammals than in cold-blooded animals. The heat regulation by the nervous system is summarized in Fig. 1.17.

The somatic nervous system is responsible for the voluntary movement of the skeletal muscle by producing heat through shivering and trembling. The involuntary

(automatic, vegetative) nervous system is accounted for by the heat regulation through the sweat glands, blood vessels (vasoconstriction, vasodilation) and brown fat. The primary function of brown fat is to generate body heat rather than ATP in hibernating animals or newborns, through an uncoupling protein (thermogenin) in the inner mitochondrial membrane.

Heat regulation is effective only within certain temperature limits. Outside these limits other capabilities (e.g. hair, fur, coat, plumage) and acquired reactions support thermoregulation. Beside the autonomic thermoregulation additional exothermic activity of fur-bearing and hairy domestic animals (horse, cattle, sheep, goat) takes place primarily by perspiration, while other animals (dog, cat, rabbit, fowls) get rid of the extra heat by panting. Sea mammals are protected from cold by fat layers the thickness of which can be up to 50 cm in whales. An additional heat regulation is the countercurrent heat exchange occurring in the circulatory system of fish, marine mammals and in the legs of birds. Arteries of this system that carry warm blood to the skin are intertwined with veins carrying cold venous blood from the skin. Heat exchange is based on the same countercurrent principle as the salt and waste excretion in kidneys, or the O_2 and CO_2 exchange in the gills of invertebrates and fish.

1.8.5 Food Content

Macronutrients include general and specific resources: carbohydrates, proteins, fats and cholesterol, fiber, water. The most important macronutrient in the food is the carbon source that is normally provided by glucose. Nutritive sweeteners, also known as caloric sweeteners or sugars, provide energy in the form of carbohydrates. Non-nutritive sweeteners are zero- or low-calorie alternatives to nutritive sweeteners. Beside oils and other lipids, dairy products and eggs have also a high fat content.

The nitrogen source is primarily consumed as protein, e.g. egg, dairy products, meat, poultry, fish, vegetables, legumes (soybean, peanut), yeast. Animal meat naturally contains proteins, lipids, including various amounts of cholesterol. Cholesterol serves as a basis for steroid biosynthesis and can be overproduced from acetyl-CoA. When the excess acetyl-CoA is neither burned in the citrate cycle due to the lack of oxaloacetate nor converted efficiently to fatty acids and triglycerides, it produces ketone bodies (acetoacetate, hydroxymethylbutyrate and acetone). Acetoacetate reacts with another acetyl-CoA to give 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) catalyzed by the HMG-CoA synthase (committed step of cholesterol biosynthesis) and further processed to cholesterol. Most of the cholesterol (∼75 %) is produced by the liver from acetyl-CoA and is carried by the bloodstream as low density lipoprotein (LDL) to other tissues and is known as “bad cholesterol” due to its high concentration, low solubility and risk to form plaques in the blood vessels (http://www.livestrong.com/article/364326-overproduction-of-cholesterol/#ixzz2HOjRd5UW). A smaller portion of cholesterol (∼25 %) is known as “good cholesterol” produced by other cells and carried to the liver as high

density lipoprotein (HDL). The formation of cholesterol deposits called xanthomas in areas of buttocks, knees, elbows and tendons should not be confused with tumor formation. Remarkably, the overproduction of cholesterol and fatty acids causes massive liver enlargement, the decrease of the white adipose tissue and upsets lipid homeostasis (Shimano et al. 1996).

Vitamins and minerals are taken mainly in the form of vegetables and fruits. The calcium content is provided by dairy products, soy, vegetables, fruits, legumes, grains, nuts and seeds, canned fish with bones. The daily intake of calcium depends on age and ranges from 0.5 g (1–3 year old) to 1.2 g (70 year and older).

1.9 Cellular, Tissue and Organ Homeostasis

The term homeostasis was originally used to describe the stability of the internal environment i.e. the extracellular fluid of an organism. However, the constancy of body cannot be maintained without the stability of the cells, tissues and organs. Consequently the conditions also have to be regulated in cells, tissues and organs and kept within narrow limits.

1.9.1 Cellular Homeostasis

Cellular homeostasis involves any process that takes place to maintain the internal steady state at the level of the cell. Intracellular homeostasis is maintained by chemical reactions during the convergent breakdown of larger food molecules to smaller ones, the provision of metabolic energy serving the needs of divergent pathways of biosynthesis, motion and transport processes. Metabolic processes are driven by enzymatic reactions, consequently these processes are regulated through their enzymes by:

- negative feedback control i.e. the end product of a metabolic pathway inhibits the catalytic activity of the committed step of the pathway,
- regulatory proteins interact and control the enzyme activity,
- covalent modification can turn on or off the enzyme activity (e.g. phosphorylation, dephosphorylation, zymogen activation by cutting off a portion of an inactive protein at its N-terminal end),
- ionic adaptation is performed mostly at the cellular-environmental interface of cellular osmolyte systems in water stressed organisms. The ionic adaptation is restricted to end products of nitrogen metabolism (polyols, amino acid derivatives, urea, methylamines),
- ionic exchange, including hydrogen ions affecting pH (e.g. K^+-Na^+ ATPase as an electrogenic pump, Ca^{2+}-H^+ pump, lower pH in lysosomes to favor hydrolytic reactions),

– membrane electric potential caused by the loss of resting potential maintained by the K^+-Na^+ ATPase,
– ion channels, transporters,
– cellular volume homeostasis.

Changes in cell function always occur in the context of the whole organism. Regulation requires the communication of a complex network among cells, different tissues and organs.

1.9.2 Tissue Homeostasis

Key players in tissue homeostasis are:

– ***Scar formation***: healing upon mechanical tissue damage takes place by scar formation to restore normal tissue and organ function. If the regeneration process is not limited it can lead to chronic diseases such as cirrhosis of the liver, renal interstitial disease, pulmonary fibrosis.
– ***Tissue growth factors***. Growth factors (nerve growth factor, epidermal growth factor, platelet derived growth factor, insulin-like growth factor) represent a special type of chemical messengers that regulate the division and development of organisms and the regeneration of damaged tissues by exerting primarily local (autocrine, paracrine) and to a lesser extent hormonal effects.
– ***G proteins***. These proteins are expressed in different tissues and play a crucial role in signal transduction and intracellular second messenger production.
– ***Ion channels***. The opening and closing of ion channels control the function of sensory tissues (sight, smell, hearing).
– ***cGMP as a second messenger***. cGMP transmits signals to the smooth muscle and to sensory tissues.

1.9.3 Homeostasis of Organs and Organ Systems

The main organs of mammalian homeostasis are the heart, lungs, liver, pancreas, kidneys, muscles, skin, hypothalamus, pituitary glands and the brain that controls basic functions of behavior. Without detailing the functions of organs, from homeostatic point of view the most important organs that maintain balance are the kidneys, the liver and the brain.

As a typical example of organ homeostasis the cardiac pumping ability and coronary blood flow is given. When the pumping capability of the heart muscles is weakened, the blood flow to the muscle of the heart will also be reduced. This then leads to further weakening of pumping and less coronary blood flow. The positive feedback regulation deteriorates the whole blood circulation. To interrupt the positive feedback loop and restore at least partially the cardiac function doctors prescribe

digitalis to inhibit the sodium-potassium pump mentioned earlier. In turn the higher intracellular sodium concentration reduces the activity of the Na^{+}-Ca^{2+} pump. As already noted this pump elevates the intracellular Ca^{2+} concentration. The increased calcium concentration favors the action potential bringing about cardiac contraction and results in a positive inotropic effect i.e. a higher contractibility of the heart.

1.10 Dimensions of Health

From homeostatic point of view there are at least five major dimensions of health that deserve mention:

1. Physical health that refers to the state of the body ensured by:
   - healthy eating habits to keep the body in homeostatic condition,
   - water household essential for cleansing,
   - exercise to maintain fitness, endurance and immunity,
   - medical checkup to notice homeostatic imbalances at their early stages, and arrest illness,
   - daily sleep (at least 7 h),
   - avoidance of toxic and addictive substances (smoking, drugs).
2. Emotional stability: to accept and cope with own or others feelings, strength, shortcomings, to handle stress, to build strong ties among family and friends, to handle with grace the unfriendly behavior of others.
3. Intellectual stability: to stimulate creativity, skills and knowledge by realistic planning, exploring opportunities, recognize demands and expectations, dealing with conflicts realistically.
4. Social stability: interaction with people, accept and understand ideas of diverse cultures, build contact and communication skills, adopt positive self image.
5. Spiritual stability: refers to belief and personal values, acceptance or rejection of God, serving the needs of others, donations, living religious life.

1.10.1 Factors Influencing Homeostasis

It depends on individuals which external changes are more likely to upset the homeostatic balance:

– Life style: pattern, rhythm of life, physical activity
– Personal – environmental hygiene
– Nourishment (spice, fat, cholesterol)
– Human relations
– Destructive obsessions (alcohol, smoking, drug abuse)
– Environmental effects

- physical, chemical, irradiation
- noise, air-, water-, soil pollution
- effects of animal and plant life

– Social impact: family, workplace, institutions
– Genetic impact, biological capabilities
– Health provision

Estimations suggest that the three major factors: life style, genetic makeup and environmental and health provision contribute almost equally to the well-being of a person. Life style has somewhat more impact on health followed by the genetic impact and environmental effects and health provisions of the society. There are several other factors that may lead to homeostatic imbalances.

1.10.2 Frequent Causes of Death

The loss of homeostasis may lead to illness and disease and ultimately to death. Based on data provided by World Health Organization (http://www.who.int/mediacentre/factsheets/fs310/en/index.html) in 2008 the major causes of death of global proportion were:

Ischemic heart and vascular diseases
Stroke and other cerebrovascular diseases
Respiratory and pulmonary diseases
Diarrhoeal diseases
HIV/AIDS
Oncological diseases
Tuberculosis
Diabetes mellitus
Road traffic accidents

The leading causes of death differed significantly in low-income countries:

Respiratory infections
Diarrhoeal diseases
HIV/AIDS
Ischemic heart disease
Malaria
Stroke and other cerebrovascular diseases
Tuberculosis
Premature and low weight birth
Birth asphyxia and birth trauma
Neonatal infections

Industrialized countries have systems for assessing causes of death, while many developing countries are missing such systems. Consequently the numbers of deaths from specific causes have been gathered by WHO from incomplete data.

In high-income countries the list of major causes of death is quite different and determined more reliably:

Ischemic heart and vascular diseases
Stroke and cerebrovascular diseases
Trachea, bronchus and lung cancers
Alzheimer and other dementias
Respiratory and pulmonary diseases
Colon and rectum cancers
Diabetes mellitus
Hypertensive heart disease
Breast cancer

The most significant change in the list of major causes of death in high-income countries is the death caused by cancer (lung cancer, colon and rectum cancer, breast cancer). The increased incidence of tumorous diseases and death caused by cancer is of growing concern. In this book the oncological aspect focuses primarily on cell growth, tumor formation and metastatic tumor spread.

1.11 Control of Processes

Those processes that correspond to regulatory rules are said to be controlled. The control has two basic forms: steering and regulation. Steering is an unidirectional connection. The headquarters is giving the commands, signs or information to the subordinated system, but the directed system has no means at all to influence the operation of the centre. To the contrary regulation allows feedback.

1.11.1 Steering

Certain parameters are set from the existing momentary "real" to the "must" value irrespective of the present state of the directed system. The steering is complete when the "real" value corresponds to the dictated "must" value. Steering is not a characteristic type of biocontrol and will not be discussed.

1.11.2 Regulation

The system is also set to the "must" value, but on the bases of feedback, taking into consideration momentary conditions.

The phenomenon of regulation can be recognized everywhere in life, e.g.:

- financial regulation
- law
- climate
- river, water level, water quality control
- agriculture, fishery, sylviculture, hunting
- food-chain, food,
- regulation of processes
- tourism,
- biohazard, etc.

Biohazard The first notable conference on the potential biohazard and regulation of biotechnology was held in Asilomar in February 1975 initiated by the Noble laureate Paul Berg. Scientists of this conference have asked their governments to regulate the application of molecular biology and to ban molecular cloning until nontransmissible vectors (plasmids, bacteriophages or other viruses) were able to grow in only specific hosts to avoid pandemic biological catastrophes. As a result the National Institute of Health Guideline on Recombinant DNA safety consideration and UNEP's Technical Guidelines for Safety in Biotechnology Biosafety Protocol have been accepted.

1.11.3 Mechanisms of Regulation

Processes that are uncontrolled or subject to steering or dictatorship are doomed to destruction. Positive regulation not only upsets the stability of the system, but can also push it to the brink of ruin. The concepts of biological regulation correspond to those taken from the electronics, as the rules and ideas of regulation are of universal validity irrespective of the medium they take place. There are two types of life systems: conformers and regulators. In conformers the environment determines their parameters. Regulators try to keep their parameters constant over a relatively wide range of ambient conditions.

1.11.3.1 Negative Feedback Regulation

In negative feedback regulation the organism has set points to which different parameters (temperature, volume, pressure, etc.) have to be adapted to maintain the normal state and the stability of the body. The momentary value refers to the values at the time the parameters have been measured. When a parameter changes it has

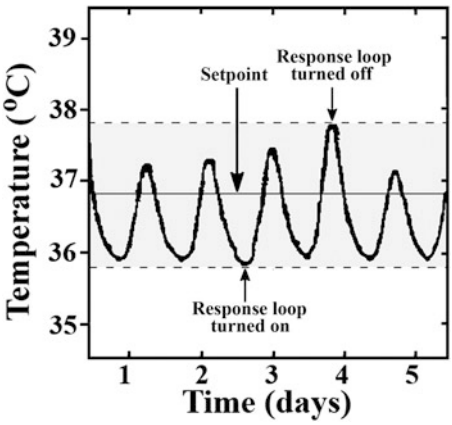

Fig. 1.18 Homeostatic oscillation of human body temperature around the set point. Normal functional range is indicated by the grey area. The set point of the body temperature is a characteristic value indicating that the process is regulated

to be turned back to its set point ("must" value). Oscillations are characteristic to negative feedback regulation shown by the example of thermoregulation within the physiological operation range (Fig. 1.18).

1.11.3.2 Positive Feed-Forward Regulation

Contrary to the homeostatic negative feedback loop where the response loop slows down the stimulation and provides a safe return to the optimal range, in positive feed-forward the stimulus results in further stimulation and prepares the body for a significant change. A positive feed-forward loop is exemplified by the delivery of a baby when it moves to the lower part of the uterus and initiates labor. The cervical stretch initiates oxytocin release in the posterior pituitary causing uterine contractions and pushes the baby against the cervix. This in turn causes cervical stretch, oxytocin release and stronger uterine contractions. The positive feed-forward regulation continues till the birth of the baby when the cervical stretch ceases and stops the positive feed-forward loop. There are only a few biological processes in nature driven by positive feedback. Beside the hormonal feed-forward regulation of birth of mammals, algal bloom, synchronized appearance of day-flies or locusts invasion could be mentioned.

1.12 Levels of Bioregulation

Biological self-regulation takes place at molecular → cellular → organism → population → global levels. As this book deals primarily with the regulation of organisms, less attention has been paid to molecular, cellular, population and global levels of bioregulation.

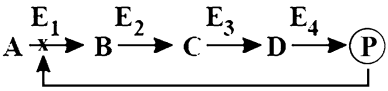

Fig. 1.19 Feedback inhibition of biosynthetic pathways. A, B, C and D represent metabolites, P is the end product, E_1 the regulating enzyme, and E_2, E_3 and E_4 other enzymes of the metabolic process. The response loop counteracts the production of P by shutting off the regulating enzyme E_1 of the committed step

1.12.1 Molecular Regulation

1.12.1.1 Inhibition of Biosynthetic Processes by End Products

The rules of biological cybernetics particularly the negative feedback apply also to cellular processes known as metabolism. Positive feedback implies the danger to destroy biological systems as it is enhancing the signal exponentially and rapidly. More importantly, negative feedback is keeping the signal level constant contributing to the stability of the system and helps to accommodate to external changes. Metabolic pathways are normally regulated at the first, so called committed step, by inhibiting the enzyme to prevent the overproduction of the end product (Fig. 1.19).

1.12.1.2 Regulation by Limited Proteolysis

This type of regulation is also known as covalent modification or zymogen activation exemplified by the inactive enzyme synthesized in the stomach as pepsinogen and activated by the relatively high H^+ ion concentration (pH 3) to pepsin. Factors of the blood clotting cascade, the pre-proinsulin > proinsulin > insulin, the trypsinogen > trypsin, chimotrypsinogen > chimotrypsin, procarboxipeptidase > carboxipeptidase or proelastase > elastase covalent modifications could be mentioned as examples of metabolic regulations acting by limited proteolysis.

1.12.1.3 Regulation of Gene Expression

Structural genes that code for enzymes and are needed to activate chemical reactions are transcribed to RNA then translated to proteins. The regulatory gene codes for the regulatory protein called repressor and is located at the 5′ upstream end of the regulatory sequence of the bacterial promoter. The repressor is able to bind to the operator region of DNA and to inhibit the transcription of the downstream structural genes. The binding of repressor to the operator will prevent the binding of RNA polymerase to the promoter region and as a consequence transcription will

not occur. The presence of another chemical and its binding to the repressor is necessary to allow RNA polymerase binding to the promoter and the transcription of the downstream structural genes and the utilization of special food molecules. The best known example of regulation at the level of gene expression is represented by the lac operon model of *E. coli*. The design of gene expression can be summarized by either removing a restraining element, which permits expression from a high-level promoter, or by providing a stimulatory element, which facilitates expression from a low-level promoter (Savageau 1998). It was suggested that these control mechanisms of gene expression are based on error minimization (Shinar et al. 2006). The error-minimization hypothesis offers a reasonable explanation for the Savageau demand rule stating that genes often needed in the natural environment tend to be regulated by activators, while rarely needed genes are regulated by repressors. In both cases, DNA sites are bound for most of the time in order to minimize errors. Error-minimization was also found in the cell cycle of CHO and *Drosophila* cells. The number of non-overlapping replication and repair subphases correspond to the number of chromosomes (Banfalvi et al. 1997; Rehak et al. 2000; Szepessy et al. 2003) suggesting that these checkpoints with minimized replicative and maximized repair activity are in conformity with the theory of error minimization.

1.12.2 Cellular Regulation

1.12.2.1 Unicellular Organisms

When unicellular organisms came into being in the ancient ocean they were surrounded only by their external environment. In prokaryotes and eukaryotes DNA is the control center of cells. In the nucleus of eukaryotic cells the DNA is packaged into chromosomes.

Regulations cover a broad spectrum of cellular changes involving:

- Membrane structures and compartmentalization
- Rate of enzymatic reactions (inhibition, activation, K_m, metabolic feedback)
- Enzyme regulation (inductive and constitutive enzymes, zymogen activation, covalent modification)
- Genetic control: DNA > hnRNA > mRNA > inactive mRNA > protein

The general view of cellular regulation through epigenetic control, gene regulation and enzyme regulation is depicted in Fig. 1.20. Epigenetics is the study of inherited changes in the phenotype (appearance) or gene expression of species caused by mechanisms other than changes in the underlying DNA sequence (hence named in Greek *epi* = above) genetics. Each mammalian cell of the body has the same genes, yet performs distinct functions. This is achieved by epigenetic control of gene expression; the switching on and off genes.

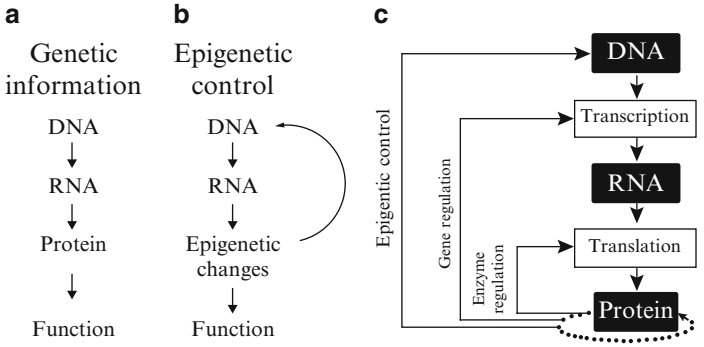

Fig. 1.20 Genetic information and epigenetic control of genetic information. Genetic information (**a**), epigenetic control (**b**) and cellular regulation through protein (enzymatic), genetic and epigenetic control (**c**)

1.12.3 Organismal Regulation

Multicellular organisms (plants, animals) consisting of eukaryotic cells protect their cells from the external environment by an aqueous internal environment. The internal environment serves to provide maximum efficiency and balance for the organism. Animals are multicellular organisms consisting of different cell types. Organs have specialized functions and are normally part only of one organ system. Organ systems consist of organs responsible for major functions of the organism.

1.12.3.1 Regulation by Hormonal and Nervous Systems

The anterior pituitary gland is regulated by the hypothalamus and secretes six hormones: three of them control the reproductive systems (follicle-stimulating hormone, luteinizing hormone, prolactin), the other three hormones regulate growth and metabolism (growth hormone, adenocorticotropin, thyroid-stimulating hormone). The posterior pituitary stores the antidiuretic hormone vasopressin and oxytocin. The antidiuretic hormone (ADH) regulates water balance, oxytocin stimulates the contraction of the uterus and the ducts of the mammary glands. The hypothalamic-pituitary-adrenal axis will be discussed in the subchapter "Major regulatory systems of organisms".

1.12.4 Bioregulation at Population Level

The Hardy-Weinberg law describes the approximate frequencies of genotypes in a population. The law states that the population will be in genetic equilibrium

when it meets five major criteria: large enough size, with no significant mutations, neither immigration nor emigration takes place, no natural selection occurs. This regulation concerns primarily the variation in a population. When the population maintains the same size it is regarded to be stable. In dense populations individuals living closer together are better protected from predators, but at the same time face a higher probability of getting diseases. Factors regulating the growth of a population imply biological and environmental factors. Further factors reducing the size of a population beside predation and diseases are parasites, competition, shorter life span, less parental care, smaller young. The growth rate of the population is inversely proportional to its density. Ecosystems with many species and interactions among species are normally subjected to disease and competition types of regulation. Small, isolated habitats (ecological islands) are particularly endangered by the disturbance of density independent regulators. Among these regulators we find primarily physical factors, such a weather (severe winter, storms, flood), eruptions at volcanic islands (e.g. Krakatoa, Indonesia) (Breining 2007), earthquakes, tsunamis, pollutants, nuclear tests creating widespread radioactive contamination (Bikini Atoll in the Pacific Ocean) (Arnold and Smith 2006; Foster 2009).

1.12.4.1 Biosphere

Certain aspects of homeostasis seem to be maintained at global level. In James Lovelock's Gaia hypothesis the living matter on Earth is regarded as a vast superorganism with the entire planet maintaining its homeostasis. Major global cycles (carbon, nitrogen, water, metal cycles) recycle and help to reuse these elements in the biosphere. Without these cycles life on Earth would not be sustainable. The destabilization of the ozone layer of Earth's atmosphere is of global concern as it used to absorb more than 90 % of Sun's ultraviolet rays, but not anymore above the southern hemisphere. Other long-term global changes have been reviewed in the subsection devoted to "Global aspects of ocean as an osmolyte system". As emphasis is placed on the stability of the inner environment of individuals, details of homeostasis of populations and biosphere are outside the scope of this book.

Gaia Hypothesis Earth in ancient Greek religion has often been personified by the goddess Gaia. The goddess Gaia personificated Earth in ancient Greek religion. The Gaia hypothesis (Gaia theory, Gaia principle), proposed that organisms coevolve i.e. interact with their inorganic surroundings on Earth. This interaction forms a self-regulating, complex system involving the biosphere, atmosphere, hydrosphere and pedosphere (outermost layer of the Earth) that contributes to the diversity of living organisms and maintains the self-regulating homeostasis on Earth.

1.13 Regulation of Multicellular Organisms

1.13.1 Autonomic Regulation

Self-government of homeostasis can be achieved by: the storage of metabolites to maintain their constant level, evolution of stabilizing mechanisms that regulate the speed of metabolic processes, autoregulation by the autonomic nervous system to make regulation automatic, complexity of the organisms of increasing independence from the environment.

A typical example of autoregulation is the blood flow regulation, the ability of organs to maintain their constant blood (food, energy, oxygen, hormones) supply under various conditions of perfusion. The blood circulation of some organs is under strict autoregulatory control. The blood circulation of kidneys, brain and coronary blood flow are under tight control. Both the renal blood flow and the glomerular filtration rate change within a relatively wide physiological range to keep renal autoregulation relatively independent from the arterial blood pressure between 80 and 160 mmHg. Cerebral regulation of blood flow is achieved primarily by the dilation and constriction of smaller arteries and arterioles. The complex physiological control systems of autoregulation cannot be discussed. The already mentioned autoregulation of coronary circulation is related to the oxygen supply. Corresponding to the oxygen consumption of the heart muscle the coronary circulation is regulated by vasoconstriction and dilation over a wide range of blood pressure. Cardiovascular disorders are manifested as arrhythmias, insufficiency of coronary circulation, heart failure, stenosis, atherosclerosis, occlusion and stroke (thrombus formation in the circulation), edema, vasospasm, hyper-, and hypotension, hypoxia, organ dysfunction, etc. The skeletal muscle and the splanchnic blood circulations are moderately autoregulated. The cutaneous blood circulation has a poor autoregulatory capability. Organs with moderate or missing autoregulation of circulation are less frequently subjected to cardiovascular disorders.

1.13.2 Local Regulation

Signals reach their target cells through, circulation (hormones), axons (nervous system), diffusion (local action) and junctions (neighboring cells). Beside the long-distance reflex control (nervous, endocrine, cytokines) there are local pathways that control neighboring cells through the diffusion of signal molecules. The degradation of these diffusion molecules is fast, they do not diffuse far, with an effective range limited to their immediate surroundings, to nearby cells and their movement can be hindered by the extracellular matrix. Among the paracrine agents are growth and clotting factors, retinoic acid (active form of vitamin A) that regulates gene expression of embryonic development (Duester 2008) and allostatin, the paracrine

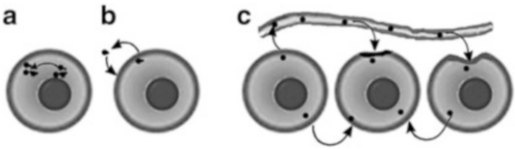

Fig. 1.21 Intracrine (**a**), autocrine (**b**) and paracrine (**c**) regulation

growth regulator of *corpora allata* in insects. Paracrine signaling responses also to allergens and is involved in tissue repair.

Local regulations are termed intracrine, autocrine and paracrine effects (Fig. 1.21). The term intracrine is not generally accepted as all reactions taking place inside the cell are known as metabolism. The autocrine regulation means that the cell is producing and releasing substances that can act back on the same cell. Paracrine signals act on nearby cells.

Chemical and electric coupling of cells by cell junction provides intercellular nexus between specialized cell types (White and Paul 1999; Kelsell et al. 2001; Willecke et al. 2002), by connecting the cytoplasm of neighboring cells allowing the passage of ions and molecules. This type of local regulation is found throughout the animal kingdom. Electrical coupling provides the fastest connection happening within milliseconds or even less. The electrical synapse is a gap junction between two neurons. As the electrical synapse was discovered before the gap junction it retained its original name.

Gap junction communication appears to be an important structural element in the local regulation of differentiation (embryonic, tissue and organ development, tissue restructuring), in electric coupling of neurons or in the simultaneous contraction of heart muscle cells. Gap junctions are not expressed in adult skeletal muscle and in mobile cells such as sperm cells and erythrocytes. More information on gap junction and adhesion molecules will be provided in Chap. 2 under the subtitle "Cell junctions".

1.13.3 Systemic Levels of Regulation

Organ systems underline the importance of physiology that has greatly improved the understanding of body functions and homeostasis. In fact homeostasis can be regarded as an introductory to physiology. Physiology expects basic cellular, biochemical and anatomical knowledge. As we move from homeostasis to cell and tumor growth, cell biology and medical terms as well as laboratory techniques will be of increasing importance and their use unavoidable.

To maintain systemic homeostasis the following organs work together as organ systems:

– Nervous system (brain, spinal cord, nerves, sensory organs). Homeostasis uses the nervous system to monitor several other systems of the body.

- Cardiovascular system (heart, blood vessels, lymph) transports nutrients, oxygen, hormones, waste products, controls temperature, fluid and balances pH.
- Respiratory system (lungs, trachea, air passages) maintains breathing, exchanges oxygen and gasses in the lung, helps to control pH and acid-base balance of the blood.
- Digestive system (mouth, esophagus, stomach, intestines, liver, pancreas) digests, absorbs food, supplies blood with nutrients and eliminates indigestible remains. Transporting functions of the body are maintained by the coordinated action of more systems including digestive, cardiovascular, lymphatic, respiratory and urinary systems.
- Lymphatic system (lymphocytes, monocytes, antibodies) defends against foreign colloidal and nanoparticles in the bloodstream, absorbs fat, controls fluid balance.
- Integumentum. The external protection of the body is secured by accessory organs such as hair, nails, sweat, oil glands, covering and protecting the body. The integumentary system controls temperature, synthesizes vitamin D.
- Skeletal system. Skeletal support and motion is provided by the bones, muscles, cartilage, ligaments and tendons. This system is responsible for body and internal movement, postures, heat production, body temperature, stores, minerals, produce blood cells, protects internal organs.
- Reproductive system. Reproductive functions are maintained by the male and female reproductive systems producing gametes (egg and sperm cells).
- Urinary system (kidney, bladder and ducts). Removes nitrogenous and other organic wastes from the blood by excretion, controls fluid balance, water-salt and acid-base balance.
- Endocrine system (hypothalamus, pituitary, thyroid and other glands)
- The nervous and the endocrine systems constitute the coordination centers of the body. Coordination takes place through the sensory input of internal and external receptors that activate the musculoskeletal system responsible for motion and induce endocrine glands to produce hormones. Homeostasis relies on feedback to keep the internal environment of the body constant. Negative feedback mechanism is utilized in both nervous and endocrine systems.

Homeostatic imbalance causes physiological changes that can lead from minor health problems to major diseases. Such malfunctions of lipid and cholesterol imbalances are atherosclerosis and cardiac disorders. Urinary malfunctions are kidney stones and gout. Disturbance in mineral homeostasis are manifested as osteoporosis, kidney stones. Among further homeostatic imbalances hypo- and hyperglycemia, diabetes, dehydration, high or low blood pressure, respiratory diseases, cirrhosis, hypo- and hyperthyreosis etc. could be mentioned.

1.14 Major Regulatory Systems of Organisms

1.14.1 Immune System

The cellular interaction and cooperation is best exemplified by the immune system consisting of two major parts, the innate and the adaptive immunity. The innate or inborn immune system is formed by monocytes (macrophages and dendritic cells) present in all animals. Phagocytes mentioned often under different names in different tissues and organs work efficiently but nonselectively without discrimination of invaders (Table 1.7). In a process known as antigen presentation phagocytes stimulate lymphocytes to produce antibodies. Lymphocytes belong to the major players of the other part of the immune system known as adaptive immune system that developed in jawed vertebrates and is highly specialized against different types of invasions. T lymphocytes are white blood cells and play a central role in cell-mediated immunity to fight infection. T cells are produced in the bone marrow, then move to and mature and develop in an organ in the chest called the thymus. After maturation, T-cells are present in the blood and in lymph nodes. B lymphocytes produce antibodies that tag invading cells for destruction and prevent viruses from infecting cells.

Interactions with foreign cells take place with freely moving phagocytic and non-phagocytic cells of the immune system of the body. To defend the invaders the immune cells communicate with other cells to produce cytokines and chemokines that stimulate lymphocytes and attract more phagocytes to the site of invasion. The development of bipotential cells to monocytes (dendritic cells, macrophages) neutrophils and mast cells is depicted in Fig. 1.22. Non-professional phagocytic cells include epithelial cells, endothelial cells, fibroblasts and mesenchymal cell.

The causative agents of inflammatory processes are colloidal and nanoparticle size invaders and dead cells caused by inner shock or trauma (Fig. 1.23). Immune cells of the blood circulation are macrophages, polymorphonuclear cells and

Table 1.7 Professional phagocytes in different organs and tissues

Organs, tissues	Names of phagocytes
Thymus	Monocytes (macrophages, dendritic cells)
Liver	Kupffer cells, monocytes
Lung	Monocytes, mast cells
Spleen	Monocytes, sinusoidal cells
Blood	Neutrophils, monocytes
Bone marrow	Monocytes, sinusoidal cells, lining cells
Bone	Osteoclasts
Intestinum (Peyer's patches)	Macrophages
Lymphoid tissue	Monocytes, dendritic cells
Nervous glial tissue	Microglial cells
Skin	Langerhans cells, monocytes, mast cells

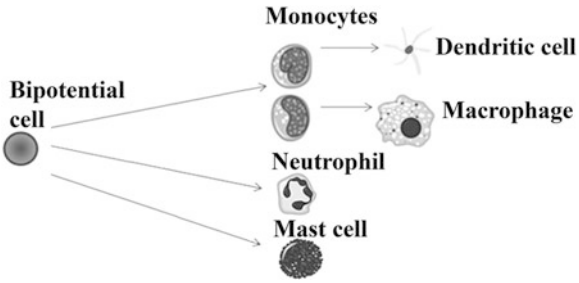

Fig. 1.22 Professional phagocytes. Bipotential cells have the potential to develop either to lymphocytes and NK cells or to phagocytes. Monocytes, neutrophil and mast cells belong to phagocytes

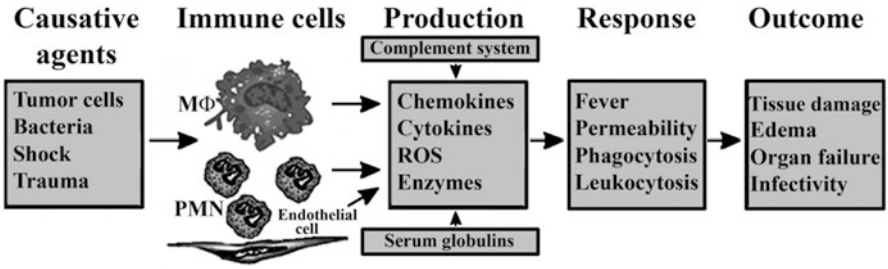

Fig. 1.23 Inflammatory processes during septic invasion of the body. *MΦ* macrophage, *PMN* polymorphonuclear cell. PMN means multinuclear leukocyte, usually neutrophylic granulocyte with its nucleus subdivided into lobes connected to each other by chromatin strands

epithelial cells lining the circulatory system, including the heart, arteries, veins and capillaries. The endothelial lining is produced by endothelial stems cells belonging to one of the three types of stem cells found in bone marrow. The thin walled endothelium lines the inner surfaces of blood vessels, lymphatic vessels and the inner cerebral and spinal surfaces. Ependymal cells are endothelial cells lining the membranes of the cerebral ventricles and the central canal of the spine. Based upon its microscopic appearance the endothelium has been subdivided into continuous, fenestrated and discontinuous endothelium. Continuous endothelial cells are connected by tight junctions constituting the blood-brain barrier. In other organs the gap junctions are channels some 1.5–2 nm in diameter between cells and transport materials from one side of the cell to be released to the other side. Due to their higher permeability fenestrated (*fenester* = window) endothelial cells allow the rapid exchange of larger molecules in the endocrine glands, fenestrated capillaries of the glomeruli of the kidney and fenestrated cells of the gastrointestinal villi. Discontinuous or sinusoidal endothelial cells with gaps present in bone marrow, spleen and liver provide fast equilibrium between the materials of the blood plasma and the tissues beneath the endothelial cells.

Endothelial cells play a key role in angiogenesis, a multi-step development of new blood vessels during tissue regeneration and in pathological processes such as tumor formation. Endothelial cells alone and in cooperation with lymphocytes produce chemokines (cytokines, lymphokines), reactive oxygen species (ROS) and

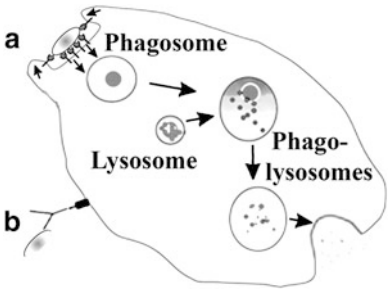

Fig. 1.24 Combined diagram of phagocytosis and destruction of a bacterial cell. (**a**) phagocytic engulfment. (**b**) binding of bacterium to the antibody recognized by the receptor of the phagocytic cell

enzymes to defend invasion by permeability changes, phagocytosis, fever etc. As a result of the fight against invaders that are destroyed, the organism survives, but tissue damages, edemas, scars of fibrous tissues, organ failures and infectivity can be the consequences. The destruction of harmful objects, such as bacteria by professional phagocytes may take place either by receptor mediated endocytosis (Fig. 1.24a) or *via* antibody coupling to the receptor that in turn binds the foreign antigen (Fig. 1.24b). Phagocytes play a crucial role in the destruction of millions of dead or apoptotic dying cells every day to secure the stability and maintain the homeostasis of the organism. Programmed cell death generally belongs to the normal development and cell depletion due to a wide range of stimuli, relatively mild in nature. Necrosis, the second major pattern of cell death, occurs in response to extreme physiological damages or as a result of genetic abnormalities.

1.14.1.1 Shift in Balance from Cell Division to Cell Death

The homeostatic balance can be shifted from tumorous cell growth to immunogenic cell death. The main immunogenic determinants of dying cells are:

- changes in plasma membrane permeability leading to increased phagocytosis,
- transfer of heat hock proteins (HSP70 and HSP90) through the plasma membrane,
- cross-presentation of tumor-derived peptides to CD8+ T cells,
- CD80 membrane receptor protein is activated by the binding of CD28 or CTLA-4. The activated protein is involved in the costimulatory signal essential for T-lymphocyte activation and cytokine production. CD80 co-stimulatory molecule facilitates *in vivo* tumor regression (Thomas and Wen 2006),
- stimulation of lysis by natural killing (NK) cells,
- maturation of dendritic cells (DC), functioning as phagocytic and antigen processing and presenting cells,
- release of proinflammatory cytokines by dendritic cells,
- release of inflammatory cytokines by necrotic cancer cells,
- internalization of foreign antigens trigger maturation and migration of dendritic cells,

- release of interleukins (IL-6, IL-8, IL-10), tumor necrosis factor (TNF-α) and high mobility group box 1 (HMBG1) protein initiate proinflammatory response,
- p53 activation in cancer cells as a response to DNA damage leading to tumor cell apoptosis,
- stimulation of the immune system through expression of NKG2D type II transmembrane protein,
- triggering NK-mediated lysis,
- formation of end-stage degradation products during late stages of cell death,
- cellular components with proinflammatory properties released from the cell debris such as RNA molecules of viral origin are recognized by the toll-like receptor protein TLR3 that induces the activation of NF-kB to increase the production of type I interferon and lead to dendritic cell stimulation, DNA molecules from bacterial infection interact with TLR9, or nucleotides,
- late-stage apoptosis associated with the induction of pentraxin-related protein PTX3, which interacts with the immunological synapse formed by dendritic cells and apoptotic bodies. PTX3 modulates the immune response triggered by dying cells,

The homeostatic balance of cell growth can be lost either by the overproduction (tumor formation) or loss of cells by cell death. As tumor and metastasis formation are of major interest, cell death will not be given further consideration.

1.14.2 Hormonal Regulation

In the reflex control cells at a distant site control the regulation. In negative feedback the response counteracts the stimulus by shutting off the response loop. The hypothalamic-pituitary-adrenal axis is providing the major hormonal negative feedback control for homeostasis. This complex consists of a set of interactions and feedback mechanism among them the hypothalamus, the anterior (glandular) lobe of the pituitary gland (adenohypophysis) and the subrenal gland (Fig. 1.25a).

Hormones that stimulate hormone secretion are called tropic hormones. Tropic hormones may also stimulate the proliferation of endocrine cells. Tropic amplification involves corticotropin releasing hormone (CRH) produced by the hypothalamus, ACTH released by adenohypophysis and corticosteroids excreted by the cortical part of the subrenal gland (Fig. 1.25b). Corticosteroids carried by the blood are switched back by negative feedback to the hypothalamus and suspend its corticotropin release. The feedback regulation is based on this returning loop. The hypothalamic-pituitary-adrenal axis is correcting deviations from the ideal state better known as set point through the compensation of processes representing opposite tendencies. The location of hypothalamus in the brain is shown in Fig. 1.26.

Hormones released into the circulation act either through second messenger systems recognized by external cell surface receptors or internal cytosolic or nuclear receptors. Major types of second messengers are:

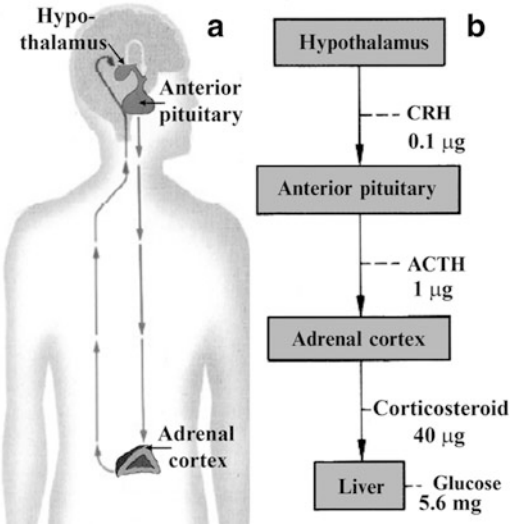

Fig. 1.25 The hypothalamic-pituitary-adrenal-axis (**a**) and its hormonal signal amplification (**b**)

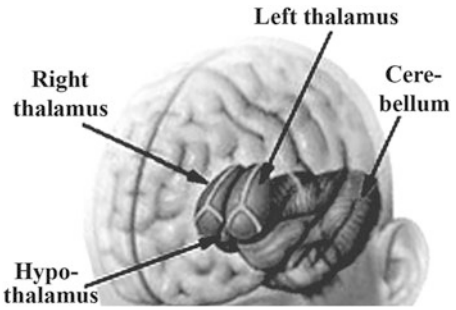

Fig. 1.26 Location of thalamus, hypothalamus and cerebellum in the brain

– hydrophobic molecules (diacylglycerol, phosphatidylinositol) diffusing from the plasma membrane into the intermembrane space,
– hydrophilic molecules (cAMP, cGMP, IP3 and Ca^{2+}) located within the cytosol, or
– gases (nitric oxide, hydrogen sulfide) diffusing through the cytosol and across cellular membranes.

Major steps of the second messenger mechanism involve:

1. Activation of membrane bound receptor by the agonist
2. Activation of G-protein and production of effector
3. Stimulation of second messenger synthesis by the effector
4. Activation of intracellular process(es) by the second messenger

The hormonal regulation of metabolic pathways through cAMP induced covalent modification is viewed schematically in Fig. 1.27.

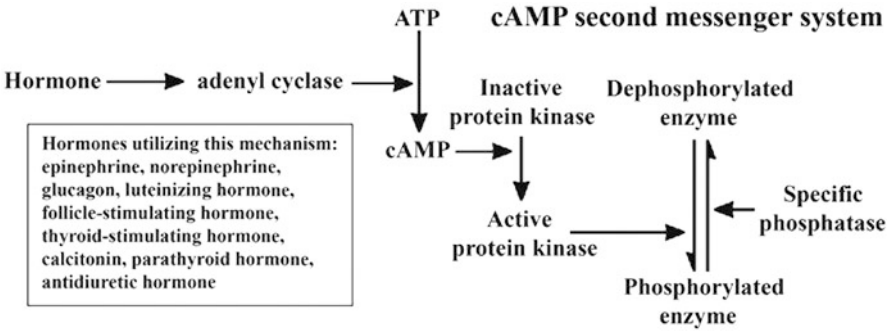

Fig. 1.27 Hormonal regulation of metabolism by the cAMP system. Hormones (*listed in the box*) bind to their specific cell surface receptors and initiate the formation of cAMP from ATP catalyzed by the adenylate cyclase enzyme. cAMP initiates a chain of phosphorylations through cAMP induced protein kinase (protein kinase A). Both phosphorylation (e.g. glycogen phosphorylase) or dephosphorylation (e.g. glycogen synthase) can activate enzymes, while the opposite process would inactivate these enzymes

1.14.3 Nervous System

In spite of the diversity of the animal kingdom all species share many conserved features suggesting that they were already present in their last common ancestor. The diversity of different life forms and habitats required a neuronal circuitry that was able to cope with relatively large internal and external changes of the body without giving up coherence or loosing homeostasis.

Last Universal Common Ancestor (LUCA) The theory of common origin proposes that all organisms on Earth descended from the ancestral gene pool of the unicellular ancestor that gave rise to life on Earth some 3.5–3.8 billion years ago (Doolittle 2000). Cloned and synthetic organisms do not belong to naturally evolved species and are subject to much ethical debate.

The nervous system, particularly the autonomic nervous system also known as visceral, involuntary or vegetative nervous system is involved in homeostatic regulation. The autonomic nervous system is located in the *medulla oblongata* in the lower brainstem and is divided into two subsystems: the parasympathetic and the sympathetic nervous system (Fig. 1.28).

The two principal divisions of the central nervous system are the brain and the spinal cord. The peripheral nervous system contains only nerves, including cranial nerves with the exception of cranial nerve II (*nervus opticus*) and connects the central nervous system to the rest of the body. The three main components of the nervous system are (a) the afferent pathway that provides input from the body

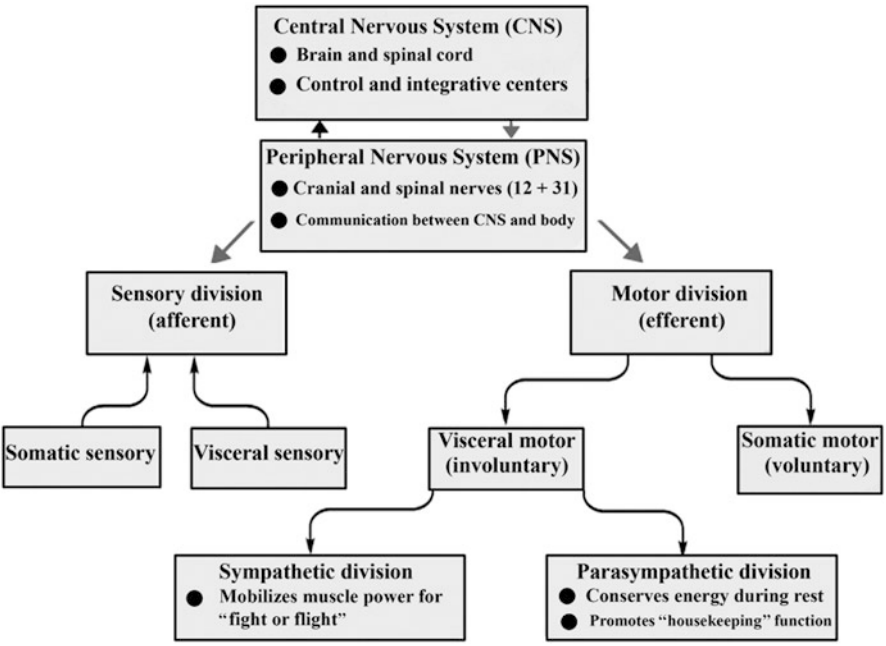

Fig. 1.28 Divisions of the nervous system. The scheme shows the major divisions involving the central and the peripheral nervous systems with particular emphasis on the involuntary autonomic (visceral) nervous system playing major role in homeostasis

through the sensory neurons, (b) the central nervous system with decision making interneurons and (c) the efferent output carried by the signals of the motor neurons to the effector muscles and glands.

(a) *Sensory input* implies

- Light – photons
- Chemicals (food, poison, mate) molecules
- Sound, touch, pressure waves

Light is sensed by:

- Photoreceptors
- Simple light detectors
  - eye-cups (planarians, medusas, cnidarians, some snails, some invertebrates)
  - eyelets, ocelli (dorsal in most insects, lateral ocelli or stemmata in larvae of some insects)
  - ommatidia (cluster of photoreceptors in the compound eyes of insects, mantis shrimp, millipedes)
  - eyes (evolved around 540 million years ago as light detecting organs. Varied and complex eyes provide the most important sense for many animals and humans).

Chemicals are sensed by chemoreceptors localized in several places on the surface of the body of simpler animals and concentrated at the head of more complex animals (nose, mouth).

Sound and touch are sensed by:

- Mechanoreceptors usually containing cilia that trigger nerve impulses when moved
- Simpler animals: distributed throughout the surface of the body
- Complex animals:
  - Surface (e.g. skin)
  - Mechanosensory organs (lateral line neuromast in fish, insect bristle, inner ear sensory neurons)

Pressure waves come from moving air molecules, moving water molecules, touch of physical contact (predator/prey/mate).

(b) *Interneurons*. These nervous cells are present only in more complex animals in the central nervous system (brain, spinal cord). Cellular structures located between the brainstem and the cortical part of the brain are known as the limbic system and are the most highly developed structures in mammals. Key parts of the limbic system are the hypothalamus and the pituitary gland. These two parts of the so called mammalian brain, particularly the hypothalamus regulate homeostatic functions of the body such as temperature, balance, blood pressure, heartbeat rate, blood sugar level. The limbic system also directs emotional reactions related to survival.

(c) *Motor division.* Rapid response is provided by motor neurons triggering usually movement. The neuroendocrine glandular response takes more time (up to hours). The motor division of the peripheral nervous system has two subdivisions: the somatic and the autonomic visceral nervous system.

  - The somatic nervous system includes all nerves that control the muscular system and external sensory receptors. External sensory organs are receptors. Muscle fibers and glands are effectors. Motor neurons of the somatic system are distinct from those of the automatic nervous system. Inhibitory signals cannot be sent through the motor neurons. A simple reflex arc of the somatic nervous system is an automatic involuntary movement to a stimulus (e.g. balance, blinking, stretch reflex).
  - The involuntary or autonomic nervous system is that part of the peripheral system that consists of two subsystems and controls internal organs. The autonomic nervous system controls muscles in the heart, the smooth muscle in internal organs such as the intestine, bladder, and uterus. It plays a major function in balancing homeostasis. The sympathetic subsystem of the autonomic nervous system is involved in the fight or flight response. The operation of parasympathetic division has an opposite tendency and is involved in relaxation.

Placing the control pathways of hormonal and nervous systems in a broader perspective the complexity of the reflex control increases in this order:

Single endocrine → single nervous → neurohormone → neuroendocrine reflexes

Common elements of the reflex control are:

Stimulus → Sensor (receptor) → Afferent path → Integration (center)
→ Efferent path → Effector (target cell or tissue) → Response (feedback loop).

1.15 Regulatory Mechanisms: Receptors, Reflexes

Regulatory mechanisms control systems to keep the internal environment relatively stable and maintained within relatively narrow limits, despite external environmental changes. In the homeostatic control mechanisms the receptor is sensing the stimulus that responds to environmental changes. This sensation sends information to the center of the control. The control center takes appropriate measures in response to the stimulus. In animals the control center of most of the homeostatic mechanisms is the brain. The incoming information through the afferent neurons is processed in the center that sends signals to the effectors (muscles, organs, other structures). Upon receiving the signal, correction takes place at distant tissues and organs to correct the deviation. Of the two major types of feedback regulations the applicability of positive feed is limited, negative feedback dominates the regulatory mechanisms (Marieb and Hoehn 2007). Under healthy conditions control mechanisms prevent the occurrence of homeostatic imbalance. The most often occurring homeostatic imbalances that can cause diseases are related to destabilized metabolism (hyper-, hypoglycemia, hypercholesteremia, gout), to dehydration, toxins, genotoxic agents causing mutations and can lead to cancer.

1.15.1 Receptors

Receptors are proteins that upon binding biologically active molecules (ligands), amplify signals and activate cell responses. Receptor structures are embedded in either the cell membrane (cell surface receptors), in the cytoplasm or in the nucleus and selectively bind specific signaling molecules and upon their binding result in specific physiological effects. After ligand binding receptors are modulated by competition, blocking and activating agents, undergo conformational changes resulting in signal transduction, cellular signaling, up- and down-regulation of gene expression, differentiation, proliferation and regulation of metabolic processes to maintain dynamic homeostasis (homeodynamics). Some ligands known as antago-

nists block receptor binding without inducing responses. While reflex responses are initiated by cells at a distant site, in local control cells initiate cellular responses in the vicinity of the change.

1.15.2 Types of Receptors

1.15.2.1 Cell Surface Receptors

Cell membranes participate in several cellular processes such as cell adhesion, signaling, ion conductivity, attachment through surface, cell wall and intraskeletal components. Plasma membrane receptors may be peripheral or transmembrane proteins that bind peptide hormones, neurotransmitters, antigens, complement fragments, autoimmunoglobulins, or extrinsic apoptotic signals. There are more than 20 families and over 1,200 human plasma membrane receptors. Known human plasma membrane receptors are listed in the Receptome database (Ben-Shlomo et al. 2003).

1.15.2.1.1 Peripheral Membrane Proteins

The association of these proteins with the surface of the lipid bilayer may result in the release of signals that activate transmembrane proteins. Peripheral membrane proteins are relatively rare relative to other more common receptor types that cross the membrane. These proteins are attached only temporarily to the cellular membrane, to integral membrane proteins or penetrate the peripheral regions of the lipid bilayer. Peripheral membrane proteins often constitute the regulatory subunits of ion channels, transmembrane receptors. The interaction between these monotopic membrane proteins and cell membrane may take place through:

(a) an amphipathic-helix localized parallel to the membrane plane,
(b) interaction by a hydrophobic loop of the protein,
(c) lipidation by a covalently linked membrane,
(d) electrostatic or ionic interaction with membrane lipids.

A typical peripheral membrane receptor is the elastin receptor. Other examples of binding of peripheral proteins to lipid bilayers are phospholipase A2, phospholipase C, alpha and beta hydrolases, cholesterol oxidase, carotenoid oxygenase, lipoxigenases, alpha toxins, sphingomyelinase C, glycosyltransferases, ferrochelatase, dihydroorotate dehydrogenases, glycolate oxidase, etc.

1.15.2.1.2 Transmembrane Proteins

A transmembrane receptor can be a single pass protein and may consist of more than one subunit or can have a seven transmembrane structure. Integral membrane

proteins have three basic domains:

– extracellular or ligand binding domain,
– transmembrane domain with hydrophobic stretches of amino acids,
– cytoplasmic or intracellular domain containing the effector region.

Transmembrane receptors associated with signaling are all integral membrane proteins that can transmit signals through the lipid bilayer. Metabotropic receptors are coupled to G proteins acting *via* secondary pathways such as enzymes (e.g. adenylate cyclases), ion channels, phospholipases, postsynaptic density (PZD) domains. The PDZ domain is a common structural motif consisting of 80–90 amino-acids, signaling proteins of bacteria, yeast, plants, viruses and animals.

Transmembrane proteins can be classified into three categories:

(a) G protein-coupled receptors (GPCRs) are composed of seven transmembrane alpha helices containing extracellular and intracellular loops. The binding site is usually located at the extracellular or between the extracellular loops (Congreve and Marshall 2010). Major GPCR receptors are: the rhodopsin-like, secretin receptor family, glutamate/pheromone metabotropic, fungal mating pheromone, cAMP and frizzled/smoothened receptors.
(b) Inotrop receptors are ligand-gated ion channels that permit the entry of ions through the activated ion channels. Ligand-gated ion channels have a heteropentameric structure with four transmembrane alpha helices (Cascio 2004).
(c) In enzyme linked or catalytic receptors the binding of the ligand triggers enzymatic activity at the cytoplasmic side of the homodimer receptor (Dudek 1999; Alexander et al. 2007). This family includes receptor tyrosine kinases (erythropoietin receptor, insulin receptor, insulin-like growth factor, other growth factor receptors, cytokine receptor). Tyrosine kinase receptors consist of homodimers. Monomers cross the membrane *via* alpha helices and contain the extracellular ligand binding cavity and the intracellular catalytic activity.

1.15.2.2 Intracellular Cytoplasmic Receptors

Intracellular receptors are located inside the cell rather than on the cell membrane. Lipophilic steroid hormones diffuse inside the cell and bind to intracellular second messengers. Classes of intracellular receptors are the cytoplasmic, nuclear receptors, the IP_3 receptor located on the endoplasmic reticulum. Examples of intracellular receptors are transcription factors, neurosteroids (sigma-1 chaperone protein), IP_3, nuclear receptors. Nuclear receptors belonging to this category are composed of C-terminal ligand binding domain and N-terminal ligand-binding domain consisting of 12 alpha helices. The ligand binding domain contains beside the twelve alpha helices an antiparallel beta sheet. Nuclear receptors are sensing steroid and thyroid hormones to regulate the expression of specific genes controlling the homeostasis through the development and metabolism of the organism.

1.15.2.3 Receptors of the Central and Peripheral Nervous System

These receptors are sensing the concentration (presence or absence) of a stimulus and initiate changes in the organism. Such cellular structures mediate between chemical agents and act on the nervous tissue initiating physiological responses. Sensory nerve terminals respond to various stimuli. These organs with nerve endings also respond to stimuli projected on skin, eye, ear, nose, mouth, viscera.

Regarding implications of receptor theory, drug effects have been classified into agonist, antagonist and inverse agonist (Negus 2006). The biochemical definition of receptor agonist comes from Ariëns and Stephenson (Ariëns 1954; Stephenson 1997), who also described that the ligand binding the ability of a drug to form a complex with the receptor can be distinguished from the efficacy of this complex to initiate a response.

Other theories of drug and receptor interactions suggest:

(a) The effect of the drug is proportional to the number of receptors (occupation theory), the number of the receptors to a given signal can be up- or downregulated.
(b) The activation of receptors is not proportional to the number of receptors occupied, but to the rates of dissociation and association between the drug and receptors within a unit time (rate theory). According to this theory agonists associate and dissociate fast, partial agonists show intermediate association and dissociation, agonists associate fast, but dissociate slowly.
(c) According to the induced fit theory the approaching of the drug to the receptor alters the conformation of the binding site to form the drug-receptor complex. Receptor types are summarized in Fig. 1.29.

1.15.3 Reflexes

The term of reflex was originally used by the French philosopher René Descartes to explain the principle of nervous activity based on the involuntary movements. Such human involuntary motor actions were recognized earlier by Galen in the second century. The involuntary reflex action is characterized by the stimulation of the sensory apparatus, conduction of these impulses through the nervous system to the brain and back to the muscles. By the time the reflex is realized, it is already over as it is performed without consciousness. Besides its spiritual meaning the term reflex gained application not only in the nervous activity, but also in mechanics, kinematics, and hydraulics. In the eighteenth century the reflex was freed from its metaphysical and mechanistic terminology and was applied to the involuntary and specific activity of internal organs.

Biological reflexes are responses to perturbing stimuli that act in the body to return to homeostasis. A reflex arc is a neural pathway that controls an action. Some of the reflexes are subconscious such as the regulation of the blood glucose

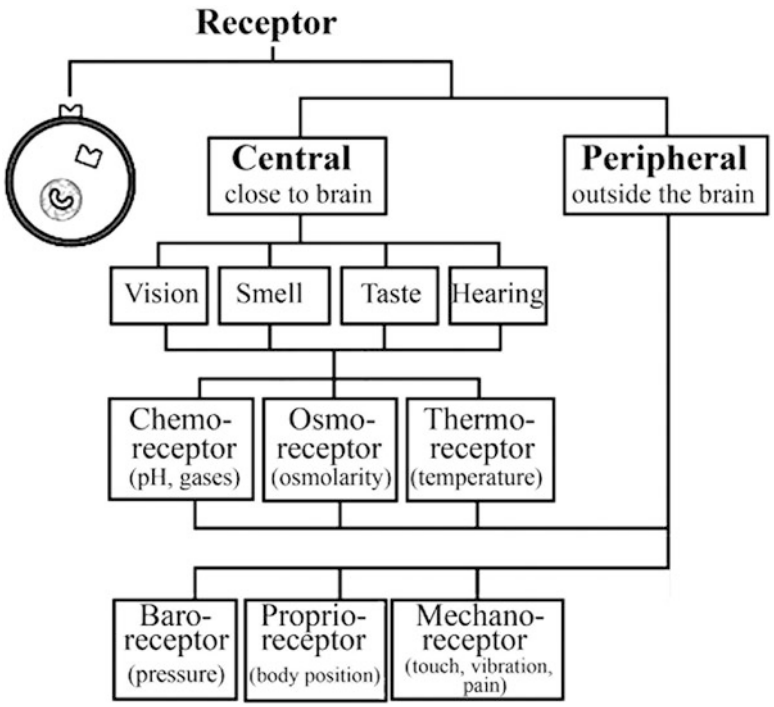

Fig. 1.29 Cell membrane receptors and receptors of the central and peripheral nervous system. Cell membrane and intracellular receptor proteins bind endocrine and neuroendocrine hormones (*round cell* at the *upper left corner*). The basic patters of central and peripheral nervous control pathways of specialized cells and functions are related to the conversion of various external and internal stimuli of the body to electric signals

level, others may be noticeable as trembling and shivering in response to dropping body temperature or conscious such as touching with the finger a razor blade and immediately withdrawing the hand.

An important discovery was the understanding of the reflex arc, the neuronal pathway that the nerve impulse follows. There are two types of reflex arc: automatic reflexes affecting inner organs and somatic reflexes affecting muscles. The reflex is said to be monosynaptic when it consists of only two neurons, one sensory and one motor neuron (e.g. patellar, Achilles reflex). In lower animals interneurons can be located outside the spinal cord (e.g. crayfish). Polysynaptic reflex arcs contain one or more interneurons connecting afferent and efferent neurons. The simplest unit of nervous activity is the reflex arc that starts with the detection of an environmental stimulus through the sensory nerve endings, followed by the traveling of the impulse *via* the sensory neuron to the spinal cord. In the spinal cord the sensory neuron synapses with a motor neuron that generates response signal to the stimulus. The response signal travels along the motor neuron to the muscle or gland. In this simple reflex the brain is not involved (Fig. 1.30). More complex reflex arcs involve

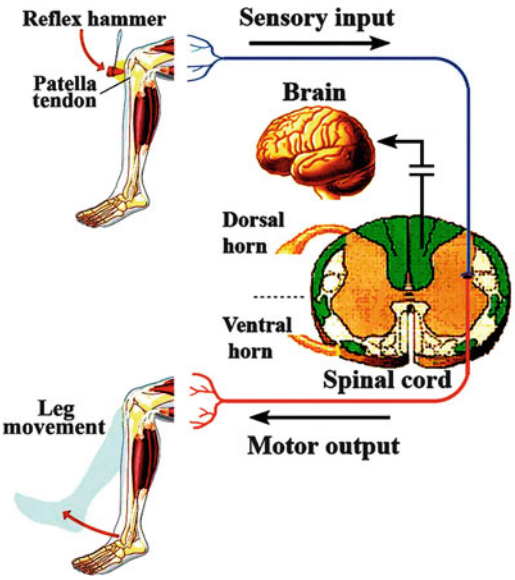

Fig. 1.30 Patellar (knee-jerk) reflex and the Bell – Magendie law. The Bell-Magendie law states: The dorsal root is responsible for carrying the sensory information and the ventral root for carrying the motor signals

the interpretation of the central nervous system. In these reflex arcs interneurons transmit the information from the spinal cord to the conscious area of the brain for analysis and decision making. The primary motor cortex is located in the posterior portion of the frontal lobe of the brain and controls the voluntary movements of specific parts of the body. Neurons of the tract referred to as pyramidal tract neurons conduct impulses from the primary motor cortex down the spinal cord. The *medulla oblongata* (myelencephalon) deals with the involuntary functions of the autonomic nervous system such as breathing, rate of heartbeat, blood pressure.

The patellar reflex (Fig. 1.30) is a simple tendon reflex occurring when the patella tendon located just below the kneecap (*patella*) is struck with a reflex hammer. This causes the lower leg to kick almost instantaneously, completely independent of the brain, i.e. the information does not go to the brain and the reflex will be realized only from the secondary movement signals of the leg. The Bell – Magendie law explains how the sensory (afferent) nerve fibers enter the posterior roots of the spinal cord, while the efferent (motor) fibers leave the anterior roots of the spinal cord in a monosynaptic reflex (Fig. 1.30). The Bell – Magendie law states that the nerve impulses are conducted in only one direction with the dorsal (posterior) roots containing only the incoming sensory information and the anterior (dorsal) root conducting the motor signals. In this simple spinal arc the reflex travels to the spinal cord and back taking the shortest distance. Another simple reflex arc is the "withdrawal" reflex upon pulling away from painful stimuli. These monosynaptic reflexes contain only one synapse crossing the sensory and motor neuron. Polysynaptic reflexes occur more often and are more common involving interneurons and integration centers in the spinal cord, in the brainstem or in the cerebrum, the center of the consciousness.

1.15.3.1 Endocrine, Nervous, Neuroendocrine Reflexes

Simple nervous feedback loops as already mentioned consist of only two major components:

1. Initial stimulus that generates a response
2. Counteraction (negative feedback) or reinforcement (positive feedback) *via* the response loop.

Simple endocrine reflex consist of at least four components, less than those of the nervous reflexes.

The components of a *simple endocrine reflex* are:

Internal or external change → Sensory center → Efferent hormonal signal → Effectors

Complex nervous reflexes consist of six or more components. Parts of such a nervous reflex are:

1. Internal or external change
2. Receptor: located at the end of the sensory neuron and react to the stimulus
3. Sensory (afferent) neuron: conducts the nerve impulse along the afferent pathway to the central nervous system
4. Integration center: consists of one or more synapses in the central nervous system (CNS). The nerve impulse passes down to dendrite, through the nerve cell body, and down the axon. At the end of the axon, the impulse encounters a fluid-filled space separating the end of the axon from the dendrite of the next neuron or from a muscle cell. This space is the synapse. A synapse located at the junction of a neuron and a muscle fiber is a neuromuscular junction.
5. Motor (efferent) neuron: conducts the nerve impulse from the integration center to the effector (muscle, gland).
6. Effector: responds to the efferent impulses by contraction (muscle fiber) or by secreting a product (gland).

Complex neuroendocrine reflexes consist of even more components:

Internal or external change → Receptor → Afferent sensory path → Nervous integrating center → Efferent neuron or neurohormone → Endocrine integrating center → Efferent center (2nd hormone) → Effectors.

It is still debated whether all conscious responses that always show the same pattern should be considered reflexes. Thus distinction should be made between reflexes and reactions. Reactions differ from reflexes in the sense that they are voluntary actions to an environmental stimulus and may have different responses, e.g. cold can be prevented by heating, taking a bath or putting on more clothes.

1.16 Metabolic Pathways

Upon various stimuli series of chemical reactions occur within the target cells. The pathways of the metabolic network consists of a series of reactions the number of which is almost incomprehensible, resembling the streets of a metropolis, with the notable exception, that arrows of the pathways indicate chemical reactions rather than streets. The metabolic strategy of consecutive reactions is to couple energetically unfavorable endergonic step(s) with energetically favorable (exergonic) step(s) so that the overall energy change for the coupled reactions is favorable. Organisms maintain homeostasis by keeping the concentrations of most metabolites at steady state, utilizing four major network motifs: linear pathways, spiral pathways, metabolic cycles and complex branched pathways.

1.16.1 Linear Pathways

Linear pathways represent the simplest network motif and represent a good starting point to gain insight how cellular networks operate. The simplest linear pathway is one where the kinetics is driven by the mass-action. Beside linear reactions (e.g. glycolysis) there are spiral pathways (e.g. β-oxidation of fatty acids), metabolic cycles (e.g. citrate cycle) and branching pathways (e.g. metabolism of amino acids) that contribute to the efficiency of the chemical work, maintain the thermodynamic steady state and minimize the entropy (molecular randomness) in living cells. Although, a single pathway or a set of linear pathways could by no means represent the full range of reactions of the metabolic network, nevertheless the glycolysis as one of the oldest metabolic route is a good example of linear pathways (Fig. 1.31).

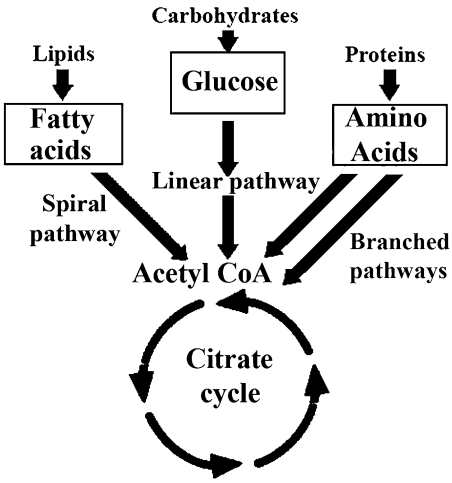

Fig. 1.31 Linear, spiral, cyclic and branched metabolic pathways. Linear pathway in the scheme is represented by glycolysis, spiral pathway by the beta oxidation of fatty acids, cycle by the tricarboxylate (citrate) cycle and branched pathways by the conversions of amino acids

1.16.2 Metabolic Cycles

Similarly to streets and avenues of cities that are connected through circles without going back to the city center, metabolic cycles connect linear pathways and other cycles. Metabolic cycles are among the most important biochemical pathways providing continuous supply of metabolites for anabolic and catabolic processes. The best known examples among the metabolic cycles are the citrate cycle (also referred to as tricarboxylate, TCA, Krebs cycle and Szent-Györgyi – Krebs cycle) that lies at the heart of carbohydrate metabolism and the urea cycle (Krebs and Johnson 1937; Holmes 1980), responsible for the removal of urea converted from the more toxic ammonia. Other metabolic cycles are less well-known, although from metabolic point of view equally important (Horecker 1976; Wood 1985; Meister and Anderson 1983).

The compilation of the skeletal models of metabolic cycles resulted in a cyclic network named the "metabolic clockwork" (Fig. 1.32) with the citrate cycle occupying the central position and regulatory role in this metabolic clockwork (Banfalvi 1994). The term clockwork sounds rather mechanical and is used mainly to give an idea of an interwoven network of chemical reactions which do not proceed as isolated systems. Several known series of reactions belong to the metabolic clockwork (Fig. 1.32).

The metabolic clockwork:

1. relates a variety of metabolic cycles that occur in cells,
2. contains the citrate cycle regarded as the central element of the clockwork,
3. permits the description of new cycles (chemical reactions, enzymes, substrates),
4. considers the tricarboxylate hemicycle to be the most conserved part of the citrate cycle since citrate as a tertiary alcohol could not be oxidized without the breakage of citrate itself and thus had to be converted to the secondary alcohol isocitrate to be able to continue its oxidation,
5. indicates that the number of cycles may vary with cell type.

1.16.3 Spiral Pathways

They are similar to cyclic pathways consisting of a series of repeated reactions to break down or build up a molecule, but come to an end. The same enzymes are involved in each turn of the spiral. The size of the molecule increases or decreases (e.g. fatty acid) until the largest or smallest size of the target molecule is reached, where the pathway ends. Reactions of spiral pathways form a loop with intermediates constantly regenerated. Each turn of a spiral in beta oxidation of fatty acids produces one molecule each of acetyl-CoA, $FADH_2$ and $NADH + H^+$.

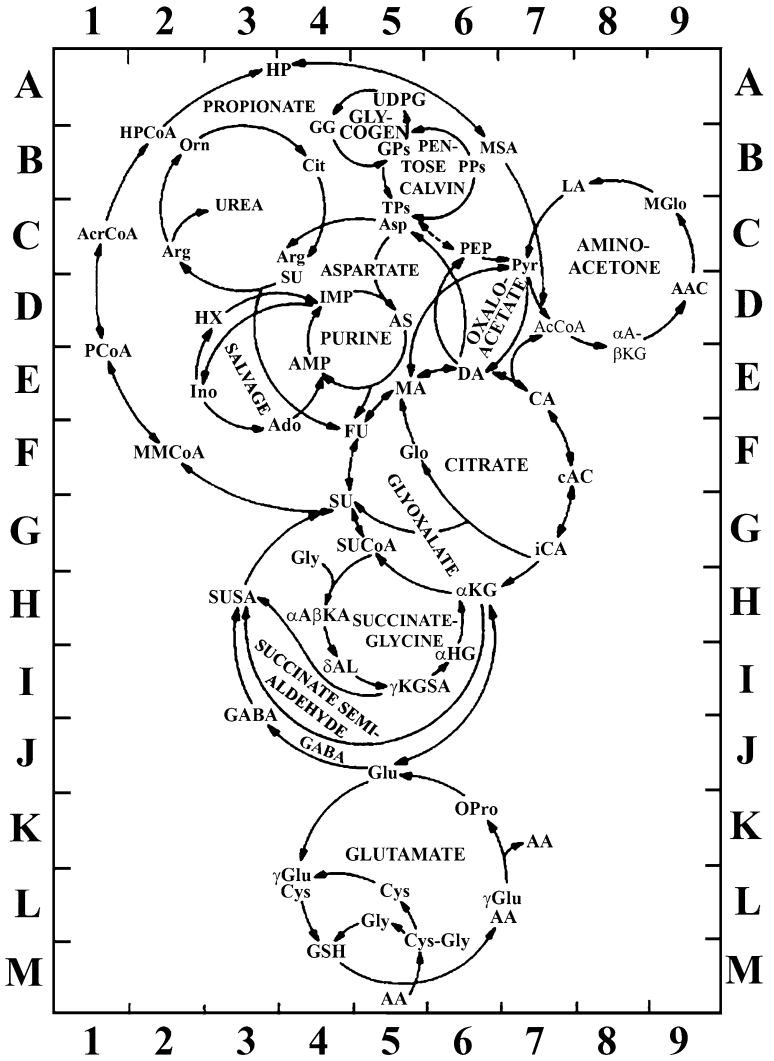

Fig. 1.32 Schematic view of the metabolic clockwork. Metabolic cycles contain the names of their metabolites in abbreviated form. Abbreviations and cycles in alphabetical order are listed below. Position of cycles are indicated in parentheses. Note: no all intermediates in a cycle may be shown. *One-headed arrows* show unidirectional, *two-headed arrows* bidirectional reactions. *1. Adenosylsuccinate cycle: AS* adenosylsuccinate, *FU* fumarate, *MA* malate, *OA* oxaloacetate, *Asp* aspartate (C-F, 5-6). *2. Aminoacetone cycle: AcCoA* AcetylCoenzyme A, *αAβKB* α-amino-β-ketobutyrate, *AAc* aminoacetone, *MGlo* methyl-glyoxale, *LA* lactic acid, *Pyr* pyruvate (C-D, 7-9). *3. GABA cycle: α-KG* α-ketoglutarate, *Glu* glutamate, *GABA* γ-aminobutyrate, *SUSA* succinate semialdehyde, *SU* succinate, *FU* fumarate, *MA* malate, *OA* oxaloacetate, *CA* citrate, *cAC* cis-aconitate, *iCA*, isocitrate (G-J, 3-6). *4. Aspartate cycle: OA* oxaloacetate, *Asp* aspartate, *ArgSU* argininosuccinate, *FU* fumarate, *MA* malate (C-F, 3-6). *5. Aspartate-purine cycle: Asp* aspartate, *AS* adenylosuccinate, *FU* fumarate, *MA* malate, *OA* oxaloacetate, (C-F, 5-6). *6. Calvin cycle: PPs* pentose phosphates, *TPs* triose-phosphates (B-C, 5-6). *7. Citrate cycle: OA* oxaloacetate, *CA*

1.16.4 Complex Metabolic Pathways

These branched pathways consist of multiple pathways that interact biochemically. These are the most frequently occurring ones in the metabolic network and provide a more complete view of metabolic processes (Michal 1999; Pitkänen et al. 2009). Here two test cases for branched metabolic pathways are mentioned. One of the representative test cases of branching points in glycolysis is the α-D-Glucose 6-Phosphate (G6P), a common form of intracellular glucose that can be converted in four different pathways. Pyruvate is another important branching point in glycolysis discussed under "Optimal systems" and "Major pathways connected with glucose metabolism" (Fig. 1.3).

1.16.5 Regulation of Metabolic Pathways

1.16.5.1 Regulation of Linear Pathways

Metabolic pathways are open systems, which operate under near-steady state conditions, with substrates constantly flowing in and products flowing out. To determine whether or not the carbohydrate metabolism is in equilibrium, the intracellular concentrations of metabolic intermediates have to be measured in

Fig. 1.32 (continued) citrate, *cAC* cis-aconitate, *iCA* isocitrate, *αKG* α-ketoglutarate, *SUCoA* succinylCoA, *SU* succinate, *FU* fumarate, *MA* malate (E-G, 5-7). *8. Glutamate (Meister) cycle: Glu* glutamate, *γ-GuCys* γ-glutamyl cysteine, *GSH* glutathione, *Cys* cysteine, *Gly* glycine, *AA* amino acid, *OPro* 5-oxoproline, (K-M, 4-6). 9. *Glycogen cycle: GPs* glucose phosphates, *UDPG* UDP-glucose, *GG* glycogen (A-B, 5). *10. Glyoxylate cycle: OA* oxaloacetate, *CA* citrate, *cAC* cis-aconitate, *iCA* isocitrate, *Glo* glyoxylate, *SU* succinate, *FU* fumarate, *MA* malate (E-G, 5-7). *11. γ-Ketoglutarate semialdehyde cycle: γ-KGSA* γ-ketoglutarate semialdehyde, *SU* succinate, *SUCoA* succinylCoA, *Gly* glycine, *α-AβKA* α-amino β-ketoadipate, *δAL* δ-aminolevulinate (H-I, 3-5). *12. Oxaloacetate cycle: OA* oxaloacetate, *PEP* phosphoenolpyruvate, *Pyr* pyruvate (C-E, 6-7). *13. Pentose cycle: GPs* glucose phosphates, *PPs* pentose phosphates, *TPs* triose phosphates (B5-6). *14. Propionate cycle: SU* succinate, *MMCoA* methylmalonylCoA, *PCoA* propionylCoA, *AcrCoA* acrylylCoA, *HPCoA* hydroxypropionylCoA, *HP* hydroxypropionate, *MSA* malonate semialdehyde, *AcCoA* acetylCoA, *CA* citrate, *cAc* cis-aconitate, *iCA* isocitrate, *α-KG* α-ketoglutarate, *SUCoA* succinylCoA (A-G, 1-7). *15. Purine (nucleotide) cycle: IMP* inosine-5′-monophosphate, *AS* adenosylsuccinate, *AMP* adenosine-5′-monophosphate (D-E, 4-5). *16. Pyruvate (pyruvate-malate) cycle: Pyr* pyruvate, *OA* oxaloacetate, *MA* malate, (C-E, 5-7). *17. Salvage cycle I: AMP* adenosine-5′-monophosphate, *IMP* inosine-5′-monophosphate, *Ino* inosine, *Ado* adenosine (D-E, 3-4). 18. *Salvage cycle II: IMP* inosine-5′-monophosphate, *Ino* inosine, HX hypoxanthine (D-E, 3-4). *19. Succinate-glycine cycle: SUCoA* succinylCoA, *Gly* glycine, *αAβKA* α-amino β-ketoadipate, *δAL* δ-aminolevulinate, *γKGSA* γ-ketoglutarate semialdehyde, *αHG* α-hydroxyglutarate, *αKG* α-ketoglutarate (H-I, 5-6). *20. Succinate semialdehyde cycle: αKG* α-ketoglutarate, *SUSA* succinate semialdehyde, *FU* fumarate, *MA* malate, *OA* oxaloacetate, *CA* citrate, *cAC* cis-aconitate, *iCA* isocitrate (G-J, 3-6). *21. Urea cycle: Orn* ornithine, *Cit* citrulline, *ArgSU* argininosuccinate, *Arg* arginine (B-C, 3-4) (With permission Banfalvi 1994)

living cells. These measurements reflect the physiological concentrations, but do not correspond to equilibrium concentrations where all reactants and products would be at their equilibrium concentrations, as physiological levels oscillate. Rate limiting steps are critical regulatory points.

1.16.5.1.1 Glycolysis

In the glycolytic pathway the phosphofructokinase 1-catalyzed reaction:

$$\text{Fructose 6-P} + \text{ATP} \rightarrow \text{Fructose 1, 6 bisphosphate} + \text{ADP}$$

is the rate limiting step, providing an excellent opportunity for the regulation of this linear pathway. Consequently, in glycolysis from the molar concentrations of fructose 6-phosphate, fructose 1,6-bisphosphate, ATP and ADP (Williamson 1965) the mass action ratio (Q):

$$\text{Q} = \frac{[\text{fructose 1, 6-bisphosphate}]\,[\text{ADP}]}{[\text{fructose 6-phosphate}]\,[\text{ATP}]}$$

can be determined for the phosphofructokinase 1 (PFK 1) and compared with its equilibrium constant (K′eq). This and many other reactions in the cell turned out not to be in equilibrium. ATP is a substrate and an allosteric inhibitor of PFK-1. Binding of ATP to the catalytic site of PFK-1 increases its activity, whereas binding to the allosteric site inhibits activity.

1.16.5.1.2 Pasteur Effect

In the absence of O_2 ATP is produced in the glucose metabolism *via* the reductive glycolytic pathway by lactic acid fermentation. This produces 2 lactate and 2 ATP molecules per glucose. Under aerobic conditions oxidative phosphorylation produces a maximum of 38 ATP per glucose and causes a drastic reduction in glucose consumption. The Pasteur effect demonstrated that it was the energy (ATP level) production and need that controlled primarily the rate of glycolysis and homeostasis.

1.16.5.1.3 Glycogen Metabolism

The concentration of glucose in human blood plasma is maintained at about 5 mM by hormonal mechanisms, insulin being responsible helping to remove and glucagon to increase glucose level in blood. Glucose is polymerized to glycogen and stored in the muscle and in the liver. The primary function of glycogen in the muscle is to supply ATP energy *via* conversion to glucose 1-phosphate → glucose 6

phosphate → glycoslysis and lactic acid fermentation. It is the liver glycogen that maintains blood glucose level. The drop of blood glucose level between meals triggers the release of glucagon by the pancreas. The hormone carried by the blood to the liver binds to its transmembrane receptors and stimulates the cAMP-dependent phosphorylation cascade, activates glycogen phosphorylase that releases glucose 1-P from glycogen (Meléndez-Hevia et al. 1993). While glycogen provides relatively fast glucose and ATP energy supply for muscles (e.g. short dashes), energy for extended periods of time is stored by fats, and released primarily by mitochondrial beta oxidation of fatty acids to acetyl-CoA channeled to the citrate cycle and terminal oxidation. Fat as storage energy is serving the long-term energy need of migrating birds, camels, camelids (llama, vicuña, alpaca), brown fat the heat supply in newborns and in hibernating mammals.

1.16.5.2 Regulation of Metabolic Cycles

The regulation of the citrate cycle is related to need for ATP. When the ATP level drops the concomitant increase of ADP and AMP takes place. Positive modulators (activators) of the cycle are the pyruvate dehydrogenase complex, the citrate synthase and isocitrate dehydrogenase enzymes. The wheel of the citrate cycle will be slowed down when the concentration of the ATP and the reducing equivalents ($NADH + H^+$ and $FADH_2$) increase resulting in the inhibition of the same three enzymes.

1.17 Maintaining Human Homeostasis

Homeostatic systems react to changes in the environment and random disturbances through a series of modifications of equal size but opposite direction to those that generated the disturbance. Among the factors that cause disturbances are allostatic factors, metabolic factors, malfunction of regulatory systems, mechanisms upsetting the biological balance of the organism and panic syndromes. The maintenance of homeostasis in the human body is securing the balance and function of organs within their normal physiological range. These mechanisms include the regulation of: body water and body fluid, osmoregulation, ion balance, acid-base (pH) balance, temperature, energy storage and balance, blood composition (sugar, fats), blood volume, hemostasis, blood pressure. These human homeostatic mechanisms will be dealt with individually.

1.17.1 Body Water and Body Fluid

Body fluid and cardiovascular homeostasis is maintained by multiple control systems with physiological (i.e., autonomic nervous system; hormonal) and behavioral

effectors coordinated by the integrative capacity of central nervous system. Thirst for water or hunger for sodium are perceptions that result from stimuli of metabolic processes signaled to and carried out in the brain. The central nervous system receives these visceral and somatic sensory inputs and after integration calculated decisions and appropriate measures are made through reflexes and receptors to optimize the fluid distribution in body compartments and to restore the hydromineral balance (Johnson 2007).

Among the mechanisms that regulate body water and body fluid balance are those that stabilize the intracellular and extracellular ion concentrations primarily sodium and potassium balance known as normal potential driven by the Na^+-K^+ pump practically in each cell of the body. The loss of normal potential maintained by the Na^+-K^+ pump has special functional implications in striated muscle, heart muscle and in nerve cells known as action potential. As the fluid homeostasis is closely related to the ion and water balance it has several common aspects such as:

- the evolution of osmoregulation both in terrestrial and water stressed animals (summarized in Table 1.1),
- coping with differences in water availability,
- transport through epithelia,
- excretion of nitrogenous waste with particular attention to vertebrate kidney and mammalian nephron functions,
- glomerular filtration and tubular resorption regulated by intrinsic and extrinsic mechanisms,
- interaction between blood circulation and excretion by the kidney.

1.17.2 Sodium Homeostasis of Blood

Excess water consumption. The maintenance of water balance is best exemplified by either drinking a larger volume (>1 l) of water or by reduced water consumption. Excess water will be filtered out by the glomerular filtration lowering to some extent the blood sodium concentration. This in turn will reduce Na^+ filtration of glomeruli acting back on nephron reabsorption of sodium generating excess water in the filtrate. Reduced sodium concentration sensed by the osmoreceptors, will lead to the secretion of less antidiuretic hormone (ADH = vasopressin), the collecting ducts reabsorb more water and results in more dilute urine. Excretion of excess water brings back the sodium concentration of the blood to its normal level. When sodium and water sources are different it is likely that the rates of their intake and loss will be regulated independently (Johnson 2007).

Reduced water consumption. In this case less water will be absorbed by the intestine, the amount of water will be lowered, the sodium concentration elevated in the blood, glomerular filtrate will be reduced, some Na^+ will be left in the filtrate after reabsorption by the nephrons. Increased Na^+ in the blood is sensed by the osmoreceptors generating more ADH, collecting ducts reabsorb more Na^+

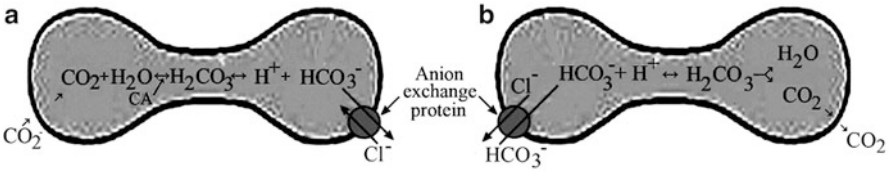

Fig. 1.33 Attendance of anion exchange protein in carbon dioxide transport and pH regulation. Blood cells are impermeable to hydrogen ions and trap them within the cell. (**a**) Peripheral blood cells take up carbon dioxide and convert it to carbonic acid catalyzed by carbonic anhydrase. Carbonic acid being a weak acid partially dissociates to protons and hydrocarbonate ions. In response to metabolism and CO_2 production in peripheral tissues the intracellular bicarbonate level increases and the anion exchanger begins to import chloride and extrude hydrocarbonate ions in a process known as chloride or Hamburger shift (named after Hartog Jakob Hamburger). (**b**) Hydrocarbonate is transported by the blood plasma, protons are carried by the hemoglobin of the red blood to the lung. In the lung the exchanger of the red blood cells work in opposite direction. Hydrocarbonate enters blood cells in exchange of chloride ions. Hydrocarbonate and proton associate to carbonic acid. Carbonic acid disproportionates to water and carbon dioxide. This way the excess of protons and carbon dioxide are removed. Carbon dioxide crosses the plasma membrane of red blood cells, passes the capillaries of the lung, enters the alveoli of the lung and is finally exhaled from the lung

in the loop of Henle, producing concentrated urine. After the removal of Na^+ and reabsorbing more water in the loop of Henle, the sodium concentration of blood normalizes.

1.17.3 pH Homeostasis of Blood

The pH of human blood is maintained within narrow limits (pH 7.38–7.42) by keeping the ratio of hydrogen ion and bicarbonate constant. Constant hydrogen ion concentration is secured primarily by the bicarbonate buffer, the major buffer system of the organism. The bicarbonate buffer is composed of carbonic acid represented by its hydrogen ions and sodium bicarbonate. Sodium bicarbonate production from carbon dioxide in red blood cells is catalyzed by the enzyme carbonic anhydrase (CA) (Fig. 1.33).

1.17.4 Regulation of Body Temperature

Heat regulation is the ability of warm blooded animals to keep their body temperature constant irrespective of the external temperature. The constant temperature is the result of the dynamic equilibrium maintained by heat production and exothermic activity. The reflex process is regulated by the hypothalamic heat center. When it is cold the vessels of the skin are constricted to prevent heat loss, increased metabolism

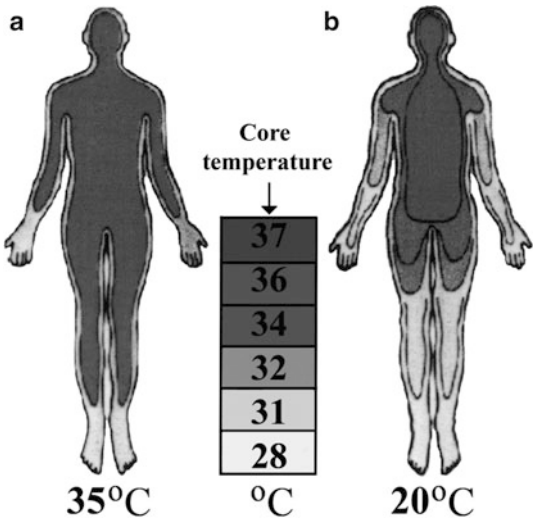

Fig. 1.34 Temperature zones of the body. (**a**) at 35 °C and (**b**) at 20 °C ambient temperatures presuming a nude body and dry air

of the muscles (shivering, trembling) produce additional heat, while the heat center initiates dermovascular expansion and reduced heat production.

The human body is able to regulate its core temperature between 36.7 and 37.8 °C under ambient temperatures between 20 and 54.4 °C (Guyton 1971). Figure 1.34 shows how the temperature of peripheral body parts is decreasing relative to the core temperature when the ambient temperature drops from 35 to 20 °C. The core temperature is optimal for body function and refers to those organs and parts of the body that are well insulated and located in the thoracal and the abdominal cavities. The core temperature is opposed by the fluctuations of the temperatures of the skin, legs, hands and other surface areas. The accepted average human core temperature is 37 °C (± 0.7) when measured internally. Oral, rectal, gut, and core body temperatures are slightly different, but well-correlated. Oral temperature is the lowest of the four. Oral temperatures are generally 0.4 °C lower than rectal temperatures (Longo et al. 2011).

The body temperature is not only regulated by central feedback mechanisms operated primarily by the hypothalamus, but also by temperature sensors. At skin temperatures higher than 37 °C sweating begins and increases above this temperature. When the skin temperature drops below 37 °C increased heat generation and conservation in man is supported by:

- reduction or cessation of sweating,
- vasoconstriction of skin vessels,
- shivering and trembling of striated muscles,
- hormonal (thyroxin) and neuroendocrine (epinephrine, norepinephrine) activation of heat production,
- brown fat cells in newborn infants (5 % of body mass) and hibernating animals generate heat by non-shivering thermogenesis in their mitochondria to avoid

hypothermia. Infants are more susceptible to cold than adults. Brown adipocytes express mitochondrial uncoupling protein 1 (UCP1 = thermogenin) enabling their mitochondria to uncouple respiration from ATP synthesis.
– and utilize substrates ($NADH + H^+$ and $FADH_2$) to generate heat rather than ATP.

1.17.5 Energy Storage

The two major energy sources available to living organisms are: sunlight and reduction-oxidation reactions. The energy cycle for life is fueled by the Sun. The major energy product for plants and animals is the highly energetic molecule ATP. The light energy from the Sun is captured by photophosphorylation. Animals gain ATP energy produced in redox reactions.

Substrate level phosphorylation. Under anaerobic conditions substrate level phosphorylation takes place in animal cells in reactions resulting in ATP by the direct transfer of a phosphoryl group to ADP.

Oxidative phosphorylation. Under aerobic conditions certain reduced forms of high energy molecules such as NADH and $FADH_2$ can donate their electrons to electron carriers of the electron transport chain which results in the production of ATP through oxidative phosphorylation.

Energy reserves of the body beside ATP are other ribonucleoside triphosphates (NTPs), primarily GTP, creatine phosphate, glycogen, fat and protein. Most of the ATP is stored in the muscles and in the liver. Major ATP consuming processes are mechanical work, transport and biosynthetic processes (Fig. 1.6). When ATP and GTP stores are exhausted, a relatively fast supply is provided by substrate level phosphorylation involving among others the high energy containing creatine phosphate. ATP and creatine phosphate serve the short-term energy need of the organism. Longer (1–2 days) energy supply is provided by carbohydrates, primarily glycogen. Under hypoxic conditions in the muscle the blood circulation cannot cope with oxygen need, the glucose released from glycogen stores undergoes exclusively glycolysis. Glycolysis without further oxidative breakdown of pyruvate is an unfavorable energy producing process. Most of the energy of the organism is stored and supplied as neutral fat. Its hydrolytic breakdown produces active acetate (acetyl-CoA) and glycerol. The burning of carbon atoms of acetate to CO_2 in the citrate cycle and the H atoms to H_2O in terminal oxidation is a relatively slow process and needs perfect blood and oxygen supply for mitochondrial oxidation and ATP production. After the fat stores are depleted the final energy provider for the organism is protein, leading to muscle loss, and to the more dangerous terminal cachexia and death.

Heat transfer processes (radiation, conduction, convection and evaporation) that are involved in the exchange of heat between an organism and its environment affect the physiological, behavioral and ecological activities and the energy budgets of organisms. These processes were summarized in Fig. 1.16.

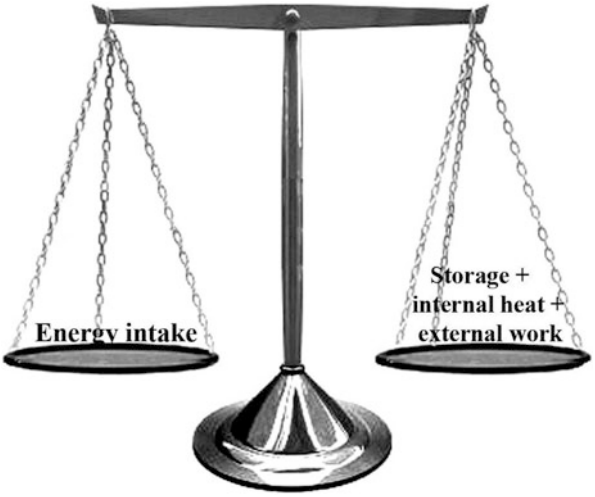

Fig. 1.35 Energy balance in living organisms with energy intake and consumption. Energy intake at one side is counterbalanced by the internal heat, external work and storage energy at the other side of the scalepan

1.17.6 Energy Balance

The regulatory process termed energy homeostasis is the balance of energy consumption by energy uptake in the form of food and its mobilization and consumption in the organism. The energy intake is properly balanced by heat production, storage energy and external work (Fig. 1.35). The neuropeptide leptin is the major mediator of long-term regulation of energy balance, suppressing excess food intake and thereby inducing weight loss (Klok et al. 2007).

1.17.6.1 Energy Imbalances

From evolutionary point of view there is a constant fight among species for food and survival. It was realized only in the last century, that what we consider regular human consumption may lead to body's energy imbalances and ultimately become manifest as diseases. Energy balance is a long-term rather than a short-term process. The two major energy imbalances are undernutrition and obesity.

Undernutrition develops in individuals suffering from inadequate provision of energy from nonprotein or protein sources. Protein-energy malnutrition includes a range of disorders of starvation and malnutrition that involves the lack of vitamins and minerals in addition to protein deficiency. Two extreme forms of malnutrition are deficiency of protein (e.g. *Kwashiorkor*) and deficiency of both energy and protein (e.g. *marasmus*).

Obesity is regarded as a chronic metabolic disease caused by the imbalance between higher energy input and lower output. Among the causes of obesity are genetic and environmental factors such as: excessive food and caloric intake, limited physical activity, genetic predisposition, familiar history, special metabolic disorders, behavioral factors. Excess consumption and compounds releasing extra energy are disturbing the biological energy balance. The overconsumption of foods, primarily natural sweeteners, oil, liquid, dairy products is the primary cause of degenerative diseases. The initial burst of energy caused also by caffeine, alcohol and several drugs is quickly abated, and followed by lethargy.

Dietary restriction can inhibit degenerative diseases including tumor formation and increase life-span. This recognition helped to clarify that the mechanisms upon food depletion have circulatory (hormonal and cytokine) consequences. Energy imbalances demonstrated that molecular characteristics of tumors determine the extent to which they are influenced by variation in host energy intake (Pollak 2012). It was recognized that a subset of tumors are growth-stimulated by excess caloric intake, and provided strong circumstantial evidence that hyperinsulinemia is one of the mediating factors.

1.17.7 Blood Composition

Blood is a connective tissue circulating inside the blood vessels. The wall of the vessel has three layers: the outer (*Tunica adventia*), the middle (*Tunica media*) and the internal (*Tunica intima*) layers. The blood consists of:

(a) plasma or extracellular fluid, corresponding to the internal environment (*milieu intérior*) of animals
(b) formed elements (red and white blood cells and platelets)

Although, the formed elements of blood, the production of blood (*haemopoiesis*), red blood cells (*erythropoiesis*), white blood cells (*leukopoiesis*) and platelets (*thrombopoiesis*) and the aging of red blood cells all play some part in homeostasis, major regulation takes place in blood plasma. As this book deals primarily with the homeostasis of the internal environment, the composition of the blood plasma deserves more attention. The blood plasma is a mixture of proteins, enzymes, nutrients and waste products, hormones and gases. Major plasma proteins are: albumins, globulins and fibrinogen. Nutrients (glucose, amino acids, lipids, cholesterol, phospholipids, vitamins, minerals) are absorbed by the digestive tract and transported by the blood plasma to the tissues and cells. As gases and particles larger than colloids would cause emboli in blood vessels, oxygen is bound to hemoglobin and carried by the red blood cells from the lung to the tissues, while carbon dioxide is transported as hydrocarbonate by the blood plasma to the lung (Fig. 1.33b). Nitrogen which has a relatively high solubility is also transported by the blood plasma. Sodium as the most abundant ion accounts for most of the osmolarity of the blood. Amino acids in the circulation come from broken down tissues and from digested proteins.

Ammonia is a toxic waste product produced during the removal of nitrogen from proteins and nucleic acid bases. Terrestrial animals convert ammonia to uric acid and urea as these are less toxic and can permeate membranes. Snails, insects, birds and some reptiles (lizards, snakes, terrestrial turtles) excrete the relatively insoluble uric acid as their major nitrogenous waste. Uric acid is precipitated, stored and left behind in eggs after hatching. In man the deposition of uric acid crystals in the joints due to their lower body temperature and to the reduced solubility of uric acid causes recurrent attacks of acute inflammatory arthritis known as gout. Urea has a high solubility and is about 10^5 times less toxic than ammonia, consequently it can be present in relatively high concentration in the blood. Nitrogenous by-products are carried by the bloodstream to and excreted by the kidneys.

Plasma proteins serve several other functions in transport, defense, and blood clotting. Their homeostatic importance comprises reserve supply of amino acids for nutrition, carrier function for other molecules, stabilization of the slightly basic character of blood (pH 7.4). Plasma proteins help to maintain the fluidity of blood and respond to vascular injury protecting against loss of blood by coagulation and invasion by microorganisms and viruses. Plasma proteins regulate the distribution of water and tissue fluids *via* the colloid osmotic (oncotic) pressure. The homeostatic function of blood dealing with its consistency (fluidity and coagulation) known as hemostasis will not be detailed.

1.17.7.1 Blood Glucose Level

The blood glucose (sugar) and the blood lipids represent the primary source and store of energy for animal and human tissues. Constant supply of glucose takes place *via* the blood stream from the intestines to the liver and the body cells and from the liver to other tissues and cells. There are two types of antagonistic hormones that keep the blood glucose levels within narrow, homeostatic rage:

– ***Catabolic hormones*** increasing blood glucose level:

  - *Glucagon* causes the liver to convert stored glycogen into glucose *via* glycogenolysis.
  - *Corticosteroids*, primarily cortisol are released in response to stress and a reduced level of blood glucocorticoids. Cortisol increases blood sugar through gluconeogenesis from non-carbohydrate carbon substrates (pyruvate, lactate, glycerol, glucogenic amino acids, and odd-chain fatty acids) and inhibit glucose uptake by cells through the insulin-regulated glucose transporter GLUT-4.
  - *Catecholamine* (primarily epinephrine and norepinephrine derived from tyrosine in the adrenal gland) level in blood is associated with stress and causes typical sympathetic (fight or flight) responses, among them increase in blood glucose level. Glucogenolysis, the release of glucose from glycogen store of hepatocytes is directly stimulated by epinephrine (also known as adrenaline) and indirectly through the inhibition of insulin release by norepinephrine (noradrenaline). Indirect effects of epinephrine are the release of

growth hormone and adrenocorticotropic hormone. Increased glucose level in response to catecholamines is a fast and intermittent effect.

- *Growth hormone* is released from the pituitary upon induction by growth hormone-releasing hormone secreted by the hypothalamus in response to epinephrine or low blood glucose level. Growth hormone increases blood glucose level by the inhibition of glucose uptake and promotion of glycogenolysis in muscle.

– ***Anabolic insulin*** is the major hormone that decreases blood glucose level. Insulin release is stimulated by glucose, amino acids, and hormones (glucagon, epinephrine, growth hormone). Reduction of blood glucose is promoted through GLUT-4 transporter and uptake of glucose by liver, muscle and other tissue cells. Insulin reduces glucose production by inhibiting gluconeogenesis and glycogenolysis. Other effects of insulin include stimulation of lipid biosynthesis, adipogenesis, glycogen synthesis, cellular uptake of K^+, phosphate and Mg^{2+}.

Abnormalities in blood glucose level are *hyper-* and *hypoglycemia.* High blood sugar level (*hyperglycemia*) is most commonly caused by diabetes with several long-term health problems affecting heart, eye, kidney and nerve functions. Low blood glucose level (*hypoglycemia*) has potentially fatal consequences from lethargy, irritability, weakness, sweating to paranoid and aggressive behavior, loss of consciousness and brain damages.

1.17.7.2 Blood Volume

The major homeostatic values that are kept constant within animal species are body temperature, pH, osmolarity, blood composition. The blood volume of an adult individual is also kept constant, the expansion of blood volume can be a natural process or can be artificially increased (hypervolemia) e.g. as a consequence of endurance exercise training.

The approximate total blood volume gradually increases with age from the premature infant of 1.5 kg, containing 134–158 ml blood to adults of 70 kg, 4,760–6,160 ml (Nathan and Orkin 1998). The body of an adult male contains about 5 l of blood, that of a women or a child less (Starr and Taggart 1989; Encyclopedia Britannica 1973). Blood represents about 7 % of the body mass in a person (Cameron et al. 1999). Estimates of blood volume are derived from plasma dilution methods. Circulating blood volumes are given as volume per kg for healthy adult animals. These volumes range between 54 ml/kg (*Rhesus* monkey) and 86 ml/kg (dog). The circulating blood volume in man is an estimated 71 ml/kg.

Evidence supports the notion that chronic hypervolemia associated with exercise training represents a net expansion of total body water (Convertino 1991). Beside the legitimate increase in blood volume under endurance training, the natural expansion of blood volume takes place during altitude acclimatization, and through heat training. The decreased amount of atmospheric oxygen at high altitudes stimulates the body into the production of the hormone erythropoietin (EPO) by the kidneys. Blood volume can be 15 % less in obese and old people. Blood volume is regulated

by the kidneys. When blood volume is too high, sodium and water with it are excreted into the urine. This process is reversed in the kidney by the retention of more sodium and increased amount of water. As blood consists of about 90 % water, reabsorption of sodium will have an immediate impact on blood volume. Artificial increase of red blood cell levels in the blood may expose to increased risk of stroke due to the increased viscosity of the modified blood. Increased blood volume is likely to cause a correspondingly higher blood pressure.

1.17.7.3 Blood Pressure

Blood pressure is the measure of force that blood exerts on the walls of the blood vessels as it passes through them. The heart pumps out blood into the thoracic aorta, originating from the left ventricle of the heart, where the arterial blood pressure is highest and then gradually diminishes across the circulation from aorta → arteries → arterioles → capillaries → to the veins back to the right atrium. The difference between aortic and right atrial pressure generates the blood flow in the circulation. In a normal blood pressure reading (120/80 mmHg) the top number is representing the blood pressure during the contraction of the left ventricle (systolic pressure), and the bottom number the diastolic pressure, that measures the pressure when the heart is resting.

Blood pressure is a function of the amount of blood pumped out by the heart and the degree of resistance of the blood flow exerted by the arteries. The circulatory system (arteries, veins, and capillaries) has a number of mechanisms that control the blood pressure. The aortic arch has inbuilt baroreceptors and chemoreceptors that convey information concerning blood pressure, blood pH and carbon dioxide levels to the lower half of the brainstem (*medulla oblongata).* This information is processed by the autonomic nervous system that mediates homeostatic responses.

Lower blood pressure can indicate low blood volume, or other conditions. High blood pressure, called hypertension is a chronic medical condition (Chobanian et al. 2003), with the most important preventable risk factor for premature death worldwide (World Health Organization 2009). High blood pressure has different stages ranging from mild, stage 1 prehypertension (120–139/80–89 mmHg) to stage 2 high blood pressure (>160/>100 mmHg). High blood pressure forces the heart to pump the blood against a higher resistance of hardening arteries causing atherosclerosis and facing the development of heart failure. Major causes of hypertension are:

- obesity, overweight
- smoking
- lack of physical activity
- high salt content of the diet
- alcohol consumption
- stress
- aging

- genetics
- familial high blood pressure
- chronic kidney insufficiency
- neuroendocrine, endocrine (adrenal, thyroid) disorders
- unknown reasons causing essential hypertension, greatly impacted by lifestyle
- secondary causes of high hypertension (kidney disease, tumors, excess amount of hormone production by the adrenal gland, birth control estrogen containing pills).

High blood pressure is the major risk factor for stroke, heart attacks (myocardial infarction), aneurism (particularly aortic aneurism), arterial and kidney diseases.

1.18 Evolutionary Aspects and Types of Regeneration

1.18.1 Tissue Regeneration

In the process of regeneration the damaged genome, cell, organism, or ecosystems are restored and the disturbed functions are regained. Regeneration is a universal ability of living organisms from bacteria to humans (Gabor and Hotchkiss 1979; Carlson 2007), with the notable difference that regeneration can be complete and the new tissue will be the same as the lost one or only partial restoration occurs. Regular cell growth is necessary for mitotic recombination, fixation of mutations, activation of oncogenes, while increased cell proliferation may accelerate aging, reduce adaptability to environmental stimuli (Weindruch and Walford 1988), accelerate the accumulation of genetic lesions and altered genetic expression, and lead to oncogene amplification (Preston-Martin et al. 1990).

Complete regeneration. This type of regeneration is thought to be a lost phenotype in mammals, as there are only sporadic examples to heal complex tissues in an epimorphic fashion, e.g. to restore a damaged limb or organ to its normal structure and function (Heber-Katz et al. 2004). Similarly to other organs, kidney and heart damages can be regenerated completely in lower vertebrates while partial proliferation and regeneration causes scarring and fibrosis in mammals.

Partial regeneration. In spite of some results obtained with rats and mammals (Becker 1972), adult mammals have only a limited regenerative capability compared to lower vertebrate embryos, larvae, adult salamanders and fish. Children up to age 10 who have lost their fingertips in accidents can regrow the tip of the digit (Illingworth 1974). Due to the unipotency of hepatocytes the human liver has the ability to regenerate from as little as 1/3rd of its tissue. Liver regeneration after partial hepatectomy is characterized by coordinated hepatocellular proliferation to restore the removed parenchyma (Higgins and Anderson 1931). This compensatory proliferation is regulated by circulating mitogenic factors (epidermal growth factor, transforming growth factor, hepatopoietin) and supported by elevated expression of cell cycle associated genes such as c-fos, c-myc, and c-Ha-ras (Fausto and

Mead 1989; Himeno et al. 1989; Michalopoulos 1990). Upon restoration of hepatic parenchyma the proliferation returns to its basal levels involving the reduced expression of transforming factor (TGF-/3) (Fausto and Mead 1989; Michalopoulos 1990). When calorie intake is restricted inducible cellular responses are preserved, but cellular proliferation is reduced and mRNA expression levels of c-fos and c-Ki-ras are lowered by controlling dietary energy (Himeno et al. 1992). Partial surgical resection of liver in rodents has proved to be a useful model to study cell proliferation (Higgins and Anderson 1931; Michalopoulos and DeFrances 1997).

Stem cells. Myocardial infarction causing heart failure remained one of the most fatal diseases. As mammalian cardiomyocytes are known to irreversibly exit the cell cycle at the time of birth, the heart was regarded to lack any regenerative capacity. Recently this paradigm has been shifted and the myocardium has been targeted by different regeneration strategies, primarily by stem cells to regenerate damaged heart muscle after heart attacks (Choi et al. 2011). However, these bone marrow-derived stem cell therapies gave mixed results (Marbán and Malliaras 2012; Hare et al. 2012).

Further and more rigorous studies are required to give a fair judgement regarding the capacity of bone marrow mesenchymal stem cells to differentiate into kidney cells (Singaravelu and Padanilam 2009) or express cardiac specific markers to determine the role of stem cell therapy for patients with ischemic heart disease (Rose et al. 2008). The long-term safety and efficacy of allogenic mesenchymal stem cells for cardiac repair also remain to be established (Vassalli and Moccetti 2011).

1.18.2 Training

The balance between optimal energy supply and consumption is considered to be the most important factor in human homeostasis. Most of the clinical symptoms are directly or indirectly related to the physiological mechanisms of energy homeostasis. There is a tendency in sport to characterize people by the quality of their homeostasis. This theory is based on the adaptation of people living under different conditions and is said to be "sport-specific" in the sense that sportsmen are likely to endure a broader range of homeostatic oscillation than people who are used to "job-specific" adaptive homeostasis. The notion that the higher the quality of life, the stronger the disturbance resistance, has been supported by the observation that athletes have increased life expectancy (Sarna et al 1993) compared to the general population. This observation matched controls who were healthy at young age disease prevention (Kujala et al. 2003).

The increasing knowledge in the field of body weight control by several new regulatory neuropeptides such as leptin, is addressing the unbalance between training load and energy availability (Saris 2001). Leptin produced by adipocytes circulating in the blood is a critical regulator of body weight. It conveys information to the hypothalamus with respect to the energy stored in the adipose tissue. High leptin level in blood suppresses appetite and initiates energy expenditure. This

hormone is also involved in the regulation of reproduction, angiogenesis, immunity, hematopoiesis and bone remodeling (Sousa et al. 2009). A substantial weight loss or in its absence, regular exercise is required for sustained improvement in carbohydrate homeostasis. Prospective studies and clinical trials underline the importance of physical activity (Pan et al. 1997; Helmrich et al. 1991; Manson et al. 1992), especially when physical activity is combined with dietary lifestyle modifications in the prevention of type 2 diabetes (Knowler et al. 2002; Tuomilehto et al. 2001; Eriksson and Lindgarde 1991; DeFronzo et al. 1987).

1.18.2.1 Body Building – Fight Against Homeostasis

During the course of overactive training the lack of energy homeostasis is the basic problem aggravated by excessive calorie intake. Body-building is an unending and constant battle against homeostasis as the person has to be disciplined in his/her attempt to fight it off. The major battleground against homeostasis is nitrogen metabolism, through the constant influx of high quality protein, absorption of amino acids and retention of as much muscle as possible, making it an expensive war.

1.19 Panic Syndromes

Panic syndrome is an anxiety disorder with recurring panic attacks and behavioral changes and ongoing worry of getting further attacks. Panic disorder is a medical condition in some patients combined with fear of public places (*agoraphobia*), although many persons afflicted with panic syndrome do not suffer from agoraphobia. Panic disorder resulting in chemical imbalance acts against homeostasis. The symptoms accompanying panic disorder are similar to and can be mistaken for a life-threatening physical illness such as heart attack. Panic disorder sufferers produce the symptoms and experience extensive chest pain that can distort the diagnosis and can be ruled out only by functional medical tests. Common symptoms of panic attack are: rapid heartbeat (*tachycardia*), hyperventilation, trembling, perspiration, dizziness, dyspnea, sweating, chest pain, nausea, chills or hot flashes, faintness, weakness, etc. The patient is reacting to these symptoms as if real danger would exist and forces him/her to a chaotic and disorderly flight. Upon observation of panic syndromes the patient does not size up the situation, but obeys the syndromes. Panic attacks may take place unexpectedly, driven by depression and anxiety or bounded to certain situations. A typical mediator of panic disorder is the increased anxiety sensitivity affecting hypochondriacal concerns (Noves et al. 2004). Partial pressure of carbon dioxide in the patient's arterial blood is an allostatic mediator in panic disorder (Meuret et al. 2009). Allostatic factors such as hyperglycemia, hyperthyroidism, mitral stenosis, labyrinthitis, pheochromocytoma, respiratory disorders, imbalance within the limbic system, can cause or aggravate panic disorder. Other non-physiological factors: stress events, excessive responsibilities, life transitions,

environmental factors, physical illness, post-traumatic stress disorder, alcoholism, alcohol withdrawal, cigarette smoking and nicotine as a stimulant, psychoactive drugs, certain medications (e.g. spinal block anesthetics), sedative abuse can also trigger panic attack in susceptible patients.

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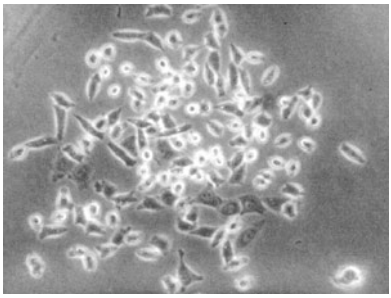
Chapter 2
Cell Cultures



Abstract This chapter describes how eukaryotic cells can be maintained under *in vitro* conditions. Most of the cells constituting the organism are non-dividing, so called resting (G0) cells, not appropriate for propagation. Only those cells can be grown that: (a) can be stimulated and driven back to the cell cycle, (b) are actively growing primary cells, (c) stem cells, (d) immortalized tumor cells. Beside cell growth technical aspects of cell cultivation (culture media and instruments) are discussed.

Keywords Cell cultivation • Organ cultures • Tissue cultures • Cell cultures • Primary tumors • Growth of cells • Culture media • Equipments • Sterile instruments • Sterile work • Deep freezing • Cell synchronization • Types of stem cells

Contents

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2.1 Cell Cultivation

At the beginning of the last century early attempts were made by several investigators and have shown that the cultivation of mammalian cells was not as easy as the artificial growth of microbes. While fermentation by microbes started some 4,000 years ago, mammalian cell cultivation is not much older than 100 years. The reason of this delay is accounted for by the lack of proper mammalian culture media.

2.1.1 Organ and Tissue Cultures

The whole organ or its part can be maintained and its tissues propagated *in vitro* to study differentiation, structure and function. The maintenance and investigation of tissues take place under *in vitro* conditions for at least 1 day either by maintaining tissue fragments or growing cultures from cells obtained from tissues.

2.1.1.1 Perfusion Culture

Culture techniques that provide continuous exchange of culture medium have been regarded superior to static and dynamic cultures since they maintain the protein expression capability and the histological architecture of the perfused organs. Liver perfusion was originally described by Claude Bernard (Bernard 1855) and adapted after a century by others (Miller et al. 1951; Hems et al. 1966; Veneziale et al. 1967). In comparison to other *in vitro* models, the structure and functional integrity of the liver is retained, consequently this experimental perfusion system has been preferred for physiological, pharmacological and pharmacokinetic investigations (Sahin 2003; Schumacher et al. 2007).

Recently the major goal of perfusion operations is to increase cell productivity by feeding bioreactors constantly and remove waste and byproducts. Large-scale high density perfusion bioreactors have been used for cell propagation, bioprocessing, human cell therapy, recombinant antibody production (Lee et al. 2005; Kim et al. 2007; Tang et al. 2007), for scaling up rat bone marrow mesenchymal stem cell (Qian et al. 2013), for human embryonic stem cell production (Fong et al. 2005) and to produce completely glycosylated proteins in slow growing perfusion cultures (Lipscomb et al. 2005). Bioreactors can be operated at high flow rates, while cells are retained by filtration, sedimentation or centrifugation. In spite of these advantages of the perfusion technique, the initial enthusiasm has lost its momentum due to the complexity of the process and the precision needed to maintain such a process for weeks. Another major problem concerns the filtration, keeping the filter from clogging over a longer period of operation. As far as sedimentation devices are concerned, they need specific settling characteristics limiting their commercial application. Centrifugal devices are difficult to keep sterile, are subject to cell damaging by shearing forces and oxygen depletion reducing quality and quantity of cell production, thus not commonly used for cell culturing.

2.1.1.2 Engineering Tissues and Organs

Tissue engineering means to grow tissues *in vitro* (outside the body) and then replace the diseased or damaged tissue within the body. Skin grafting started in the early 1980s, followed first by blood vessels then by the engineering of other tissues (cardiac, pancreatic, cartilage, bladder). As tissue engineering was undertaken for every type of tissue and organ within the urinary system, it was expected that engineered urologic tissues will have a bright future and clinical applicability (Atala 1999; Atala 2000; Atala 2009). Other studies published in this area also emphasize the importance of the future clinical implication of tissue engineering at least in urology (Zini et al. 2004). That tissue engineering is a viable approach to replace diseased or damaged tissues has been demonstrated by the successful transplantation of bioengineered trachea (Chistiakov 2010).

2.2 Cell Cultures

Cell cultures offer the advantage that:

- tissues and organs consist of different cell types. Different cell types can be separated from each other,
- to control the experimental conditions of cell growth is easier than those of intact organisms,
- in tissues it is difficult to separate the "self" properties of cells due to their interactions with other cells.

2.2.1 Adherent and Suspension Cultures

There are two basic types of cell cultures: (a) monolayer and (b) suspension cultures

(a) *Monolayer cultures*. Adherent cells grow attached to the bottom of glass or plastic surfaces and spread as a single layer (monolayer). Growth can be easily followed under inverted microscopes. The cells of the monolayer can be dissociated mechanically or enzymatically (e.g. by trypsin). The major limiting factor of cell growth is the relatively small surface area.

(b) *Suspension cultures* are obtained mainly from hematopoietic cells and consist of round shaped cells that float freely in the medium. To secure proper gas exchange in suspension cultures, agitation, magnetic stirrer and rotating spinner flasks are used. By keeping cells floating, many cell lines can be adapted for suspension culture. The passage of suspension cultures is easier, allowing fast scale-up but requires daily counting and viability check of cells. As it consists of individual cells the suspension culture does not require mechanical or enzymatic dissociation of cells. Suspension cultures can grow up to 5×10^5cells/ml density. Suspension cultures are used for bulk production of cells, batch harvesting with several applications in research.

As an example of monolayer cell culture HaCaT cells and as a suspension culture K562 human erythroleukemia cells are shown in Fig. 2.1.

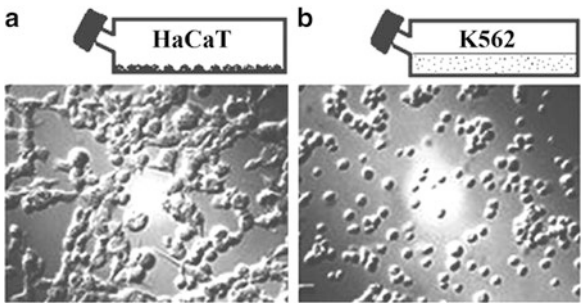

Fig. 2.1 Monolayer HaCaT cells (**a**) and K562 suspension cell culture (**b**)

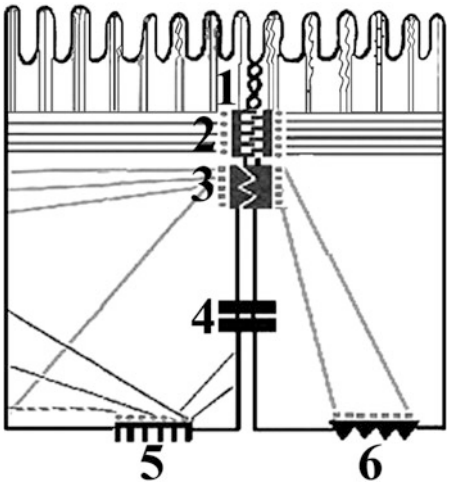

Fig. 2.2 Cell junctions. *1*. Tight junction, *2*. adherent junction (*zonula adherens*, belt desmosome), *3*. desmosome (*macula adherens*), *4*. gap junction, *5*. focal adhesion (adhesion plaque), *6*. hemidesmosome

2.2.2 Cell Junctions

The already mentioned gap junction (nexus) is a specialized intracellular connection between many cell types of animal cells and is analogous to the plasmodesmata of plant cells (Alberts 2002). Different other types of cell junctions contribute to separate or to help cellular communication. Among the different types of other cell junctions found in epithelial cells are: tight junctions (*zonula occludens*), actin bundles of the cytoskeleton (*zonula adherens*), belt desmosomes with intracellular filaments connecting cells (*macula adherens*), spot desmosomes, interconnecting filaments between spot desmosomes and hemidesmosomes.

In many animal tissues each cell is separated by its extracellular coating. However, those cells that are most frequently used for cell growth and have a higher tendency to form tumors (e.g. epithelial cells) have plasma membranes that are continuous with that of adjacent cells. Vertebrate cells can be connected through the following major types of junctions (Fig. 2.2):

- Tight junctions
- Adherent or anchoring junctions
- Gap or communicating junctions
- Desmosomes

1. *Tight junction* (*zonula occludens*). Epithelial cells join together forming an impermeable barrier, similarly to the tiles of a bathroom. The cells exposed to the lumen form the apical surface. Epithelial cells at the basis and at the sides of the tissue form the basolateral surface. The major proteins involved in the junction are claudins and occludins that associate with peripheral membrane proteins

located on the intracellular part of the membrane and anchor to cytoskeletal actin molecules. The tight junction performs two major functions:

- It prevents the passage of molecules and ions through the space between cells. Materials may enter through these surfaces by diffusion or active transport. The barrier function of tight junctions serves the maintenance of osmotic balance and prevents the passage of molecules and ions.
- The tight junction prevents the movement of transmembrane proteins between the outer and inner sides of the membrane contributing to receptor-mediated endocytosis at the apical surface and exocytosis at the basolateral surface.

The largest epithelial surface is provided by the alveoli of the lung ($\sim$80 m^2 in man). Several disorders of the lung (chronic bronchitis caused by smoking, asthma, cystic fibrosis) are related to the increased permeability of tight junctions of the airway epithelium.

2. *Adherent junctions* (*zonula adherens*, belt desmosome) also occur in epithelial tissues. Plasma membranes of neighboring cells are connected *via* strong mechanical attachments built by transmembrane cadherins and catenins connected to cadherins and actin filaments. Other principal interactions in the junction take place among actin, actinin and vinculin. Adherent junctions hold tightly together not only epithelial cells, but also cardiac cells while the heart contracts and expands. Adherent junctions are responsible for contact inhibition.

Contact Inhibition In cell cultures normal cells placed on the tissue culture dish grow only until they reach confluency i.e. the surface of the dish is covered by a single layer (monolayer) of cells touching each other.

3. *Gap junction* (*nexus*). These junctions are analogous to *plasmodesmata* of plant cells. The gap junctions consist of connexons. These cylindrical structures are constructed from six transmembrane proteins called connexins that connect across the intercellular membrane like press-buttons. Connexons are intercellular channels $\sim$1.5–2 nm in diameter permitting the passage of ions and small molecules up to $\sim$1,000 Da. Gap junctions have been found in various animal organs and tissues where fast transmission of signals is required. Gap junctions play an important role in cardiac muscles; they signal the contraction allowing heart muscle cells to contract tandem. Transmissions of gap junctions are performed by electrical synapses in the brain, electric impulses and responses of neighboring cells play role in embryonic, organ and tissue development, and wound healing. Among the few exceptions of cells that have no gap junctions are the adult striated muscle cells, sperm cells and erythrocytes. Inherited disorders of gap junctions (congenital heart defects, congenital deafness) are caused by connexin gene mutations.

4. *Desmosomes* (*macula adherens*, adhering spot) are localized patches of epithelial cells that hold two cells tightly together by a special type of junctional complex. Cells outside are connected *via* the adhesion protein cadherin and inside by the attachment plaque consisting of desmoplakin, plakoglobin, and cytoskeletal filaments of keratin. Desmosomes are present in simple and stratified squamous epithelium and in muscle tissue connecting muscle cells to one another. Pemphigus is an autoimmune disease where antibodies have been developed against constituting proteins (e.g. cadherins) of desmosomes.
5. *Focal adhesions* (cell matrix adhesions) are considered as sub-cellular macromolecules that mediate the regulatory effects (e.g. cell anchorage) of extracellular matrix adhesion on cell behavior (Chen et al. 2003). This type of adhesion provides mechanical linkage to the extracellular matrix and controls signaling proteins related to integrin binding and clustering.
6. *Hemidesmosomes* are small stud- or rivet-head-like structures on the inner basal surface of keratinocytes in the epidermis of the skin. The major difference between desmosomes and hemidesmosomes is that desmosomes link two cells together; hemidesmosomes attach one cell to the extracellular matrix through desmopenetrin cell adhesion proteins. Hemidesmosomes comprise a variety of some ten or more molecular components.

2.3 Establishment of Cell Cultures

Primary cells are obtained directly from tissues or organs. Isolation of primary cells is a medically or physiologically oriented process as cells are obtained from blood or released from tissues by enzymatic digestion (collagenase, trypsin, pronase). Most of the primary cultures have a short life span, are intolerant to freezing and undergo senescence after a limited number of doublings. Primary cultures are likely to be composed of mixtures of cell types. By turning a primary coculture to a cell culture by passaging it under sterile laboratory conditions, a selection takes place in the cell mixture with the fastest growing cells predominating. With subsequent subculturing the cell population becomes a more and more homogeneous monoculture. Cell lines that have been established from tumorous primary cultures are inherently immortal. Other, primarily rodent cells can spontaneously immortalize. These rodent cells undergo a selection crisis at around the 12th generation and only a few cells survive. Viral or plasmid transfection has also been used to immortalize cells (Freshney 2005). For safety considerations at least 20 passages are recommended to claim that a cell culture turned to a cell line consisting of immortalized cells.

The following distinctions can be made among different cell cultures:

(a) *Primary cultures* are fresh isolates of cells isolated directly from tissues, organs, tumors. With the notable exception of cells originating from tumors, primary cultures have a limited life span.

(b) *Cell cultures* are obtained after the 1st passage of primary cultures. Several passages are needed until their cellular composition is stabilized. Cell cultures differentiate to immortal cell lines. A "cell culture" generally refers to the growth of cells derived from eukaryotes, particularly animal cells.
(c) *Cell lines* are stable immortal cell cultures obtained after at least 20 passages.
(d) *Cell strains* are cells adapted to cultures, but with finite division potential (~40–50 passages).
(e) *Hybridomas*. The hybridoma technology fuses normal cells with an immortalized cell line to form hybridoma cell lines (hybridomas) that produce monoclonal antibodies.
(f) *Clones* are populations of cells originating from a single cell after its mitotic division. Division may bring some heterogeneity into the population (e.g. immortal human keratinocyte HaCaT cells). On karyotyping, HaCaT is an aneuploid line (initially hypodiploid) with unique stable marker chromosomes indicating its monoclonal origin (Boukamp et al. 1988). Several factors including origin, timing, regional niches, genetic events etc. contribute to heterogeneity.
(g) *Embryonic carcinoma cells* are a special type of stem cells derived from teratocarcinoma (a mixture of teratoma with embryonal carcinoma). These cells were isolated earlier and laid the foundation of work on embryonic stem cells. Embryonic stem cells and embryonic carcinoma cells share common properties such as pluripotency and tumorigenicity. As it has not been clarified what drives the embryonic stem cells into tumorous development, it remained a major obstacle of using them in regenerative therapy. To make the use of embryonic stem cells safe, mixed populations of tumorigenic and nontumorigenic stem cells either have to be separated or the tumorigenic subpopulation has to be selectively targeted (Knoepfler 2009).
(h) *Embryonic stem cells*. Most embryonic stem cells have been derived from human embryos that have been obtained exclusively under clinical *in vitro* conditions from fertilized egg cells and then donated for research with the written consent of the donors. Preimplantation-stage embryonic cells are transferred into a culture dish that can be coated with murine embryonic skin cells that are not dividing and serve as a feeding layer to the embryonic stem cells. Recent techniques grow embryonic stem cells without the feeding layer to avoid the transmission of viral infection and other macromolecules. Culture medium is added to embryonic stem cells. Cells subcultured for at least 20 times without differentiation remain pluripotent and are referred to as embryonic stem cell lines. Further details of embryonic stem cells will be given at the end of this chapter.

As an example the preparation of a primary cell culture, and cell line from a melanoma tumor obtained from the clinical Department of Dermatology, University of Debrecen will be detailed in the next subtitle. Melanoma is a malignant tumor of melanocytes, but less common than other skin cancers. The integument of skin is making up 1/6th of body weight, with a surface area of 1.8 m^2. Its most important function is to serve as a physical barrier to the environment, protection against

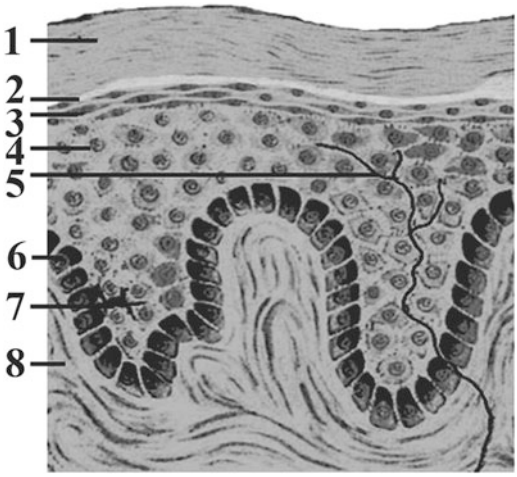

Fig. 2.3 Cross section of the epidermis of the skin. *1*. Horny layer (*stratum corneum*), *2*. light layer (*stratum lucidum*) with translucent cells seen only in thick epidermis, *3*. granular cell layer (*stratum granulosum*), *4*. spinous or prickle layer (*stratum spinosum*), *5*. free nerve ending, *6*. basal or germinativum cell layer (*stratum basale*), *7*. Langerhans (dendritic) cell, *8*. dermis

microorganisms, radiation, toxic agents and mechanical intrusion. The external layer of the skin (epidermis) is composed mainly of keratinocytes. The epidermis also contains melanocytes, Langerhans (dendritic cells) and touch sensitive Merkel cells in the basal layer (Fig. 2.3).

The pigment (melanin) producing melanocytes represent only a small proportion of the basal layer. Melanin protects against ultraviolet (UV) irradiation. After its production, melanin is accumulating in melanosomes and transferred to the adjacent keratinocytes. The basal layer is forming the dermoepidermal junction separating the epidermis from the dermis. The dermis is an area of supportive connective tissue between the epidermis and the underlying subcutis that contains sweat glands, hair roots, blood and lymph vessels, nervous cells and fibers.

2.3.1 Preparation of Primary Culture from Melanoma Tumor

An example is provided how primary cell culture can be made from a solid melanoma tumor.

Day 1

– Medium-sized melanocytic nevus obtained after surgical removal at the Department of Dermatology (University of Debrecen) under sterile conditions was placed in sterile saline and sent to the Department of Biotechnology and Cell Biology to start a melanoma primary cell culture (Fig. 2.4a). All further experiments were performed under strict control of sterility.

The selected area of the tumor (Fig. 2.4b) was cut into smaller pieces and after mincing frozen in liquid nitrogen. (Trencsenyi et al. 2009). Only tumor cells will survive, other primary cells die (Fig. 2.4c).

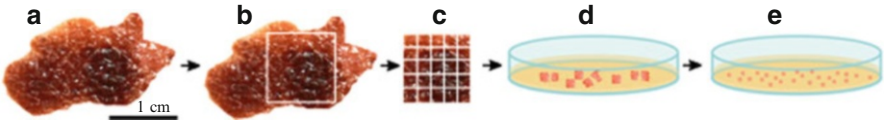

Fig. 2.4 Establishment of melanocyte cell line from melanoma tumor. (**a**) Medium-sized melanocytic nevus after surgical removal, (**b**) removal of the outer parts of the nevus indicated by the *boxed area*, (**c**) cutting the selected part of the tumor to small slices, (**d**) placing tumor slices in Petri dish and subjected to protease digestion, (**e**) resuspension of tumor cells in medium and cell growth

Day 2

- Frozen tumor pieces were further minced into $2 \times 2 \times 2$ mm pieces and incubated for 3 h at 37 °C in RPMI-1640 medium containing 10 mg collagenase I, 10 mg hyaluronidase and 30 μl DNase I per 100 ml. (Fig. 2.4d).
- After digestion, the mixture was filtered through four layers of sterile gauze, washed and resuspended in RPMI-1640 medium supplemented with fetal bovine serum and incubated at 37 °C in a 5 % carbon dioxide atmosphere (Fig. 2.4e).

Days 3–23

- After overnight incubation, nonadherent cells were discarded and adherent cells were subcultured.
- The primary cell culture was continuously grown and subcultured every day. After 20 days the new melanoma cell line designated MeDe (*Melanoma Debreceniensis*) was established.
- HMB45 (monoclonal mouse antihuman melanosome antibody) was used to test the melanoma origin of the cell line.
- Cells of the upgraded cell culture were distributed in small fractions (10^6 cell/vials), frozen in liquid nitrogen and used in further experiments.

Other tumor cells have been obtained from rat tumors generated by *in vivo* carcinogenesis. Tumor cell lines have been established from these cells named HeDe (*Hepatocarcinoma Debreceniensis*), NeDe (*Nephroblastoma Debreceniensis*) (Trencsenyi et al. 2010) and My1/De (myelomonocytic leukemia type 1) (Trencsenyi et al. 2012).

Tumor cell lines are supplied by internationally acknowledged collections:

- American Type Culture Collection (ATCC) (www.atc.org)
- European Collection of Cell Cultures (ECACC) (www.hpacultures.org.uk/collections/ecacc.jsp)
- German Collection of Microorganisms and Cell Cultures (DSMZ) (www.dsmz.de)

- Gene Transfer Protocol Library (www.bio-rad.com)
- National Cancer Institute's cancer cell lines (www.cancer.gov)

The Cancer Cell Line Encyclopedia (CCLE) is a compilation of gene expression, chromosomal copy number and sequencing data from 947 human cancer cell lines (Barretina et al. 2012).

2.4 Development of Primary Tumors

2.4.1 Primary Tumors

A primary tumor has been defined as a tumor growing at the anatomical site where tumor progression began and proceeded to yield a cancerous mass (Weinberg 2007). Distinction should be made between the original site where the tumor first arose and the site(s) elsewhere where the tumor metastasized. The original tumor is the primary tumor, but can take up residence in other tissues during a process called metastasis. As its occurrence is not expected, the early primary tumor is the most difficult to find but the easiest to remove. Although, in some cases the removal of the primary tumors is sufficient, in malignant cases a combined therapy is used including chemotherapy and radiation to make sure that the patient becomes cancer-free.

Primary tumors that are cancerous have the potential to spread to other parts of the organism meaning that they are malignant. Benign tumors grow normally slower, do not spread or represent only the initial stage that can turn to malignant transformation. Malignant or cancerous tumors show a general tendency of growing faster, being more aggressive, projecting metastases to other tissues and growing back after removal. Benign tumors of organs will be detailed in Chap. 4, those of malignant tumors in Chap. 5.

It is now widely accepted that pre-malignant lesions such as dysplasia and hyperplasia cause genetic alterations including monoclonal expansions, environmental changes (e.g. viral infections) that induce polyclonal changes and become fully malignant. At the early stage of primary tumor expansion cells are neither invasive (intruding in neighbouring tissues), nor metastatic (spreading to distant sites). It is assumed that only a restricted number of cells in the primary tumor suffer such a genetic alteration that is considered to be highly metastatic (Yokota 2000), consistent with the multifactorial model of colorectal tumorigenesis in which the steps required for the development of cancer often involve the mutational activation of an oncogene coupled to the loss of several genes that normally suppress tumorigenesis (Vogelstein et al. 1988). It is less well known that even if primary tumors do not spread they can be deadly. Such primary tumors can develop in major organs, primarily in lungs and brain by blocking the blood flow in these organs.

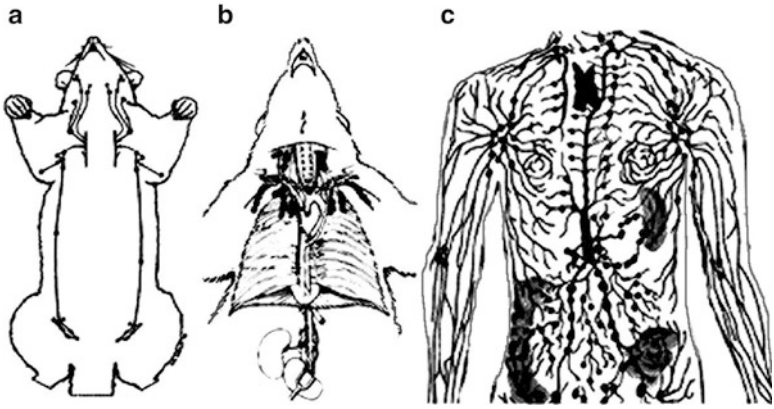

Fig. 2.5 Anatomical illustration of lymph nodes in the thoracal region. (**a**) The low number and small size of thoracal lymph nodes explain why opossums are inefficient against tuberculosis. (**b**) The lymphatic system of rats is resistant to tubercle bacilli. (**c**) The lymphatic system of the human thorax is more resistant to tuberculosis than marsupials, but less developed than those of rodents. Connections among thoracal and mammary lymph nodes are poorly understood

2.4.2 Status of Lymph Nodes

That the development of primary tumors into metastasis depends also on the status of the lymphatic system is demonstrated in Fig. 2.5 by comparing the development of thoracal lymph nodes in marsupials and mammals (Banfalvi 2012).

Mammalian lymphatic systems. There are significant differences not only among the lymphatic systems of rodents (e.g. mouse, rat) as exemplified by the ratios of hormone-dependent and hormone-independent tumors (Nandi et al. 1995). Even in closely related mammals (cat, dog) no relationship could be demonstrated between the number and the size of lymph nodes (Patsikas and Dessiris 1996). Further variability is signified by hemolymph nodes, hemolymph glands and hemal nodes typical to some mammals, not found in man.

Human lymphatic system. Small primary tumors and even sentinel lymph nodes could remain hidden and unnoticed inside the thoracal cavity. The lack of detection of thoracal lymph nodes (Fig. 2.5c) could explain the scarsity of malignancies metastasizing to the breast, head and neck, melanoma, genitourinary and gastrointestinal carcinomas (Suwatanapongched and Gierada 2006). The chain of human thoracal lymph nodes that continues in the axillary region suggests that in man and in a modified manner in higher mammals is in a closer relationship than in marsupials (Fig. 2.5a) similarly to lower mammals such as rodents (Fig. 2.5b). The interplay between thoracal and internal mammary lymph nodes could be one of the reasons of the relatively high incidence of human mammary tumors (Banfalvi 2012). Different illustrations (chain and web of lymph nodes) point to the lack of complete knowledge of lymph node anatomy not only in man but also in other mammals.

2.5 Growth of Cell Lines

2.5.1 Growth Curves

To determine the type of growth pattern (linear, exponential or cubic), growth curves are used in statistics. Cell growth is characterized by growth curves. The type of cell growth under optimal conditions has been determined to be exponential. However, cell cultures are often far from being grown under optimal conditions especially at the start of a new cell culture or at the end when cells die in large numbers. In the growth profile characteristic changes are seen as growth phases and are useful for making decision regarding future experiments.

2.5.1.1 Phases of Growth

The phases of the growth curve are not to be confused with the phases of the cell cycle (G0, G1, S, G2, M). The growth curves of mammalian cells resemble closely those obtained for the bacterial growth of outgrowing *B. subtilis* spores (Fig. 2.6).

To obtain a mammalian growth curve a small population of cells is deposited in a Petri dish containing nutrient medium and grown in CO_2 incubator as usual. Microscopic cell count is made at various time intervals. After starting the growth of the culture the first phase of cell growth is the adaptation or lag phase that is usually not longer than the doubling time (18–20 h). During the adaptation time cells are conditioning to the medium with little or no increase in cell number. Major cellular

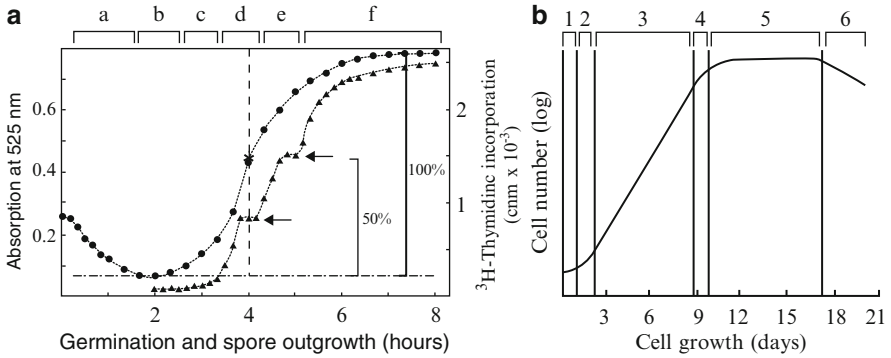

Fig. 2.6 Phases of growth curves of bacterial and mammalian cell cultures. (**a**) *Bacillus subtilis* spore germination and outgrowth: (*a*) germination (•–•), (*b*) swelling stage, (*c*) emergence, (*d*) initiation of replication and first cell cycle (▲–▲), (*e*) elongation and second cell cycle, (*f*) stationary phase (With permission Banfalvi 2011a). (**b**) Growth curve of mammalian cells. (*1*) lag phase, (*2*) acceleration phase, (*3*) log phase, (*4*) deceleration phase, (*5*) plateau phase, (*6*) degradation phase

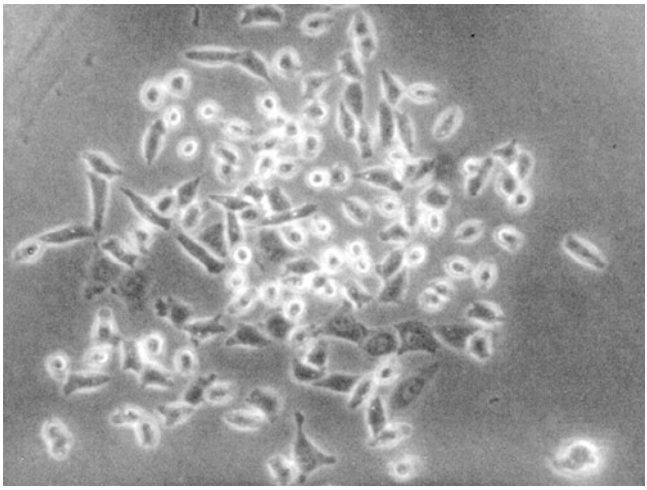

Fig. 2.7 Early acceleration phase of Chinese hamster ovary cell culture. A small population consisting of approximately 120 CHO K1 (ATCC CCL61) cells were deposited in Petri dish and cultured in DMEM medium containing 10 % fetal bovine serum at 37 °C in CO_2 incubator for 24 h. Elongated cells started to grow while round cells are still in resting phase

adaptations are related to internal cytoskeletal and enzymatic changes. A Chinese hamster ovary cell population being in early acceleration phase is visualized after 24 h culturing in Fig. 2.7. Approximately half of the population has started to grow seen as elongated cells, while the rest of the round cells are still in the adaptation (lag) period.

The lag phase of cell growth is followed by the acceleration phase that is not much longer (1–2 days) than the lag phase. Acceleration phase leads into log phase with cell growth increasing exponentially. This phase will last as long as there is sufficient nutrient supply to support the increasing cell number. Economical considerations dictate that components of the growth medium should be exhausted simultaneously rather than limited by a single or few components of the medium and deceleration phase should commence gradually. During the plateau phase the total cell number remains constant, while the viable cell number is likely to decrease, especially at the end of the plateau phase. To maintain logarithmic cell growth cells are subcultured before they reach the plateau phase. After the culture medium is exhausted cells start to die in large number and the growth curve is declining. Growth curves are useful to evaluate the growth characteristics of a cell line. From the growth curve the lag time, the doubling time of the population and its saturation density can be determined. Our protocol that follows the cell growth and division of cells through cell cycles is provided to determine the length of the cell cycle (Nagy et al. 2012).

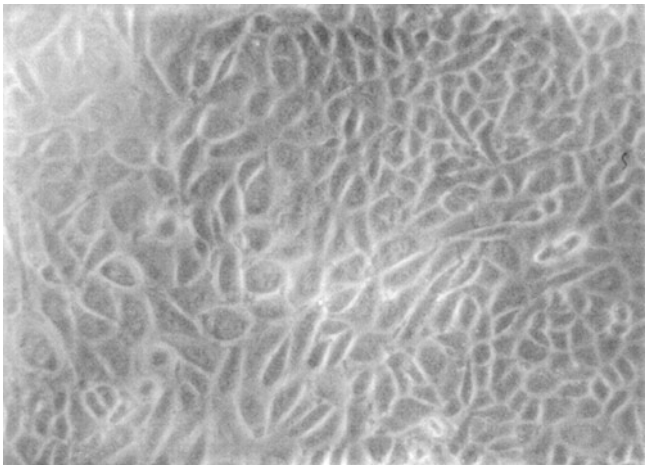

Fig. 2.8 Confluency of Chinese hamster ovary cell culture resulting in contact inhibition. A population consisting of 5×10^5 CHO K1 (ATCC CCL61) cells were deposited in a T75 flask and cultured in 10 ml DMEM medium containing 10 % fetal bovine serum at 37 °C in CO_2 incubator. After 24 h another 10 ml complete medium was given and the culture reached confluency after further incubation for 24 h

2.5.1.2 Measurement of Cell Growth

Suspension culture. It is relatively easy to count the cell number in a counting chamber (e.g. hemocytometer), or in a counter by spectrophotometry (e.g. turbidimetry), electrical resistance (e.g. Coulter counter), flow cytometry.

Monolayer culture. Cells cease to divide when they reach confluency and are said to demonstrate contact inhibition (Fig. 2.8). Cell growth curves are measured by the density of cells growing on the surface of the vessel by using an ocular grid inserted into an inverted phase contrast microscope. Cell growth is plotted on a semilog (log-linear) scale where cell density (cell/cm^2) of the culture with the cell number on the log ordinate scale, against the time on the linear abscissa scale.

2.6 Technical Aspects of Cell Cultivation

2.6.1 Cell Culture Media

Basal medium. Ross Granville Harrison, an American biologist was the first in 1907 to use successfully artificial tissue cultures by cultivating frog neuroblasts in lymph medium. His cell culture contributed significantly to the understanding of precursor and stem cells, nervous system and tissue transplant techniques (Abercrombie 1961). The Basal Medium Eagle (BME) developed in 1955 supported the *in vitro*

growth of a mouse fibroblast and a human carcinoma cell line (Eagle 1955). Eagle suspected that all mammalian cells are likely to have similar requirements for *in vitro* growth. Eagle has meticulously identified the 27 components that could not be omitted in the nutrients of mammalian cells without causing the cell cultures to die within a few days. These components included 13 amino acids, 6 salts, 7 vitamins, glucose and a small amount of human or bovine serum to approximate the minimal requirements of the inner environment of mammalian cells. This recognition widened the applicability for "monolayer" growth of normal and transformed cell lines. Monolayer cultures grow in contact with the culture vessel. BME became the basis of minimal essential medium (MEM) and Dulbecco's Modified Eagle's Medium (DME) for primary cell cultures and cell lines.

Earle's and Hanks' salts. Earle's salts utilize the buffering capability of bicarbonate/carbonic acid system and have been added for the short–term maintenance of pH in cells placed in a CO_2 incubator. These salts will rapidly increase the pH of the cell culture medium under atmospheric conditions. Hanks' salts have been designed for atmospheric equilibration and use in CO_2 environment resulting in the rapid lowering of pH of the nutrient broth called culture medium. Hanks' medium 199 gained a broad range of applicability especially for growing non-transformed cells.

Serum free, protein free, chemically defined media. These innovative specialty media that started to gain popularity in the 1980s are devoid of serum and contain either certain amino acids, or protein fractions. Protein-free media contain plant or yeast hydrolysates rather than proteins, many of them are animal-origin free. Chemically defined media contain neither serum, nor proteins, nor hydrolysates, nor any other unknown components. All the constituents of the medium are chemically well defined chemical structures. Cells grown in serum-free cultures are generally more susceptible to changes in pH, temperature, osmolarity, mechanical shearing and enzymatic effects. The following precautions should be taken during the adaptation of cells to serum free medium:

- Proteins including serum proteins bind more antibiotics, thus 5–10 times less antibiotic concentration will be sufficient in serum-free media. It is better to avoid the use of antibiotics in serum-free media. Antibiotics may mask bacterial infection.
- Seeding cultures at a higher density than normal at each passage during adaptation is recommended.
- To avoid cell clumping during adaptation carefully suspend the cells to break up the clumps.
- Slight change in cell morphology is of no concern as long as the doubling time and the viability remain constant.

The advantages of serum free media are that they allow:

- faster growth, higher production and viability of cells,
- consistent and reproducible performance,
- improved control and evaluation of cellular functions,
- better detection of cellular mediators,
- easier purification and downstream processing.

Serum free media can be selected to grow primary, insect, hybridoma, immunological, stem cells, etc.

The two most commonly used media are:

D-MEM/F12 contains: D-glucose, L-glutamine, HEPES buffer, hypoxanthine, essential fatty acids (linoleic and lipoic acid), putrescin, sodium pyruvate, thymidine, amino acids, vitamins and phenol red indicator.

RPMI 1640 consists of more than 40 components including amino acids, salts vitamins, HEPES buffer and phenol red. It was developed at the Roswell Park Memorial Institute (RPMI) (Moore et al. 1967). It has been shown to support the growth of a broad range of mammalian cell lines. RPMI 1640 medium is suited for the growth of several cell lines even in the absence of serum.

These and other media are regularly supplemented with HEPES buffer, EDTA, L-glutamine, fetal bovine serum albumin. The addition of antibiotics (penicillin, streptomycin, gentamycin) and antifungals (fungizole) is optional, but not recommended as antibiotics may mask the microbial infections of the cell culture.

2.6.2 Carbon Dioxide Incubator

Incubators are devices providing *in vitro* environments to grow and maintain cell cultures under optimal temperature, humidity, carbon dioxide and oxygen content inside the incubator. These instruments are indispensible not only in microbiology, cell biology and molecular biology to culture bacterial and eukaryotic cells but also in industry as egg incubators (chicken, turkey, goose, duck, quail, turtle, lizard, snake, etc.).

Cell culture incubators have been designed to cultivate mammalian cells typically under constant temperature (37 °C), relative humidity (95 %) and slightly acidic pH maintained by the 5 % CO_2 level. The interiors of carbon dioxide incubators are made of non-corrosive stainless steel. Some newer models contain antimicrobial copper surfaces to prevent microbial contamination. An even newer feature of CO_2 incubators to defend contamination is to use automatic heat or UV light exposure. The size of the incubators can vary from laptop size to small rooms. Commercially available carbon dioxide incubators are able to set the temperature range from 4 to 50 °C, control humidity (95–98 %) and CO_2 levels (0.3–20 %) and include a timer. Some incubators can be programmed to influence cell cycle events by changing temperature, humidity, etc. The temperature in water jacketed CO_2 incubators is regulated through the walls of the water bath, or by the radiant heat of electric coils. Inbuilt refrigerating units provide special control not commonly used. Programmable controls include: temperature alarms, CO_2 alarms, door opening alarms, password protection. Automated cell culture monitoring systems assist to manage and observe live cell imaging locally or by remote operation control over public or private networks.

2.6.3 Microscopes

It was in 1850 when the inverse microscope was invented (Smith 1852). Inverted microscopes gained application in micromanipulation and metallurgics. Inverse or inverted microscopes are also used for observing living cells at the bottom of cell or tissue culture flasks, plates or dishes.

Conventional microscopes are normally not capable to trace cells in large containers and their use is limited to glass slides. In an inverted microscope the light source and the condenser are on the top above the stage, while the four to six objective lenses of different magnification fitted to the revolving turret are below the stage pointing upward. Focusing is achieved by a dual concentric knob for coarse and fine adjustment. Inverted microscopes can be equipped with accessories, such as video cameras, fluorescent illumination, confocal scanning, etc. The capture of cellular movement through the inverse microscope by time-lapse photography and turning it to video microscopy movies also referred to as cinemicrography has been described by Rose (1963). The development of analog video-capture technology expanded the use of inverse microscopy as an analytical tool (Inoué 1997; Salmon 1995). The analog technique has been replaced by computer-based digital image-capture systems (Inoué and Spring 1997; Sluder and Wolf 2003). The ease of use, low noise and high quantum efficiency of digital micrographic systems enabled the study of dynamic events in cell biology. Further development was the establishment of time-lapse video-microscopy to visualize longer periods of dynamic processes of morphological changes (Pulkkinen et al. 1996; Rieger and Schroeder 2008), such as the rapid movement of apoptotic cells referred to as “dance of death” (Bernard and Malawista 1995; Humke 2000). Our long-term scanning system (Nagy et al. 2010) has been adapted to image continuously growing cell cultures under normal and toxic conditions schematically viewed in Fig. 2.9.

2.6.4 Cell Culture Flasks

Monolayer cultures. Vessels have normally flat surfaces on which the cells can adhere. After it has been recognized that practically any vessel with a flat bottom surface that can be sterilized is suitable for growing cells including coverslip cultures, a variety of flasks, bottles and dishes such as Carrel flasks, T-flasks, Kolle flasks, penicillin culture flasks, Erlenmeyer flasks, prescription bottles, Roux bottles, Petri dishes, etc. have been used. Recently, mainly plastic cell culture vessels (dishes, multiwell plates, flasks) in hundreds of combinations, formats and widest range of surfaces, often with gas permeable screw caps or growth surfaces dominate the market of mammalian cell culturing. The cells growing as a single layer have asymmetrical shapes and stop growing upon reaching confluency. The majority of the cell cultures isolated from vertebrates belong to this anchorage dependent category. Cells are maintained and propagated by periodic passaging. Every cycle

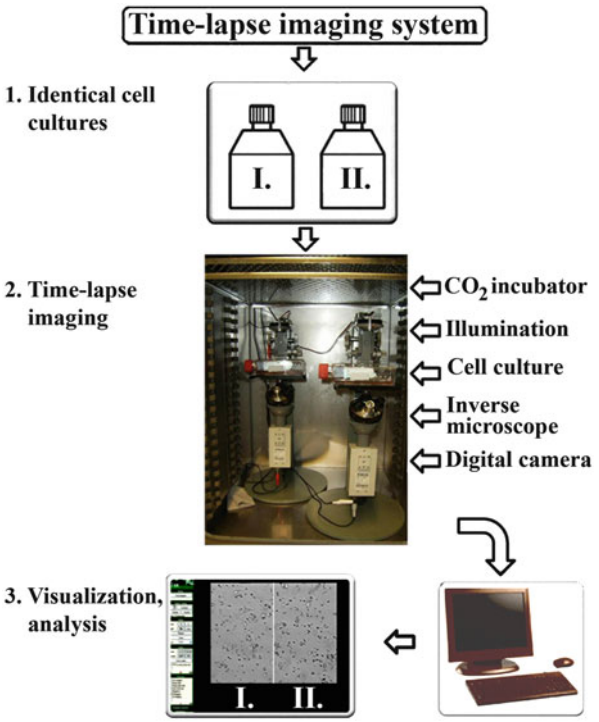

Fig. 2.9 Long-term scanning system. *1*. Incubation of two identical cell cultures in CO_2 incubator at 37 °C. *2*. Time-lapse imaging in the incubator. Arrows indicate the parts of the system at the right side of the picture consisting of the CO_2 incubator, two inverse microscopes, digital cameras, cell cultures in T flasks sitting on the inverse microscope illuminated with white light emitting diodes. *3*. Visualization of individual cells by computer and analysis of data (With permission Nagy et al. 2012)

of subculturing cells is referred to as passage. Replating or subculturing is a process repeated many times for months. To maintain cultures for years, batches of cells are kept frozen rather than subculturing them for a prolonged period of time without experimental use.

Growth of suspension cultures. These cultures are grown in culture flasks under sterile conditions. This makes subculturing significantly easier than passaging adherent cells. Cells are already suspended in the medium, thus enzymatic detachment is not needed, but sedimentation of cells to the bottom of the culture flasks has to be avoided. In culture flasks the volume of the medium relative to the surface area is drastically increased, consequently proper gas exchange is limited. Adequate gas (CO_2, O_2) supply is achieved by agitation most often using a magnetic stirrer and spinner flask (Fig. 2.10).

It is recommended to feed suspension cell cultures every day to maintain logarithmic growth and to avoid the accumulation of dead cells and cell debris.

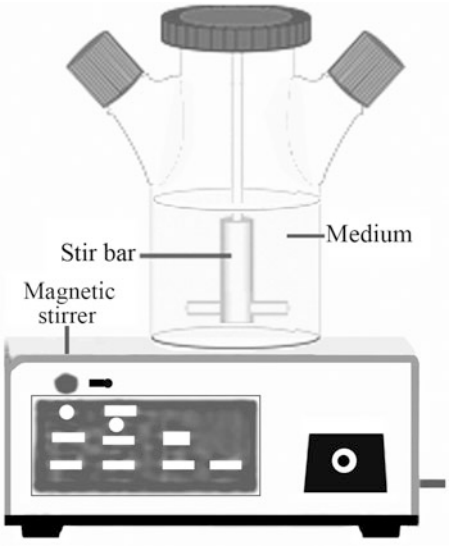

Fig. 2.10 Spinner flask and magnetic stirrer for suspension culture

Table 2.1 Recommended volumes of media for laboratory-scale spinner flasks

Volume of flask (ml)	Minimum volume (ml)	Scaling up to maximum (ml)
100	20	20 → 40[a]
200	25	25 → 50 → 100
500	100	100 → 200
1,000	200	200 → 400
2,000	400	400 → 800 → 1,600[b]

For medium-scale use the volume of spinner flasks can go up to 36 l
[a]Not recommended. Growth in T-flask is sufficient for these volumes
[b]Only under higher aeration using higher revolution of the stir bar without damaging cells

Scaling up the volume of the suspension culture is done by direct dilution i.e. adding new medium to the old one rather than replacement. Growing mammalian suspension culture in sterile shaker flasks is less popular than in spinner flasks. Shakers are more suitable to grow bacteria, their cell wall being more resistant to physical shearing. As the accumulation of cell debris in shaker cultures cannot be prevented cells are sedimented at lower gravity (100 g for 10 min) and resuspended in fresh medium, while the debris remains in the supernatant. It is recommended to use stirrer bottles with hanging stirrer bars that are designed specifically for suspension cultures to prevent cellular damage. Such spinner bottles also provide superior gas exchange and enable to scale up volume from 20 to 1,600 ml (Table 2.1) in a cell biology laboratory.

Scaling up cell cultures. Cell cultures from a suspension culture or monolayer cultures approaching confluency are prepared for suspension culture and started in a smaller (200 ml) spinner flask containing a minimum volume of 25 ml medium under constant stirring in a carbon dioxide incubator. The spinner paddle has to be

lowered as much as possible without touching the bottom of the vessel, with the spinner speed set at minimum and constant value. The screw caps of the side arms are set to provide proper gas exchange. The spinner speed depends on the type of the cell and impeller.

Logarithmic growth is maintained by scaling up volume from 50 to 100 ml and 200 ml in a 500 ml spinner flask. After switching to a larger (2 l) spinner flask scaling up can be continued from 400 to 800 ml and to 1,600 ml. Mammalian cells are sensitive to physical shearing, thus the revolution force of the magnetic stirrer can only be moderately increased at higher volumes. The impeller has to rotate freely without contacting the sides or the bottom of the vessel. Scaling up results in $3–4 \times 10^8$ cells in 1 week (Banfalvi 2011b).

2.6.5 Cell Culture Centrifuges

After cell growth adherent cultures need to be removed first from the surface of the vessel by scraping them off using a hand-held flexible natural-rubber scraper named rubber policemen or a one-piece plastic scraper. Scraping damages cells, thus the proteolytic removal of the monolayer by trypsin (or trypsin-EDTA) solution is recommended. Adherent cells obtained from monolayers are suspended in physiological solution (saline, PBS) before centrifugation. Cells grown in suspension culture are transferred from the flask to conical bottom centrifuge tubes and after centrifugation the pellet is resuspended. Centrifugation serves to remove medium, protein end products, dead cells and cell debris. Modern centrifuges have custom adapters available for culture flasks to reduce contamination to the flask and to reduce the number of transfer steps, saving time and money.

2.6.5.1 Special Types of Centrifugation

Cell separator centrifuges. These instruments have been designed with two major purposes in mind: an inlet for product acceleration and an outlet to separate mammalian cells by centrifugation in a gentle way. The cell culture enters the centrifuge through a hollow spindle feed inlet that accelerates gradually as it moves upward, minimizing the shear forces and preventing cell lysis. Hermetic outlets also prevent from air, avoid foaming and denaturation and maintain hygienic standards.

Centrifugation of viral culture. The viral sample is centrifuged onto a single layer of cells and viral growth is estimated by antigen detection. The centrifugation step greatly reduces the time of detection, enhances the sensitivity of measurement because the viral particles of the sample will be in close association with the cells. Several human viruses have been identified by viral culture centrifugation: adenovirus, cytomegalovirus, enteroviruses, herpes simplex virus, influenza virus, parainfluenza virus, rhinovirus, respiratory syncytial virus, varicella zoster virus, measles and mumps (Leland and Ginocchio 2007).

Counterflow centrifugal elutriation system. The sedimentation velocity based on cell size is operative in this technique also referred to as counterstreaming centrifugation (Banfalvi 2008; Banfalvi 2011b). The Beckman elutriation system is and advanced centrifugation device that uses an increasing sedimentation rate to yield the separation of cells in a specially designed centrifuge and a rotor containing the elutriation chamber. Centrifugal elutriation is regarded the most physiological way to obtain synchronized subpopulations covering the whole range of the cell cycle. More details will be given on centrifugal elutriation in the subchapter devoted to "Overview of cell cycle synchronization and flow cytometric control of synchronization".

2.6.6 Water Baths

Water baths may serve cell culturing to warm up media. Water baths are temperature controlled for operation with or without the shaker function. In cell biology laboratories utility water baths with analog or digital temperature control are suitable for maintaining temperature set points from ambient to 100 °C. Shaking water baths are supplied with accurate temperature control, variable water levels, easy cleaning and designed for samples that need to be temperature controlled and shaken. Reciprocating shaking bath can be used as shaking water or constant-temperature bath. The use of heated circulators for water jacketed spinner flasks to grow cells outside an incubator is limited as mammalian cells need a carbon dioxide atmosphere. Among specialty baths waterless bead baths can be useful, while fluidized sand baths operating at higher temperature (50–600 °C) have not been designed for cell biology laboratories.

It deserves mention that water baths are the primary cause of contamination in cell biology laboratories and need frequent cleaning. Instead of using water baths it is recommended to leave all material including media necessary for cell culturing under the sterile laminar-flow hood after spaying them with 70 % alcohol and turning on the UV lamp and sterile air flow before doing cell culture work. Media can be more conveniently warmed up in the CO_2 incubator, in waterless bead bath or using heating blocks.

2.6.7 Sterile Instruments

The medical term of aseptic work refers to freedom of pathogenic microorganisms. The cell biology meaning of aseptic technique is synonymous with sterile conditions to maintain cell cultures under rigorous noncontaminating working conditions to avoid expensive losses and delays. Critical factors with respect to contamination are: laboratory or work space conditions, cell lines, media and reagents, equipments and instruments, sterilization procedures and laboratory personnel. Theoretically

cell culturing could be carried out on an open bench in a low-traffic area, but practically culture work is done under sterile conditions using a horizontal laminar-flow hood or a vertical laminar-flow hood (biosafety cabinet). In fact aseptic work encompasses a wide spectrum of safety measures ranging from personal hygiene, clean work space free from contaminating air currents and draughts, aseptic operations supported by disinfectants (70 % ethanol or other disinfectants), combined with sterilization including flame sterilized opening of vessels, loops, needles, small hand instruments, sterile materials, equipments and media to laminar-flow biosafety cabinets.

Laminar-flow hoods and biological safety cabinets ("chemo hoods") are devices that act as primary barriers either to protect the material being manipulated within the hood from worker-generated or environmental sources of contamination, or to protect the laboratory worker and laboratory environment from exposure to infectious or other hazardous materials that are present within the hood (Richmond and McKinney 1993; Barkley and Richardson 1994; Coté 1998). The two major types of laminar-flow hoods use high-efficiency particulate air (HEPA) filter and blowers of nonmixing stream of air:

(a) horizontal-flow clean bench
(b) biological safety cabinet

Basic protocols on aseptic techniques that apply these safety cabinets have been detailed by Coté (1998).

2.6.8 Filtering Devices

The first devices for the filtration of large volumes of solutions have been described by Gordon (1926). In cell biology laboratories the sterile filtration of small volumes, often only a few ml-s is required. Another feature of the small disposable devices with a polypropylene or polycarbonate housing is that they enable the filtration of a wide variety of different types of samples. Without going into details, the product types of filters are: syringeless, syringe, in-line, capsule, centrifuge, venting, vacuum protection filters and other specialty devices.

Pressure filtration devices containing membranes, paper or glass fiber filter discs with sample infusion cylinders are suitable for batch filtration of samples from 20 ml. Pressure devices without sample loading cylinders serve the filtration of larger volumes up to several liters. Pressure filtration is particularly useful for the sterile filtration of serums, media that are highly viscous and difficult to filter. Cleaning or changing filters is relatively simple. While selecting the right device for filtration, the volume and membrane guidelines for sterile filtration, fluid viscosity characteristics, protein concentration, filter scalability, dimethylsulfoxide (DMSO) compatibility and mycoplasm retention have to be taken into consideration to meet the broad range of filtration challenges.

Filtration devices remove from cell culture media contaminants and agents such as bacteria, fungi, viruses, and mycoplasma that would destroy the cell culture. Sterile filtering of media is preventing bacterial contamination using 0.2 μm sterilizing grade membrane filters. For media containing animal sera, a final sterilizing grade 0.1 μm filter may be used for additional protection against mycoplasma. The serum content of mammalian cell cultures posed difficulties, as the high protein content slowed down the filtration process, made purification difficult and expensive and presented risk of biocontamination (microbial, viral, prion associated encephalitis). To avert such risks serum and protein free media have been introduced for mammalian cell cultivation. Substitutions of serum and protein in cell culture media resulted in granulated powders combined with selected cost-effective filtration systems and impacted greatly filterability and contributed to the avoidance of batch-to-batch variations of solution properties. Selection criteria for Sterilizing grade High Capacity (SHC) filters imply, that the filter must be proven inert, sterile filtration without adverse or inhibitory effects on cell growth or protein expression. SHC and similar high quality filters have been developed to provide fast throughput, flow rate, low extractables and filtration economy by eliminating expensive filter change-outs and contributed to scale-up serum free cell culturing, to the production of monoclonal antibodies, vaccines and other biological materials.

2.7 Sterile Work Under the Laminar Flow Hood

Different types of laminar boxes have been designed to provide an aseptic work area in cell biology laboratories. The three major types of laminar flow hoods are designated as Class I, II and III, to meet various research and clinical needs.

Class I hoods similar in design and air flow to chemical fume hoods offer protection primarily to the laboratory personnel and environment, and only to a lesser extent to the cultures against contamination.

Class II hoods have been developed for Biosafety Level 1, 2 and 3 (BSL-1, BSL-2, BSL-3) laboratories. BSL-1 laboratories are suitable for work involving well-characterized agents not causing diseases in immunocompetent adults. These laboratories represent only minimal potential hazard to the environment and laboratory personnel. Due to the low level of risk BSL-1 laboratories work is conducted under standard microbiological conditions with trained and supervised personnel. Biosafety Level 2 represents moderate potential hazard to personnel and environment. The laboratory personnel have to participate special training to handle pathogenic agents. Access to Biosafety 2 laboratory is limited, precautions are taken against contaminated items and infectious work is carried out exclusively in Class II biological safety laminar flow hoods. Just to mention a few potentially hazardous materials, the most important ones are primate-derived and virally infected cultures, carcinogenic or toxic reagents, radioisotopes.

Class III biosafety cabinets are gas-tight, providing the highest possible level of protection both to the personnel and environment. Major risk factors are human pathogens and BSL-4 materials including autoinoculation, infectious aerosols, infectious droplets transmitted *via* the aerosol route through the mucous membrane and for which there is no safe vaccine or therapy. BSL-4 safety facilities allow safe operation by utilizing physical and operational barriers to keep personnel from contacting with infectious agents.

Among the basic rules of sterile technique the most important ones are:

1. Set up the working area including the culture hood as far away from laboratory traffic, drafts and windows as much as possible. Place the sterile work to a room that has no through traffic. Keep the door of the sterile room closed by indicating outside (e.g. red sign) that sterile work is under way or will begin within 30 min. Do not interrupt the sterile work of others when the red sign is on.
2. Plan each experiment carefully that everything will be sterile and under the hood before the sterile work begins.
3. Minimize the potential of external contamination originating from workers (clothing, hair, hands, noses, mouths) by minimizing the number of unauthorized personnel and the time spent in the sterile room.
4. Minimize environmental contaminants (airborne particles, dust, molds, bacteria, viruses, fungi, etc.). The use of ultraviolet (UV) light is recommended to sterilize the air and exposed work surfaces.
5. Before and after use clean the work surface of the culture hood and disinfect thoroughly. For routine cleaning wipe the work surface with 70 % ethanol.
6. Place only items required for sterile work under the cell culture hood. The hood should not serve as a storage area. Items should not stand behind each other to avoid air turbulence. Spray each item with 70 % ethanol.
7. Simultaneously with external UV lamps turn on the UV lamp and the air flow inside the hood for at least 30 min before sterile work. These aseptic measures also warn colleagues that sterile work is under way. Do not enter the room; stay outside while the reduction of microorganism by UV germicidal irradiation is in progress.
8. The presence of Bunsen burners for flaming is not recommended and can be contraproductive regarding the maintenance of sterile work under the hood.
9. It is debated whether or not the cell culture hood should be constantly running. Economy dictates that it should be turned off after use.

2.8 Deep Freezing, Storage of Cells

Deep freezing or cryopreservation is a process, where cells or tissues are cooled down to sub-zero temperatures, either to −70 °C in a deep freezer or to −196 °C in liquid nitrogen and stored in frozen condition. At these temperatures the biological

activity of cells comes to a standstill. The viability of cells cannot be maintained during the cooling and the opposite resuscitation processes without cryoprotective agents.

The natural cryoprotection is due to the accumulation of small organic molecules that serve as osmolytes, reduce the shrinkage of cells and protect against ice formation. Freeze tolerance has been developed in organisms that survive the winter by freezing, ceasing biological functions, yet surviving. The best known examples of freeze-tolerant vertebrates are found among certain species of frogs, salamanders, snakes, turtles and lizards. It turned out that under natural (i.e. slow) cooling rate, freezing initiated the endogenous production of cryoprotectant glucose in frogs in the liver that is distributed throughout the tissues of the body. Rapid cooling caused substantial hemolysis in control frogs, while erythrocyte injures were significantly reduced in glucose loaded frogs (Constanzo et al. 1991). That the damage of red blood cells during freezing was due to osmotic stress was hypothesized earlier by James Lovelock, who also suggested that increasing salt concentration in cells loosing water during external ice formation would cause the ice crystals to damage cells (Mazur 1970). This theory was proved and lethal intracellular freezing was avoided by cooling cells slow enough to permit sufficient water to leave the cell and reduce intracellular ice formation to minimum during progressive freezing (Mazur 1963). While Mazur proved the validity of the theory of Lovelock (better known as Gaia hypothesis) he created a new hypothesis that when ice forms outside the cell, the residual unfrozen medium forms channels in the ice and increases its solute concentration.

In response to the higher osmotic concentration outside the cell causes the cells to lose water and to shrink. If cooling is too rapid, insufficient cell water is removed osmotically and intracellular freezing ensues (Mazur 1984). According to the recent view the cellular injury during freezing comes from: (*i*) the direct damage from the ice crystal formation in the cells and (*ii*) the secondary damage are caused by the increased concentration of solutes during progressive freezing of the extracellular fluid.

These and other experiments led to the recognition that rapid-cooling injury is related to the inadequate production and distribution of the cryoprotectant during freezing and established the basic principle of successful cryopreservation and resuscitation: slow freeze and quick thaw. Although, the protective cooling rate differs depending on size and membrane permeability, for mammalian cells protected with a cryoprotectant (glycerol, dimethylsulfoxide) a typical $\sim$1 °C/min cooling rate is recommended. Controlled programmable freezing technique has been introduced (Vutyavanich et al. 2010) and machines have been adapted primarily for the slow freezing of oocytes, sperm and stem cells. Other applications of slow programmable freezing for tissues, organs, embryos have also been developed. The cryogenic preservation of humans (cryonics) is under way. As far as resuscitation is concerned countless cryopreserved cells, vaccines, tissues, organs, embryos and biological samples have been successfully thawed, revived and used successfully, but this was not the case for cryopreserved brains or bodies of mammals.

Cryopreservation of cell lines allows continuous logarithmic cell growth to be suspended and stocks of cells stored for an unlimited period of time provided that the temperature is maintained under – 135 °C. Before cryopreservation it is recommended to:

- scale-up the cell culture to produce a large enough stock,
- keep cells in log phase during scaling up to reduce the number of cell debris and dead cells to maintain high viability (>95 %),
- harvest cells as pre-confluent cultures, below their maximum cell density before freezing,
- resuspend cells containing high concentration (30 % or more) of serum or protein. For mammalian cells 90 % serum is recommended,
- distribute cells in several fractions,
- use dimethyl sulphoxide (DMSO) or glycerol as cryoprotectant. DMSO is preferred at a final concentration of 10 %. In cell lines where DMSO would induce differentiation the use glycerol is recommended.

2.9 Cell Synchronization and Its Flow Cytometric Validation

Synchronized cells represent different stages of the cell cycle. Synchronization has been described for bacterial, plant, protozoan, yeast, fish and mammalian cells. The synchronization is based principally on two major strategies: (a) blocking cells at certain stages of the cell cycle by chemical treatment known as “arrest-and-release” approach, and (b) physical strategy, to collect cells representing subpopulations of the cell cycle. These subpopulations are then used to study different aspects of regulatory mechanisms particularly at the level of macromolecular biosynthesis such as DNA replication, gene expression, protein synthesis, posttranslational modifications, the exact study of individual phases, new drugs, etc. At the same time when the structure of the double helical structure of DNA was discovered, Watson and Crick postulated that the specific base pairing makes possible the copying for the genetic material (Watson and Crick 1953). In the same year autoradiographic studies of Howard and Pelc (1953) have shown that DNA replication in broad bean plant cells (*Vicia faba*) is limited to one discrete period between two mitoses and led to the recognition that the cell cycle consists of phases known as G1, S, G2 and M. Soon it was realized that the principal mechanism of the cell cycle is common in all eukaryotic cells. The following principal criteria need to be met for synchronization:

(a) Both normal and tumor cells should be arrested at the same specific phase of the cell cycle. This is not always the case as upon gamma irradiation the cellular and nuclear sizes are increased (Nagy et al. 2004), consequently the separation based on cell size (e.g. elutriation) is misleading. UV irradiation is blocking chromatin condensation at its fibrillary stage, nuclear structures are blurred and covered with fibrillary chromatin, disturbing the evaluation of the flow cytometric profiles of the cell cycle (Ujvarosi et al. 2007).

(b) Synchronization must be noncytotoxic. This criterion is not met by chemical synchronization (Amon 2002; Cooper 2003; Coquelle et al. 2006; Banfalvi 2011c).
(c) The metabolic block should be targeted to a specific phase and be reversible. Specific targeting is possible but toxicity cannot be excluded (Amon 2002; Cooper 2003; Coquelle et al. 2006).
(d) Large quantities of synchronized populations should be obtained. This can be achieved upon scaling up cell growth.
(e) The synchronization must be medium independent. As the existing culture media are supporting the growth of several mammalian cells, this criterion is not a critical factor during synchronization.
(f) Synchrony should be maintained for more than one cell cycle. This is possible, but not longer valid than a few (2–3) cell cycles.
(g) Synchronized populations should exhibit uniform size. This is secured only when the synchronization is based on cell size (e.g. elutriation).
(h) DNA content of the initial culture and during scaling up should be the same. The balance can be upset during toxic treatment of cells (Nagy et al. 2004; Ujvarosi et al. 2007).

The stringent conditions of cell synchronization resulted in heated debates that still exist, but are not commented. It is fair to say that several synchronizing methods have been developed, but none of them is suitable for general use for at least three reasons: (*i*) the proportion of synchronized cells in isolated populations (fractions) is not sufficiently high, (*ii*) manipulations perturb cell physiology, (*iii*) *in vitro* treatment can be toxic and can: (a) prevent DNA synthesis (thymidine, hydroxyurea, aphidicolin), (b) inhibit mitotic spindle formation (nocodazole), (c) arrest not only the cell cycle at certain points, but can kill important fractions of the cells (Amon 2002; Cooper 2003; Coquelle et al. 2006; Banfalvi 2011c).

2.9.1 Physical Separation

These synchronizing methods are based on cell density, cell size, antibody binding, fluorescent labeling of cells and light scatter analysis of flow cytometry. Differences in sedimentation velocity are exploited during sedimentation at unit gravity (Macdonald and Miller 1970; Durand 1975; Tulp and Welagen 1976), density gradient centrifugation (Mitchison and Vincent 1965; Schindler et al. 1970; Wolff and Pertoft 1972; Probst and Maisenbacher 1973) and velocity sedimentation by counterstreaming centrifugation also referred to as centrifugal elutriation (Lindahl 1956; Sörenby and Lindahl 1964; Grabske et al. 1975; Meistrich et al. 1977; Banfalvi 2008; Banfalvi 2011b). Major objection against sedimentation at unit gravity and density gradient centrifugation are reproducibility, artifact formation, heterogeneity and slowness, consequently they are outdated techniques.

Centrifugal elutriation. This method was originally invented by Lindahl and utilized counterstreaming centrifugation (Lindahl 1956). The method was modified

and renamed to centrifugal elutriation and developed into a complex Beckman elutriation system. This technique is yielding the best known separation of cells in a specially designed centrifuge and a rotor containing the elutriation chamber. The technical details and the protocol of centrifugal elutriation have been recently reviewed (Banfalvi 2008; Banfalvi 2011c), here only the major advantages of centrifugal elutriation are mentioned:

(a) centrifugal elutriation is one of the few methods that fulfills the principal criteria of synchronization,
(b) even small differences in sedimentation velocity can be exploited and homogeneous fractions can be obtained from various cell types,
(c) different populations represent different stages and cover the whole spectrum of the cell cycle,
(d) large number of cells (up to $2–5 \times 10^8$) can be subjected to synchronization allowing a fine tuning of separation resulting in many fractions and higher resolution power (Banfalvi et al. 1997). Highest resolution ($\geq$97 %) was achieved in G1 phase and gradually declining homogeneity was observed in S phase (>80 %), and in G2 (70–75 %) phase cells after centrifugal elutriation (Keng et al. 1980).
(e) isolated subpopulations can be adapted for clinical investigation (Almici et al. 1992; Berger et al. 2005; Micklethwaite et al. 2009).

Flow cytometry and cell sorting. The technology of flow cytometry was developed from pulse cytometry but became more popular than other related cytometric measurements. Flow cytometry allows the counting and examination of small (0.2–150 μm) particles (chromosomes, nuclei, cells) suspended in a stream of fluid passing through the orifice of the detecting device. In cell biology the detection of individual cells and their DNA content is of particular importance. During flow cytometry stained cells normally fixed in ethanol and contained in a thin stream of fluid intercept and scatter the light and excite the fluorochrome dye (propidium iodide, Hoechst 33342, DAPI, actinomycin, mithramycin, antraquinone, etc.). Fluorochrome light intensity serves then quantitative DNA analysis. Flow cytometers and analysers are capable of collecting multiparameter data, but do not separate cells. This additional function is done by cell sorting and requires sophisticated electronic components normally not incorporated in bench-top instruments. In flow cytometry combined with fluorescent-activated cell sorting (FACS analysis) the detectors (forward and side scatter) of the light beam (regularly laser) are directed to the stream containing the particles. Forward scatter detectors are in line with beam, side scatter detectors are directed perpendicularly to the light beam. Forward scatter analysis related to cell size and volume focuses on the identification of cells, while side scatter analysis is correlated with the inner complexity of cells (e.g. shape, granularity, and roughness of cellular membrane). Forward and side scatter analyses were used earlier to detect the presence or absence of dead cells and cell debris. Recently forward scatter analysis has been adapted to magnify apoptotic signals. An example of forward scatter analysis where the proportion of UV irradiated apoptotic cells relative to unirradiated ones is shown in Fig. 2.11.

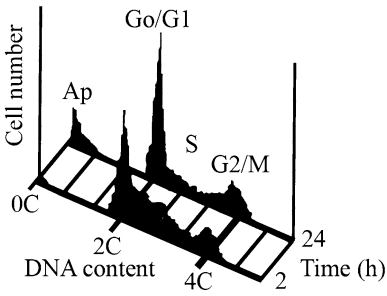

Fig. 2.11 Apoptotic changes detected by forward scatter analysis. K562 cells (5×10^5) were exposed to 4 Gy γ-irradiation. One aliquote of cells was incubated under standard conditions in a CO_2 incubator at 37 °C for 2 h, the other for 24 h, then stained with propidium iodide and subjected to forward scatter analysis. The C-values and major phases of cell cycle are indicated. *Ap* apoptotic cells (With permission Banfalvi et al. 2007, Fig. 2D)

Apoptosis in control K562 cells was under detection limit (~1 %). The development of apoptosis from its initiation to the shrinkage of cells took time and increased in gamma irradiated cells after 2 and 24 h incubation in the sub-G1 window from 4.8 to 15.6 %, respectively (Fig. 2.11.) Beside the accumulation of apoptotic cells, the increase in G1/Go and G2/M phase and the decrease of S phase population were observed after UV irradiation. Major drawback of flow cytometry is that it poses limitations in sample size and time required for synchronization.

Cytofluorometric purification of cells. This procedure of purification of cells belonging to distinct phases of cell cycle utilizes stable transfection with chimeric protein consisting of histone H2B and green fluorescent protein (Coquelle et al. 2006) and separates diploid and tetraploid cells in a fluorescent activated cell sorter (FACS) (Castedo et al. 2011).

Microchip-based flow cytometry. Flow cytometers have been developed that require much lower volumes of reagents combined with disposable chips. Small particles are pressed through a small detection device under an external hydrodynamic pressure gradient or electro-osmotic flow and controlled fluid movement.

Dielectrophoresis. This method applies laminar flow and electrokinetic forces for the separation of cells. The alternative current moves particles forward and backward generated by microelectrodes that periodically reverse the direction of the electric charge (Morgan et al. 2003; Holmes et al. 2005).

2.9.2 Chemical Synchronization

Mitotic shake-off. Cells growing in monolayer can be completely detached by gently shaking and isolated from the supernatant by centrifugation. As cells spend only 1–2 h in mitosis, this population is relatively small (less than 10 %) even in exponentially growing cell cultures. The number of mitotic cells can be increased

by mitotic blocking agents (e.g. hydroxyurea, colchicine). Mitotic selection can be repeated and the harvested mitotic cells combined. Either alone or in combination with mitotic blockers mitotic shake-off is an excellent synchronizing method (Grdina et al. 1984).

Membrane elution – Baby machine. This method is similar to the mitotic-shake off in the sense that when in a monolayer a cell divides, one cell has enough space to settle down, while the other new born, baby cell in G1 phase is released to the medium (Thornton et al. 2002; Cooper 2002) and can be bound to a membrane and harvested continuously without chemical treatment.

Cell cycle blocking agents. Cells are blocked in specific stages of the cell cycle upon exposing them to agents that interfere with different biosynthetic processes (Merrill 1998). Classes of agents synchronize cells at:

- Mitosis through spindle poisons (colchicine, vinca alkaloids, nocodazol) and topoisomerase II inhibitors (razoxane)
- DNA synthesis – S phase (thymidine block, hydroxyurea, aphidicolin)
- G1 phase (serum starvation)
- Combination of blocking agents, e.g. thymidine-nocodazol, thymidine-colcemid, cytosine arabinoside-colcemid, serum deprivation combined with aphidicolin treatment.

Synchronization of embryonic cells. Serum deprivation of embryonic stem cells before transplantation turned out to decrease the rate of cell death after transplantation (Zhang et al. 2005). To decide whether G0 or G1 cells function better as donor cells northern blot analysis showed that cells after serum reestablishment were in mid-G1 stage (Memili et al. 2004).

Synchronization at low temperature. When exponentially growing cells, where most of the cells are in S phase are subjected to lower temperature (30 °C), cells complete DNA synthesis, but do not initiate a new cell cycle and become arrested in G1 phase. This method was recommended to study S phase-dependent replicative and repair processes (Enninga et al. 1984).

Major objection against cell cycle blockaders is that they are likely to perturb the cell population in an unpredictable manner.

Synchronization of unicellular organisms. When temperature sensitive bacterial cell cultures have been shifted from permissive to nonpermissive temperature, the existing replication forks could be completed without the initiation of new ones. Subsequent shift to permission temperature initiated the growth of a synchronized culture (Withers and Bernander 1998). The other method adapted the "baby machine" methods by binding newborn cells to a membrane (Helmstetter and Cummings 1963; Helmstetter et al. 1992). Sporulation of bacteria and outgrowth of spores has been utilized for the synchronization of *Bacillus subtilis* (see Fig. 2.6a). The synchronization of yeast cells has adapted: feeding and starvation, magnesium exhaustion, heat shock, arrest and release, mutants arresting cells at specific cell cycle stages, periodic feeding and dilution techniques (Walker 1999; Manukyan et al. 2011).

2.10 Stem Cells

2.10.1 Fertilization

Fertilization in humans unites the sperm and ovum. After ovulation the egg cell leaves the ovary and enters the fallopian (uterine) tube, where fertilization takes place (Fig. 2.12a). The first cleavage on day 2 produces a two-cell stage (Fig. 2.12b), followed by the second cleavage generating the four-cell stage on day 3 (Fig. 2.12c). At uncompacted morula stage on day 4 the embryo contains 8 cells (Fig. 2.12d) followed by the compact morula stage with 16 cells on day 5 (Fig. 2.12e). Further divisions on 5–7 are known as early and late blastocysts. The zygote as late blastocyst will implant in the uterine wall after 8–9 days (Fig. 2.12f) and will reside there over the course of 9 months. Normal cells are often subdivided according to their embryonic origin and can arise from one of the three embryonic layers known as endoderm, ectoderm and mesoderm.

2.10.2 Pluripotent Embryonic Stem Cells (ESCs)

The fertilized egg cell gives rise to all embryonic somatic cells and germ cells. The cells of the zygote are totipotent up to few early cells of the morula stage. Embryonic cells reach blastocyst stage 5–7 days post fertilization. Pluripotent stem cells are descendants of totipotent stem cells and develop into the three germ layers:

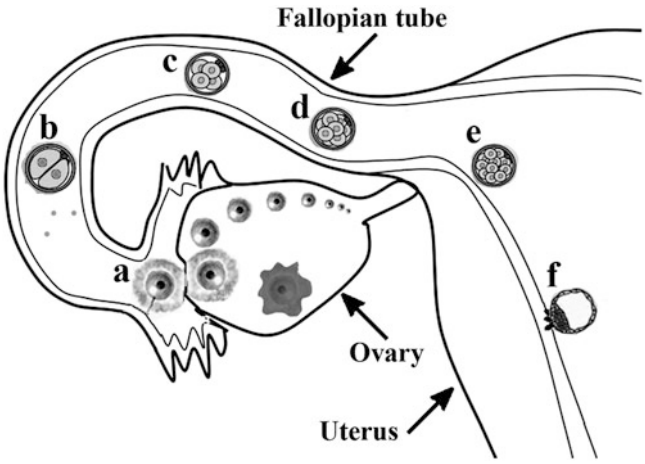

Fig. 2.12 Early steps of human embryonal development. (*a*) Fertilized egg (zygote), (*b*) two cell stage, (*c*) four cell stage, (*d*) eight cell morula stage, (*e*) 16 cell morula stage, (*f*) implantation of blastocyst in the uterus (This figure is an adaptation of Human Fertilization, png from Wikipedia, the free encyclopedia. http://en.wikipedia.org/wiki/Fertilisation)

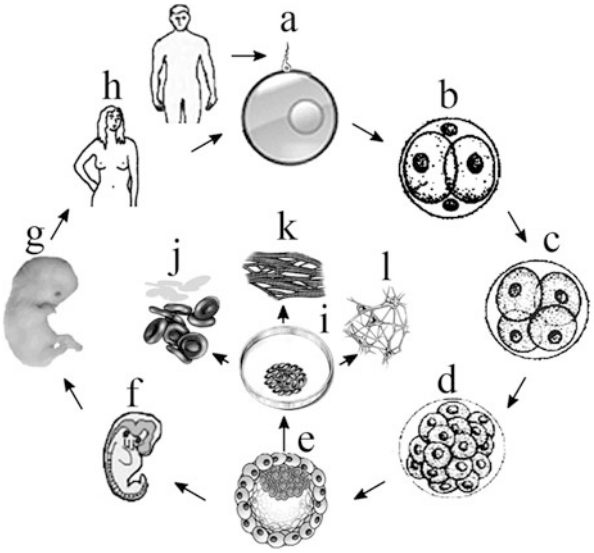

Fig. 2.13 Origin of human embryonic stem cells. (*a*) fertilized egg, (*b*) totipotent two cell stage, (*c*) totipotent four cell stage, (*d*) totipotent morula, (*e*) pluripotent blastocyst, (*f*) fetus, (*g*) embryo, (*h*) adult, (*i*) cultured pluripotent embryonic stem cells originating as inner cell mass cells within a blastocyst, (*j*) differentiated blood cells, (*k*) differentiated muscle, (*l*) differentiated neural cells

endoderm, mesoderm, and ectoderm. Multipotent stem cells produce cells of a particular lineage or closely related family of cells (Fig. 2.13). Extraembryonic membranes and the placenta do not belong to the embryonic development.

Cells removed from the inner cell mass of the blastocyst are known as pluripotent stem cells (Fig. 2.13e). Blastocyst cells can be cultured in cell biology laboratories for a long period of time to produce stem cells and can differentiate into any of the 220 cell types in the adult body developing from three primary germ layers (Thomson et al. 1998). After further *in vivo* development of blastocysts the fetal cells can be cultured as primordial germ cells. *In vitro* cultured germ cells acquire similar properties to embryonic stem cells. The isolation of embryoblasts or its inner mass raises ethical issues and exemplifies the two sides of the debate arguing that: (a) such research is inherently wrong as it does not recognize the embryo as a human being, (b) others have no moral objections to it (Suckiel 2008; Baldwin 2009; George and Lee 2009; Douglas and Savulescu 2009; Doerflinger 2010).

2.10.3 Adult Stem Cells

These are undifferentiated cells present among differentiated cells, in tissues and organs that help regeneration and differentiation to yield some or all of the major specialized cell types of the tissue or organ. Adult stem cells serve to maintain

and repair that particular tissue where they are found. Adult stem cells are not pluripotent, but multipotent and produce a limited number of cell types.

Typical examples of adult stem cells are represented by:

(a) Hematopoietic stem cells of the bone marrow form blood cells: red blood cells, lymphocytes (T and B), natural killer cells, granulocytes (neutrophils, basophils, eosinophils), monocytes (macrophages, dendritic cells).
(b) Bone marrow stromal cells (mesenchymal stem cells) give rise to cartilage cells (chondrocytes), bone cells (osteocytes: osteoclasts, osteoblasts), fat cells (adipocytes) and fibrous connective tissue (tendon).
(c) Neuronal stem cells generate nerve cells (neurons), non-neuronal (glia) cells (astrocytes, oligodendrocytes, ependymal cells, Schwann cells, satellite cells).
(d) Epithelial stem cells are lining the digestive tract (absorptive, goblet, paneth, enteroendocrine cells), the ventricles of the brain (ependymal cells).
(e) Skin stem cells in the basal layer of epidermis and hair follicles. Epidermal cells produce keratinocytes migrating to the surface of the skin.

2.10.4 Induced Pluripotent Stem Cells (iPS or iPSCs)

These can be artificially derived from adult non-pluripotent cells by forced expression of specific genes. The transformation of somatic cells into embryonic-like stem cells served the prospect that prohibitive and controversial issues such as the use of embryos can be replaced by the reprogramming of adult cells that can differentiate into tissues other than those expected in a particular tissue. The aim of this new strategy is to reprogram cells into other cell types that have been damaged or lost. The major goal of reprogramming is to turn adult somatic cells to stem cells through the induction of embryogenic genes and to produce induced pluripotent stem cells. However, there remained serious concerns regarding the reprogramming such as adult cells have low proliferative rates, contain many mutations that can either turn into cancer or to premature cell death. The importance of inducible pluripotent stem cell research has been acknowledged in 2012 when Shinya Yamanaka and the other acknowledged stem cell researcher John Gurdon were awarded the Nobel Prize in Physiology or Medicine "*for the discovery that mature cells can be reprogrammed to become pluripotent*" (Takahashi et al. 2007; Gurdon et al. 2003).

Stem cell therapy is at the forefront of basic and clinical research and clinical trials to heal aging cells, to replace damaged tissues and organs (e.g. heart failures, spinal cord injuries, neurodegenerative disorders such as Parkinson's disease) with stem cells turning into new tissues. However, stem cells generating new tissues may not only stimulate the growth of local cells, but can proliferate in an uncontrolled manner. The safe use of stem cell therapy has to be explored before its commercial application with unanticipated effects would cause too much damage beside the already known risk of unapproved stem cell treatment. The major danger of stem cell transplantation to damaged tissues and organs is that these cells could turn to out-of-place tissues and tumors.

2.10.5 Cancer Stem Cells

During the recent stem cell debate (Gilbertson and Gaham 2012), it has been proposed that cancer may have its own stem cells responsible for sustaining long-term tumor growth through the production of transient populations of highly proliferative cells (Chen et al. 2012), as most of the tumor growth come from a few cells similar to stem cells (Beck et al. 2011). Cancer stem cells are supposed to be those cells within the tumor that renew themselves and drive tumor formation. The cancer stem cell hypothesis suggests that neoplastic clones represent a rare fraction of cells with stem cell properties that can proliferate indefinitely.

The existence of cancer stem cells is based on experiments with immunodeficient mice that tolerate the growth of human cancer cells and produce tumors that represent a biologically distinct set with stem-cell-like properties (Bonnet and Dick 1997; Singh et al. 2004; Ricci-Vitani et al. 2007; Schatton et al. 2008). The cancer tumor cell hypothesis has been weakened by the observation that after the transplantation of single human cells into highly immunocompromised mice, only one in four tumour cells derived from melanoma initiated a tumor (Quintana et al. 2008). Although, evidence has been provided for both the clonal origin of cancer and through the accumulation of genetic mutations leading to malignant transformation, but the idea that malignant cells ultimately originate from cancer stem cells is not quite established. Although, the cancer stem cell hypothesis and the role of these stem cells remained a topic of heated debates, the identification of cancer stem cells in murine tumors provided further evidence for this concept (Medema 2013). Nevertheless, more evidence is needed to confirm the cancer stem cell hypothesis that may give a new perspective not only to the understanding but also to the treatment of cancer.

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Chapter 3
Loss of Homeostasis

Abstract The loss of homeostatic balance involves the discussion of tissue adaptations, among them decreased stress tolerance (atrophy), increased stress tolerance (tissue injury and death, hypertrophy, hyperplasia, dysplasia, neoplasia, metaplasia), pathological hypertrophic syndromes (*cor bovinum*, *cor pulmonale*) and abnormal hypertrophic enlargements of body parts. Other moderate imbalances deal with heloma and callus formation. Major regenerative irregularities will touch upon hepatomegaly and cirrhosis.

Keywords Tissue adaptation • Stress tolerance • Atrophy • Hypertrophy • Tissue injury • Tissue death • Hyperplasia • Dysplasia • Neoplasia • Metaplasia • Pathological hypertrophies • Heloma • Callus formation • Liver regeneration • Hepatomegaly • Cirrhosis

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Contents

3.1 Loss of Biological Balance

The Greek philosopher Heraclitus (540–480 BC) stated that "Nothing endures, but changes". Constant changes are intrinsic to all things including the human natural condition. Hippocrates (460–375 BC) contrasted the healthy balance of "elements" (earth, air, fire and water) with illness and disease caused by the systematic disharmony of these "elements" (Moal 2007). Walter Bradford Cannon postulated that homeostasis, the synchronized adjustments of the internal environment of specific physiological parameters (blood pressure, temperature, pH, etc.,) must be kept within physiological steady-state ranges and also recognized the threats to homeostasis. These threats may come from extreme changes of the external or from the internal (e.g., pain, infection) environment, with serious physical, psychological and with emotional consequences (Goldstein and Kopin 2007). Cannon emphasized the importance of negative feedback systems and warned that, no matter what caused the danger to the maintenance of homeostasis, the responses within the organism would be the same. The importance of emotional stability to maintain homeostasis has also been recognized by Cannon, who suggested that real emotional shock could cause not only disease but lead to fatal conditions. Extreme cases have been reported from primitive tribes, referred to as voodoo death, evil eye, pointing to the bone, faith healing, after breaking social or religious taboos (Cannon 1942; Zusne and Jones 1989). As these terms belong to anomalistic psychology that deals with paranormal phenomena (sightings of ghosts, hallucinations, dreams, ecstasy, magnetism, supernatural malobservations and misinterpretations) (Leudar and Thomas 2000), without anything paranormal being involved, they have little to do with the loss of homeostasis. Nevertheless, physiological studies acknowledge that sudden death can be caused by situations against which there is no defense. The reaction of hopelessness was shown by some wild rats confined to swimming in a jar in a situation against which there was no defense and hopelessness

turned to complete giving up (Richter 1957). In such hopeless situations the predominance of the parasympathetic rather than the sympathetic nervous system was postulated by Cannon (1942). The physiological, i.e. natural sudden death often has cardiac causes, with abrupt loss of consciousness (Myerburg 2005). Airway obstruction (respiratory arrest), toxicity, poisoning, anaphylactic shock are other examples of sudden death. In sudden death normal negative feedback mechanisms of homeostasis become overwhelmed by destructive positive feedback mechanisms that take over (Marieb and Hoehn 2007).

Several diseases contribute to the disturbance of homeostasis causing homeostatic imbalance or loss of homeostasis. The efficiency of control systems decreases gradually in every organism with age. Beside the gradual decline of regulation known as aging, the lack of regulation can completely destabilize the inner environment and cause death, while inefficient regulation may turn to illness. Known examples of homeostatic imbalances have been observed in the following homeostatic functions:

– Metabolic imbalances can develop when organs (liver, pancreas, kidney, etc.) do not function properly. Major categories of metabolic imbalances are:
  - disorders of carbohydrate metabolism (hyper-, hypoglycemia, diabetes, fructose intolerance, lactose intolerance, gangliosidoses, mucopolysaccharidoses, glycogen storage diseases),
  - mitochondrial diseases (Kearns-Sayre syndrome),
  - peroxisomal dysfunctions (Zellweger syndrome),
  - lysosomal storage diseases (>70, e.g. Tay-Sachs disease, sphingolipidoses, gangliosidosis, Gaucher and Niemann-Pick disease),
  - disorders of amino acid metabolism (phenylketonuria, glutaric acidemia type I, maple syrup urine disease, alcaptonuria),
  - disorders of fatty acid oxidation (medium-chain acyl-CoA dehydrogenase deficiency),
  - disorders of porphyrin metabolism (acute intermittent porphyria),
  - disorders of steroid metabolism (congenital adrenal hyperplasia),
  - purine and pyrimidine metabolism (gout, Lesch-Nyhan syndrome),
  - urea cycle disorders or defects, caused by a mutation in a deficiency of one of the six enzymes in the cycle (e.g. ornithine transcarbamylase deficiency),
  - lipid storage diseases inherited from one or both parents who carry defective genes that regulate a particular protein and are either autosomal recessive or X-linked recessive traits (Gaucher disease, Niemann-Pick disease, Fabry disease, Fabre's disease, GM2 gangliosidoses, leukodystrophies, acid lipase deficiencies).
– Dysfunction of digestion (constipation, diarrhea, intestinal gas, bloating).
– Chronic inflammation. Acute inflammation is the short-term immune response to trauma, infection, and allergy that has been sufficiently addressed. In chronic inflammation the response is either not satisfactory, not completely turned off or extinguished. Joint pain is causing inflammatory autoimmune diseases in

the synovial fluid such as rheumatoid arthritis, osteoarthritis (degenerative joint disease), ankylosing spondylitis, psoriatic arthritis. Other chronic inflammations cause muscle strain, bursitis, lyme disease, Sjörgen's syndrome (damage to tear and salivary glands causing the eyes and mouth to become dry), food allergy to citrus, corn, dairy products, gluten, yeast, egg, tomato, potato, pepper, eggplant, etc.

- Hemostasis: disorders can be hemophilia (insufficient blood clotting), formation of unwanted blood clots can cause fatal heart attack or pulmonary embolism, defect in platelet plug formation (Von Willebrand disease).
- Electrolyte imbalances (K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-, PO_4^{3-} present as minerals in the body) affect the amount of body fluids primarily blood and urine:
  - dehydration (lack of water supply, excessive sweating),
  - water gain – water retention can be caused by excess salt intake (high sodium imbalance, hypernatremia primarily in infants and elderly), high blood pressure, fluctuation of hormones (e.g. second half of the menstrual cycle), edema causing sudden weight gain, capillary damage and leaks, congestive heart failure (fluid buildup to the legs or fluid congestion in the lungs), kidney disease (water retention in the legs), lymphatic damage (extended periods without exercise or physical activity, block of lymph nodes by cancer), liver damage particularly cirrhosis causing increased blood pressure in the liver and accumulation of liquid in the abdominal area.
- Septicemia is an inflammatory stage of the whole body (presence of pathogens in the bloodstream), caused by infection (bacteria, fungi, viruses or parasites) often associated with fever, hypothermia, tachycardia, confusion, edema (Levy et al. 2003).
- Circadian disruption: prolonged sleep restriction with concurrent disruption of the circadian cycle can alter metabolism and increase the risk of obesity and diabetes (Buxton et al. 2012). Not all aspects of chronic sleep restriction have been thoroughly studied, but cumulative data evidence its detrimental role on metabolic processes (Killick et al. 2012).
- Imbalances of skin are relatively mild and reversible (athletes' foot, skin infections, allergies, cold sores, contact impetigo, psoriasis, boils, carbuncles, etc.). Severe burns may lead to electrolyte imbalances and dehydration.

3.2 Tissue Adaptation

The basic principle of tissue adaptation is that changes in the relative level of physical stress induce predictable adaptive responses in all biological tissues (Mueller and Maluf 2002). Physical stress is defined as the force applied to a given area of biological tissue (Tipler 1982). Exercise interventions are able to modify physical stress and decrease impairments, functional limitations, disability, and pain in a variety of patient populations. Exercises, postural instructions, orthotic devices

and modalities, that modify physical stress have been shown to reduce impairments, functional limitations, disability, and pain of patients (Brown and Holloszy 1991). Tissues can give characteristic responses to physical and mental stress. Among the tissues muscle besides being the force generator, is also the major shock absorber protecting bones, cartilage and ligaments from excessive stress (Radin et al. 1975). The thresholds for tissue adaptation can range from loss of adaptation (injury, death) to decreased or increased tolerance or to maintenance (Mueller and Maluf 2002). The fundamental principles of physical stress have been described and five major responses to physical stress will be discussed as: decreased stress tolerance (e.g. atrophy), maintenance, increased stress tolerance (hypertrophy), injury, and death (Mueller and Maluf 2002).

3.2.1 Decrease in Stress Tolerance

In many societies life is stressful, but the majority of the population has developed a stress tolerance and only a small population (∼10 %) is subjected to "overstress" and suffers from low stress tolerance due to its improper lifestyle. Overstress that was considered earlier a mental problem has biochemical and physical consequences such as muscle tension, headaches and stomach problems. Regular physical exercise is the best stress reliever.

3.2.1.1 Atrophy

It occurs when tissue degeneration exceeds tissue production in response to reduced stress levels, but hormonal changes, membrane permeability and changes in the properties of the tissue may also reduce stress tolerance. The physical stress level has been regarded as a composite value depending on the magnitude, time (frequency, duration, rate) and direction (tension, compression, shear, torsion) of the stress.

3.2.2 Increased Stress Tolerance of Tissues

3.2.2.1 Hypertrophy

In this mechanism tissues with larger cells become more tolerant to physical stress by producing more tissue than is degenerated. Hormonal and membrane changes can also improve hypertrophic stress tolerance. During weight training the size of skeletal muscle cells increases. Anabolic steroids have a similar effect, they enable muscle cells to increase in size and strength.

Pathological cases are described under "Pathological hypertrophies".

3.2.2.2 Tissue Injury

Increasing levels of physical stress can amount to the upper highest level of tolerance referred to as maximum stress threshold that can already cause some damage without causing noticeable dysfunction. The tissue can still fully recover upon rest. When the maximum stress threshold is exceeded tissue damage can turn to discomfort, pain and impaired function. High radiation levels may have not only short-, but also long-term effects, e.g. astronauts face a slightly increased risk of cancer in later life due to occasional solar flares from the Sun.

3.2.2.3 Tissue Death

The fundamental principal is that extreme deviations from the maintenance stress range exceeding the adaptation levels tissues lose their viability and die. Tissue death can also occur when damaged tissues are subjected to further extremely high or extremely low stress levels preventing their recovery (Mueller and Maluf 2002).

3.2.2.4 Hyperplasia

It also belongs to adaptive cellular changes involving the physiological proliferative increase in cell number. In hypertrophy the adaptive change is an increased cell size. Hyperplasia differs from neoplasia in that hyperplasia is regarded as a physiological adaptive cell change where adaptive growth remains under normal control mechanisms. Hyperplasia can be defined as an abnormal increase in cell number in a tissue or organ, excluding tumor formation, where the bulk of the tissue or organ is increased. Elevated number of cells is demanded to compensate cellular damages, skin loss, chronic inflammatory processes, hormonal dysfunction, etc. Hyperplasia can be induced for higher athletic performance by increasing the number of muscle fibers through specific power training instead of increasing the size of muscle fibers (Antonio and Gonyea 1994). Types of hyperplasia are:

- *Benign prostatic hyperplasia* – prostate enlargement. It is often interchangeably and incorrectly named benign prostatic hypertrophy. As it is caused by an increased number of cells it is in fact hyperplasia. In the development of benign prostatic hyperplasia androgen hormones (testosterone, androsterone) are considered to play a permissive role. Estrogens interfere with the hormonal stimulation of the prostate gland and decrease the prostate's size.
- *Cushing's syndrome* – hormonal disorder caused by long-term exposure to elevated levels of cortisol produced by the hyperplastic adrenal gland.
- *Hemihyperplasia* – with one side of the body affected sometimes generating limbs with different lengths.
- *Hyperplasia of the breast* – with increased risk of breast cancer.
- *Intimal hyperplasia* – response of a vessel to injury. Blood flow affects development of intimal hyperplasia after arterial injury in rats (Kohler and Jawien 1992).

- *Focal epithelial hyperplasia* – wart-like growth in mucous tissues (mouth, throat) caused by human papilloma virus. Also known as Heck's disease causing cancer.
- *Sebaceous hyperplasia* yellowish benign development on the skin particularly on face.
- *Compensatory liver hyperplasia* – caused by acute injury to restore liver function. Up to 75 % of the liver can be damaged or removed surgically and with full regeneration taking place through hepatic hyperplasia. Living-donor liver transplantation is based on compensatory liver hyperplasia.

3.2.2.5 Dysplasia

Dysplasia refers to abnormal developmental change of phenotype (size, shape and organization) of immature cells restricted to the original tissue often turning to an *in situ* neoplastic process. Dysplastic cells are of unequal size, abnormally shaped, show strong pigmentation (hyperchromatism) and contain unusually high number of dividing (mitotic) cells. Increasing degree of dysplasia is related to the likelihood of carcinoma development (Ridge et al. 2008). Examples of dysplasia:

- epithelial dysplasia of the cervix can be detected by an abnormal papilloma smear,
- dysplasia of blood-forming cells (immature cells in bone marrow, decreased number of mature functional cells in blood).

Dysplasia is the earliest form of pre-cancerous lesion as this compensatory mechanism does not restore homeostatic balance. With dysplasia we arrived to another type of homeostatic imbalance that is related to abnormal development where the loss of homeostasis does not result in instant death of an organism, but is the beginning of a neoplastic process.

3.2.2.6 Neoplasia

It is an abnormal proliferation process where the tissue has lost its homeostasis and proliferation turns first to a benign tumor or to a cancer with abnormal cell proliferation that cannot be regulated by normal signs or stimuli. Proliferation that is a normal response to compensate abnormal conditions can turn to neoplasia with abnormal proliferation.

3.2.2.7 Metaplasia (Cell Type Conversion)

The origin of embryonic stem cells and how they are differentiated to different tissue cell have been schematically viewed in Fig. 2.13. This normal process is contrasted by the cell type conversion of metaplasia where the reversible replacement of one differentiated cell type by another mature differentiated cell type takes place. Such changes can be part of a normal maturation process or

generated by an abnormal stimulus. The cell type conversion that occurs *via* a natural maturation process cannot be considered carcinogenic. The physiological metaplasia can be explained by placing a cell from its established microenvironment to another one, where the cell undergoes adaption e.g. by changing to another type that is more suitable to the new environment. Examples of chronic physical and chemical irritation causing pathological metaplasia are: cigarette smoke that causes mucus-secreting respiratory epithelial cells to be replaced by squamous epithelial cells (squamous metaplasia), can eventually turn to a cancerous process. Barett's esophagus squamous epithelial cells of the lower esophagus can change to columnar epithelium cells upon gastro-esophageal reflux. Urinary bladder epithelium can change to squamous epithelium induced by bladder stones. Metaplastic conversion takes place when undifferentiated endothelial podocytes involved in renal filtration turn to differentiated mesenchymal cells.

3.3 Pathological Hypertrophies

Physiological hypertrophy is the nontumorous enlargement of an organ or tissue resulting from an increase in the size rather than the number of constituent cells. Pathological hypertrophy is a compensatory mechanism that allows to cope with pathogenic stimuli, but as it is independent and out of proportion to the rest of the organism it is accompanied by diseases.

3.3.1 Hypertrophied Heart

In physiological hypertrophy (athlete's heart) the mass and pumping capability of the heart muscle is increased as a consequence of healthy exercise or pregnancy (Mone et al. 1996). Trained athletes may have 60 % more left ventricular muscle than untrained people. Aerobic training helps an athlete's heart to pump out larger volumes of blood per beat due to the increased size of the ventricles with less beats per minute, while anaerobic training is the one that causes the thickening of the myocardial wall (McMurray 1998). Physiological cardiac hypertrophy is reversible characterized by normal cardiac morphology without fibrosis or apoptosis (Bernardo et al. 2010). Among the signaling pathways the insulin-like growth factor/phosphoinositide-3-kinase (PI3K) pathway and the upregulation of heat shock transcription factor 1 have been reported to mediate physiological cardiac growth (McMullen and Izumo 2006; Sakamoto et al. 2006).

In pathological hypertrophy the mass of the heart increase can go up to 150 %, without increasing its pumping ability. In response to stress (hypertension, heart muscle injury, myocardial infarction, heart failure, neurohormones), muscular hypertrophy of the heart can cause cardiovascular diseases such as primary myocardial dysfunction associated with cardiomyopathies. The most frequently occurring cardiac muscle enlargements are ventricular hypertrophies.

3.3.1.1 Cor Bovinum

Cor bovinum (Latin, for ox heart) refers to an extreme hypertrophy of the **left ventricle** of the heart due to volume overload, caused by tertiary syphilis but currently more often due to chronic aortic regurgitation, hypertensive and ischemic heart disease. Left ventricular hypertrophy can be the consequence of cardiovascular disease, high blood pressure, aortic stenosis, aortic insufficiency, mitral insufficiency, excitation of heart by drinking large quantities of fluids (notably beer).

3.3.1.2 Cor Pulmonale

Cor pulmonale is the enlargement of the **right ventricle** of the heart caused by a primary disorder of the lungs or of the pulmonary blood vessels. In *cor pulmonale* pulmonary hypertension is representing the common link between lung dysfunction and heart disorder. Primary abnormalities of the left side of the heart or congenital heart diseases are not considered *cor pulmonale*, as they develop secondary to a wide variety of cardiopulmonary diseases.

Major causes of *cor pulmonale*:

- Chronic obstructive pulmonary disease
- Chronic blood clots in the lungs
- Cystic fibrosis
- Scarring of the lung tissue
- Severe curving of the upper part of the spine
- Sleep apnea, pauses occur during breathing due to airway inflammation
- Valvular heart diseases involving valves of the heart (aortic, mitral on the left and tricuspidal valves on the right) can also cause pathological hypertrophy.

At the molecular background of the development of pathological cardiac hypertrophies changes in cardiac gene expression have been suspected that are thought to provide the heart with a means to compensate for increased hemodynamic load (Katz 1990). Maladaptive hypertrophy is commonly associated with upregulation of fetal genes, fibrosis, cardiac dysfunction and increased mortality (McMullen and Jennings 2007).

Atrial enlargement (left, right atrium) of the heart occurs less frequently. Obesity is the most important risk factor of left atrial hypertrophy.

Cardiac dilation also belongs to cardiomegaly wherein mainly cardiac chambers are enlarged, but does not belong to hypertrophy. In hyperplastic aortitis at tertiary stage of syphilis the vessels that supply the aorta itself with blood, the *vasa vasorum* show hyperplasic thickening of aortic walls that restricts blood flow and causes ischemia. As the disease progresses, syphilitic aortitis leads to life threatening aortic aneurysm, with major concern of rupture and instant death unless treated immediately.

3.3.2 Other Abnormal Hypertrophic Enlargements of Body Parts

- adenomegaly – gland enlargement
- dactylomegaly – abnormally large fingers or toes
- elephantiasis – hypertrophy of certain body parts (usually legs and scrotum); the disease of lymphatic filariasis is elephantiasis – edema with thickening of the skin and underlying tissues caused by thread-like nematodes (roundworms)
- splenomegaly – an abnormal enlargement of the spleen
- hepatomegaly – abnormal enlargement of liver
- giantism (gigantism) – excessive size; usually caused by overproduction of growth hormone by the pituitary gland
- acromegaly – enlargement of bones of hands, feet and face; often accompanied by headache and muscle pain and emotional disturbances; the cause of increased growth hormone by the anterior pituitary gland is usually a benign tumor development after the skeleton and other organs finish growing.

3.3.3 Hormonal Hypertrophy

Training-induced changes in acute testosterone responses may be important factors for strength development and muscle hypertrophy (Ahtiainen et al. 2003). The low level of testosterone (e.g. in older women) could be a limiting factor in strength development, while higher testosterone level could mediate interactions with the nervous system contributing to the hormone induced hypertrophy of the muscle (Häkkinen et al. 2000).

3.3.3.1 Insulinomas

These relatively rare and often benign pancreatic neuroendocrine tumors are caused by beta cell hypertrophy characterized by hypoglycemia, high level of insulin in blood, enlarged and hyperchromatic beta-cell nuclei (Lodish et al. 2008).

3.4 Heloma (Corn, Clavus)

The Latin names of soft and hard corns are *heloma molle* and *heloma durum*, respectively. Soft corns occur between adjacent toes, hard corns on dry, smooth hairless surfaces of the skin. Corns are formed during the rubbing motion of

pressure pointing against the skin. Growing corns intensify the pressure and can cause damage in deeper tissues eventually cause ulceration i.e. sore on the skin, accompanied by the disintegration of tissues. Ulceration is a typical condition of diabetes when the skin becomes insensitive and corn development can damage nerves of the peripheral nervous system and lead to diabetic neuropathy. Diabetic foot ulcer is among the major complications of diabetes occurring in ∼15 % of diabetic patients and precedes 84 % of all lower leg amputations (Brem and Tomic-Canic 2007).

3.5 Callus Formation

The callus is also a thickening of skin exposed to prolonged rubbing, but unlike a corn, the thickening is evenly distributed without a dense central core. Calluses occur on the soles of the feet, but can also be present on other parts of the body (hands of people handling tools, instruments, equipments, etc.). The physical advantage of calluses is that they provide a protecting cushion against pain. Calluses can develop near the base of the toes caused by friction of shoes. Callus formation can also be related to walking problems or abnormality in gait or foot structure causing constant stress. Calluses and corns tend to return even after removal, but this is less likely when foot padding and proper shoe inserts are being used.

3.6 Liver Regeneration

3.6.1 Immortal Liver

The liver is probably the most complex organ in the body, containing at least seven different cell types and carrying out over 5,000 functions (Ciecierski et al. 2005). The metaphoric significance of the immortality of liver is cited by this passage from the myth of Hesiod (eighth century BC):

"He (*Zeus*, *god* who ruled the Olympian world) bound devious *Prometheus* (a Titan) with inescapable harsh bonds, fastened through the middle of a column, and he inflicted on him a long-winged eagle, which ate his immortal liver, but it grew as much in all at night as the long-winged bird would eat all day" (Caldwell 1987) (Fig. 3.1). The liver was regarded immortal as for the ancient Greeks, as it was the seat of the soul and intelligence, beside its regenerative power (West 1966; Chen and Chen 1994).

The mysterious regenerative capacity of liver after injury or resection remained a fascinating enigma. Partial hepatectomy is the strongeststimulator of hepatic

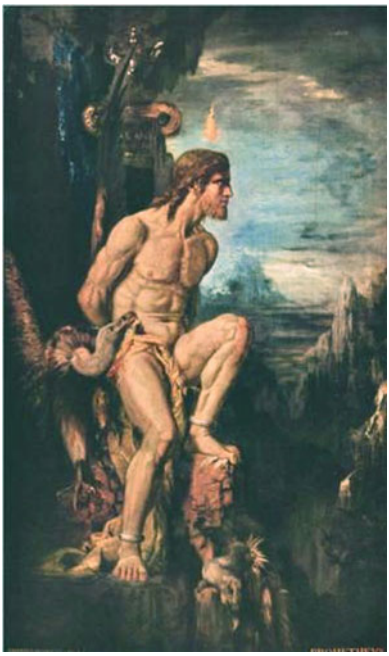

Fig. 3.1 Prometheus by Gustave Moreau (1868)

regeneration proved by studies of resections in rat models (Court et al. 2002). The regeneration of liver cells was confirmed by sequential partial hepatectomies and proved that:

(i) all cells of the liver including hepatocytes, biliary epithelial cells and endothelial cells equally contributed to the regeneration,
(ii) regeneration was fast and finished within a few days,
(iii) liver cells have a practically unlimited capacity for proliferation and
(iv) liver cells are not terminally differentiated.

Regeneration has been defined as orchestrated response induced by specific external stimuli (gene expression, growth factor production, and morphologic structure). Among the several factors and cytokines, hepatocyte growth factor, epidermal growth factor, transforming growth factor-alpha, interleukin-6, tumor necrosis factor-alpha, insulin, and norepinephrine, appear to be the most important players in the regeneration process (Michalopoulos and DeFrances 1997). The essential circuitry required for the regeneration process has been categorized into three networks: cytokine, growth factor and metabolic networks (Fausto et al. 2006). Although, a variety of genes, cytokines, growth factors, and cells are known to be involved, the exact mechanism of liver regeneration and the interactions among cells and cytokines are still not known. As an explanation many pieces of the puzzle have already been elucidated, but many smaller pieces are still missing, and the multiple interlocking edges with other pieces of the puzzle give a blurred picture

of liver regeneration (LaBrecque 1994). Complex knowledge about the molecular and cellular mechanisms of liver regeneration is both conceptually important and directly relevant to clinical problems (Pahlavan et al. 2006). Up to now the complex process affecting liver regeneration is not completely understood. The clarification of the regeneration could provide novel strategies in the management of liver transplantation from donors to recipients (Taki-Eldin et al. 2012). Furthers studies of liver regeneration after partial hepatectomy are expected to provide:

(a) deeper understanding of mammalian cell proliferation *in vivo*,
(b) molecular insights into the self-renewal of mature cells,
(c) information regarding the connection between regeneration and stem cells.

3.6.2 Hepatomegaly

The swelling of the liver (hepatomegaly) as an abdominal mass is not a disease, but a medical condition. Hepatomegaly is a sign of an underlying problem that can have several causes such as infection, liver toxicity, metabolic disorder, congestive heart failure, hepatic tumors (occasionally combined with jaundice).

3.6.2.1 Causes of Hepatomegaly

As there are many other causes of hepatomegaly, here only the major types and a few examples belonging to these types are listed:

– Metabolic reasons of hepatomegaly:
  - Amyloidosis
  - Glycogen storage diseases
  - Lysosomal aberrations
  - Fatty acid metabolic irregularities
  - Carnitine palmitoyltransferase I deficiency

– Infective diseases
  - Infectious mononucleosis (glandular fever) caused by Epstein-Barr virus and pseudoglandular fever caused by cytomegalo virus
  - Acute hepatitis (tender hepatomegaly in about 10 % of the cases)
  - Malaria, amoebiasis, leptospirosis, actinomycosis

– Tumors
  - metastasis (most common cause)
  - hemangioma, hepatocellular carcinoma, myeloma, leukemia, lymphoma, carcinoma

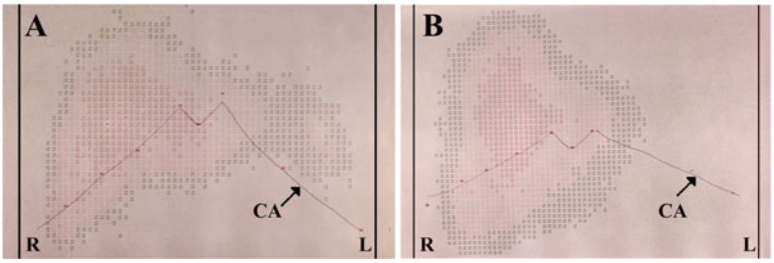

Fig. 3.2 ^{113m}In colloid liver scintigraphy of normal liver (**a**) and hepatomegaly (**b**). *Red numbers* indicate high, *yellow numbers* medium and blue numbers low level of radioactivity. University doctoral dissertation. Figs. 21 and 22. Department of Internal Medicine, Medical University, Szeged. *R* right side, *L* left side of the patient, *CA* costal arch. The costal arch is the lower margin of the chest formed by the bottom edge of the rib cage (With permission Banfalvi 1971)

– Cirrhosis
  - biliary, portal, cardial
  - haemochromatosis
– Drugs, toxins
  - alcohol, poisons
– Inborn causes of hepatomegaly:
  - haemolytic anemia
  - polycystic diseases
  - Cori's (Type III glycogen storage) disease

3.6.2.2 Diagnosis of Hepatomegaly

In several tumor types metastasis is confined to the liver. Approximately 50 % of the patients with liver metastases have clinical signs of hepatomegaly or ascites. Nevertheless, as demonstrated by the major types of hepatomegaly, it is a symptom rather than a disease, and its treatment depends on the identification and control of the underlying condition that can be indeed a disease and should be handled accordingly. The diagnostic tools of hepatomegaly involve blood tests, liver-function tests and ultrasound detection. For liver scintigraphy (Fig. 3.2) the short-lived indium isotope (^{113m}In, half-life 99.5 min) has been replaced by technetium ^{99m}Tc (half-life 6 h) and single-headed scanners by multi-headed gamma cameras. In spite of its inferior sensitivity, but due to the lower cost of gamma camera and its flexibility scintigraphy is still a useful diagnostic tool to capture the emitted radiation of the radiotracer to create two-dimensional images. Single photon emission computed tomography (SPECT) utilizes 1–3 detectors rotating around the patient providing more accurate 3D anatomical information. Positron emission tomography (PET)

with several detectors produces a three-dimensional image of the body or its processes. The PET system detects coinciding pairs of gamma rays of a positron-emitting radionuclide administered to the patient as a biologically active molecule.

The glucose analogue ^{18}FDG is preferentially used to follow the metabolic activities of liver and other organs and to trace cancer metastasis as PET scans. The imaging of this radiotracer and other types of molecules will be discussed in Chap. 5, devoted to metastasis.

3.6.3 Cirrhosis

While hepatomegaly has been defined as an undefined liver status with liver enlargement, cirrhosis is the consequence of a chronic liver disease with incomplete regeneration of the damaged liver tissue by fibrotic scar tissue resulting in reduced liver function. There are several known and some unknown (idiopathic) causes of cirrhosis, but the most common ones are alcoholism, hepatitis B and C, fatty liver disease, viruses, toxic metals (e.g. iron and copper accumulating in the liver), autoimmune liver disease. Only a small number of people with cirrhosis get liver cancer.

3.6.3.1 Development of Cirrhosis

The blood supply of liver differs from other organs. Most of the blood supply of the liver comes from the intestinal veins through the portal vein and only a smaller amount from the liver arteries. The portal vein is divided into smaller veins, the smallest ones called sinusoids being in close contact with liver cells that line up along the sinusoids. This close connection provides an intensive exchange of substances between blood and liver cells. After passing the sinusoids the blood is collected into increasingly larger veins and after leaving the liver as the largest hepatic vein returns the blood to the heart. During the formation of cirrhosis this close connection between blood and liver cells is damaged or obstructed and liver cells are unable to remove or add substances from and add to the blood. The obstruction increases the portal pressure and due to the portal hypertension the blood returns to the heart through other veins that bypass the liver. The lower blood flow will support less liver cells, the reduced number of liver cells loose contact with blood and accumulate toxic substances and the bypass of blood flow results in cirrhosis. Cirrhosis is aggravated by the destruction of bile canaliculi running between the liver cells and line the sinusoids. As a result liver is unable to eliminate bile that would be necessary for the digestion of lipids in the intestine. Major causes leading to cirrhosis are:

– Alcoholic liver disease – blocking protein, fat and carbohydrate metabolism, hepatotoxicity measured by lactate dehydrogenase (LDH) and lower ratio of

aspartate aminotransferase/alanine aminotransferase (ASAT/ALAT) activities. Reduced ASAT/ALAT ratio (between 1 and 2) is associated with cirrhosis, high transaminase ratio (>2) is indicative of hepatocellular carcinoma or alcoholic hepatitis (Sorbi et al. 1999).

Transaminases Transaminases of diagnostic importance were earlier known as glutamate-oxaloacetate (GOT = ASAT) and glutamate-pyruvate (GPT = ALAT) transaminases. The liver and the heart have high ASAT activities. ALAT activity is highest in liver. Upon tissue damage of the heart (e.g. myocardial infection) the ASAT activity becomes extremely high with a smaller elevation of ALAT. During hepatocellular damage both transaminase levels are elevated, but the ALAT level is higher than that of the ASAT. Consequently, transaminase levels are suitable not only for the diagnosis of hepatic diseases but also for cardiac infarction.

- Non-alcoholic steatohepatitis has no alcohol history and is associated with other liver damages caused by insufficient intake of protein (*marasmus*), coronary artery disease, diabetes, obesity, corticosteroid and hepatotoxic drugs and toxins. The chronic inhalation of organic solvents may also increase transaminase levels and lead to cirrhosis. Toluene inhalation e.g. significantly increased serum ALAT and ASAT levels (Tas et al. 2011).
- Chronic liver inflammation caused by viruses (*hepatitis C, hepatitis B*).
- Biliary cirrhosis diagnosed by serum alkaline phosphatase, cholesterol and bilirubin elevations and characterized by pruritus, skin hyperpigmentation, hepatomegaly, bile duct lesions.
- Sclerosing cholangitis – with *steatorrhea* (presence of excess fat in feces), *pruritus* (itching), fat soluble vitamin deficiencies, bone and ulcerative bowel diseases, dilation of bile ducts.
- Autoimmune hepatitis causing inflammation, scarring and cirrhosis.
- Hereditary hepatitis – familial cirrhosis, hyperpigmentation, hemochromatosis, diabetes, cardiomyopathy, iron overload. Alpha 1-antitrypsin deficiency is an autosomal recessive trait. Lyposomal acid lipase deficiency is a rare autosomal recessive disorder leading to cirrhosis, liver failure and death. Cystic fibrosis is also an autosomal recessive disease affecting not only the liver, but also the lungs, pancreas and intestine.
- Cardiac cirrhosis of right sided heart failure with concomitant liver congestion.
- Wilson's disease – increased copper content in liver.
- Carbohydrate deficiencies – galactosemia, glycogen storage disease type IV.

Similarly to hepatomegaly, cirrhosis is a symptom rather than a disease. Cirrhotic liver damage will have several important implications as many critical functions of the liver will be impaired, among them blood clotting (bleeding from esophageal varices), detoxification of drugs (due to the destruction of smooth endoplasmic

reticulum of liver cells), glucose and lipid (as lipoproteins) supply, increased risk of infection, hepatic encephalopathy with life threatening complications (confusion and coma). The most common complication of cirrhosis is fluid retention in the abdominal cavity known as *ascites* contributing to a significantly reduced quality of life of the patient. At this stage cirrhosis becomes an irreversible process with the only option of liver transplantation.

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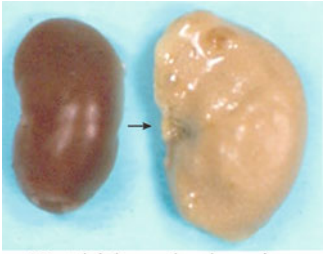
Chapter 4
Tumor Development



Rat kidney leukemia

Abstract This subchapter deals with the properties of tumors that occur in humans and experimental animals. The chapter starts with the definition of tumors, followed by the characterization of benign tumors, premalignant and malignant tumors and their prevention. In the subchapter of tumor staging the classification of tumors will be overviewed. Tumor models will include animal, mainly rodent models. Tumor tests (biopsy, imaging techniques, blood and bone marrow tests), techniques of tumor screening, carcinogens, antitumor agents and benign tumors of different organs (gastrointestinal, lung, renal, brain, heart, spleen and brain) will cover the rest of the chapter.

Keywords Tumor growth • Benign tumors • Premalignant tumors • Malignant tumors • Cancer prevention • Primary tumor models • Animal tumor models • Classification of tumors • Tumor staging • Tumor tests • Biopsy • X-ray imaging • Ultrasound images • Blood tests • Bone marrow test • Positron emission tomography (PET) • Carcinogens • Viral oncogenes • Tumor screening • Laboratory methods • Genetic tests • Antitumor agents • Tumor formation • Benign tumors

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Contents

4.1 Characterization of Benign and Malignant Tumors

To describe tumor growth a large number of biological effects have to be taken into account. Although, mathematical models are able to reproduce experimental data of tumor cell development with satisfactory qualitative agreement (Ambrosi

and Mollica 2002), major attention will be paid to cell populations and how these populations behave under different chemical and nutritional conditions. The growth itself has not been uniformly defined, as it can be described as:

(a) the volumetric increase of the mass of cells without reference to increasing cell number as in cell development and reproduction,
(b) increase in population as in mitosis,
(c) division of the mother cell into two daughter cells.

The term of cell growth is similar, but not identical with proliferation. Proliferation is the reproduction or multiplication of similar forms of cells. Although, we have already made distinction between the volumetric increase (e.g. hypertrophy) and hypotrophy (e.g. cirrhosis), to avoid ambiguity in further discussion cell growth will be regarded as increased number of cell division.

4.1.1 Tumor Growth

4.1.1.1 Definition of Tumors

Any type of anomalous growth of cells in the body is called tumor, irrespective of an uncontrolled fast growing cancerous (malignant) tumor that may spread to other parts of the body or a slow growing, mostly harmless non cancerous (benign) neoplasm.

4.1.1.1.1 Neoplasia

Tumor formation is the abnormal growth or division of cells. Current medicine uses tumor as a synonym of neoplasm. Tumors can be solid or cysts filled with fluid. Cysts can be formed without the involvement of neoplastic cells. Not all neoplasms cause solid tumor formation, although the cell number may increase (e.g. leukemia). Based on the speed and on the mass, the tumor (neoplasm) can be benign, pre-malignant (carcinoma *in situ*) or malignant (cancer). The term benign tumor (neoplasm) is derived from the Latin word meaning swelling. Originally the medical definition of acute inflammation was defined by the Roman Celsus (∼30 BC–38 AD) and meant the swelling of flesh with four characteristic signs: *tumor, dolor, calor, rubor* (swelling, pain, heat, redness). Recently tumor rather than swelling is being used for tumors, as other non-neoplastic swelling processes exist, such as inflammations caused by trauma, infection or other factors. The word tumor is synonym for cystic growth and solid neoplasm, bearing in mind that not all neoplasms are progressive such as nevus, heloma, callus, carcinoma *in situ*. Different diseases included is neoplasia make its definition difficult. Probably the best definition comes from the British oncologist Willis: “A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with

that of the normal tissues, and persists in the same excessive manner after cessation of the stimulus which evoked the change" (Willis 1952).

4.1.1.1.2 Carcinoma In Situ

This is can be an early form of cancer containing proliferating neoplastic cells in their natural habitat (*in situ*) without penetrating into surrounding tissues, yet considered a precursor form of cancer and if left unattended it will transform to a malignant neoplasm depending on its location. Bowen's disease, that is a carcinoma *in situ* of the skin, causes the accumulation of neoplastic epidermal cells within the epidermis only, without penetrating into the deeper dermis.

4.1.1.1.3 Cancer

Tumors and cancers are often confused with one another. As already mentioned neoplasms are benign, non-cancerous tumors. Cancers can be in pre-malignant or malignant stage. Cancer is by definition malignant. ***Malignancy*** means that the tumor can invade and destroy nearby tissues and move to other parts of the body. Malignancy may cause more than 100 different types of cancerous diseases affecting most of the organs of the human body with each type unique regarding its symptoms, causes and therapy. Different types of benign tumors will be discussed at the end of this chapter.

4.1.2 Characterization of Tumor Cells

The preparation of cell cultures and the *in vitro* growth of tumor cells have been reviewed in Chap. 2. Although, tumors occur with relatively high incidence both in humans and in animals especially in old age, the number of malignant cases is relatively low. Most of the tumors are localized and as they pose little risk to their hosts, they are regarded as benign tumors.

4.1.2.1 Types of Benign Tumors

The most important characteristics of benign tumors are that they usually do not threaten life, can be treated or after removal they normally do not grow back, do not invade neighboring tissues and do not spread to other parts of the body. The cells of benign tumors resemble normal cells, may function as normal cells and stay in the same tissue as healthy cells. Benign tumors are regularly surrounded by a fibrous capsule that distinguishes them from the healthy tissue and makes it easy for the surgeon to remove them without the danger of tumor regrowth. Nevertheless, benign

tumors also need regular supervision as they may pose serious threat when they reach a critical mass that might interfere with regular function (e.g. press against nerves or blood vessels causing pain). In endocrine tissues tumors can overproduce hormones that affect the biological activity of the organism. Major types of benign tumors are:

- *Adenomas* arising from glandular epithelial tissues (pituitary, adrenocortical, basal cell, bile duct, follicular, hepatocellular, nipple adenomas, adenoma of the colon known as polyp) are normally not cancerous only if they develop into adenocarcinomas.
- *Fibromas* are tumors of fibrous or connective tissues potentially affecting any organ. More harmful are uterine fibroids that are common and may cause urinary incontinence, pelvic and vaginal pain and discomfort. Fibroma has two major types: hard fibroma consists of more fibrous elements, while soft fibroma contains loosely associated connective tissues appearing in armpits, neck, groin, eyelids. Fibromas normally do not cause symptoms and can be easily removed by surgery, but represent esthetical nuisances to its bearers. Of the several fibromas only a few examples are mentioned: myxo-, angio-, cystic, tendon sheet, nonossifying, pleomorphic, chondromyxoid, collagenous, perifollicular fibromas. They can turn rarely to cancerous fibrosarcomas.
- *Hemangiomas* are benign tumors comprising an increased number of blood cells often seen on the surface of the skin. They require removal (e.g. laser surgery) only if they interfere with the intake of food, with hearing or vision.
- *Lipomas.* Most of them are small soft-tissue (adipose tissue) tumors that are movable to some extent. It is debated whether they may become malignant. Lipomas are named after the tissues that are affected, the most common type is found under the surface of the skin.

4.1.2.2 Premalignant or Precancerous Tumors

This type of tumor growth is about to become malignant. Non-invasive tumors stay separated within the same tissue and do not grow or invade normal tissues. These non-invasive tumors are also called carcinoma *in situ* (“in the same place”) or pre-cancers.

Examples of precancerous tumors are:

- *Actinic keratosis linked to solar damage.* Mainly fair-skinned and older people are susceptible to this type of cell growth. DNA lesions and the risk of malignancy are proportional to the Sun exposure.
- *Cervical dysplasia.* Although, dysplasia itself is not regarded as a tumorous process, but when caused by papilloma virus it is likely to turn to a malignant transformation in the lining of the cervix or the uterus. Especially endangered are women between age 25 and 35 years. As cervical cancer generally develops only over a period of years, regular screening helps early detection and treatment of early precancerous changes and prevents cervical cancer formation.

– *Lung metaplasia.* Cell type conversion may occur in the bronchi of the lung most commonly caused by smoking. Glandular cells of bronchi may become cancerous squamous epithelial cells.
– *Leukoplakia* is a condition where keratosis develops primarily in the oral mucosa associated with smoking. Leukoplakia can also define conditions of the genitals and the urinary tract. Leukoplakia has been described as a precancerous state (Pindborg et al. 1997; Reibel 2003; Ishida et al. 2007).

4.1.2.3 Malignant Tumors

Characteristic features of malignant growth are that it can:

(a) pose threat to life,
(b) be removed but can grow back,
(c) free itself from normal discourse with its normal neighbors,
(d) evolve endlessly through new adaptive changes,
(e) move into and invade the terrain of other cell populations of nearby tissues and organs,
(f) spread to other parts of the body (metastasis).

Cancer cells can be distinguished from normal cells microscopically as they:

– grow faster in a specific tissue than normal cells (the cell cycle of tumor cells is shorter, resulting in more mitoses and prone to mutations). Faster growth of tissue samples serves cytological differentiation of benign from malignant tumors.
– have a higher nucleus-to-cytoplasm ratio (during fast growth the chromatin is likely to be less condensed; smaller cells are more invasive and have a better path-finding ability to break away),
– have more prominent nucleoli (the transcription of ribosomal genes and the generation of ribosomal subunits is essential for the increased rate of protein synthesis in cancer cells).
– have less specialized structures (due to the loss of certain gene functions).

Malignant tumors are characterized by two major properties: invasiveness and spread. Invasiveness means that the cancer grows into neighboring healthy tissues (Fig. 4.1). The idea that the cancer is non-invasive (Fig. 4.1a) in the initial stage of tumor growth, but may became invasive (Fig. 4.1b), probably means that part of the cancer remains encapsulated, while other part grows out into other normal tissues. However, this does not mean the lack of invasiveness and is probably the reflexion of the beginning of the invasion as one cannot expect that the malignant spread takes place at the same time in each direction. Although, it is generally known that benign tumors can progress to malignancy, the early steps of this malignant process are hard to be detected. When the invasion continues, more and more cells lose their tissue characteristic traits, acquire altered chromosome structure, become invasive

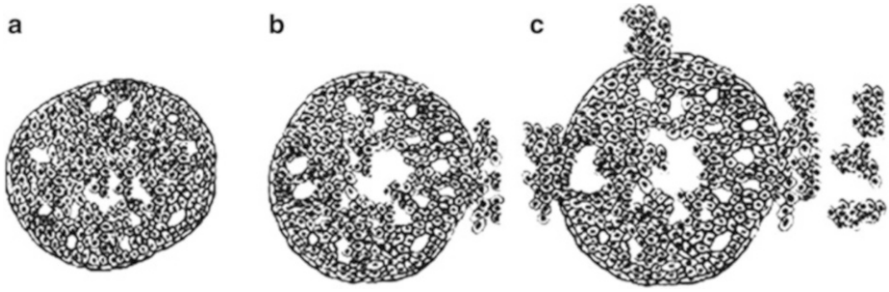

Fig. 4.1 Development from non-invasive tumor to malignant spread of cancer. (**a**) Pre-cancerous non-invasive tumor (carcinoma *in situ*), (**b**) early stage of invasiveness (cancer), (**c**) invasive tumor spread (metastasis)

and metastatic. As a result of these changes the encapsulation ceases and malignant cells leave the localized non-invasive tumor and can spread in different directions (Fig. 4.1c). Malignant tumors invade the surrounding tissues and are likely to enter the circulatory system of the body and seek areas of proliferation at distant places from the original primary site. The spread of tumor cells to secondary areas of growth is a process named metastasis. One would expect that malignant cells become more differentiated than benign tumor cells, but just the opposite happens. Malignant cancer cells are thought to be less well differentiated than benign cells and may lack specific enzymes characteristic to the primary differentiated tissue they started to evolve from. This variability in phenotype can be correlated with changes in genotype seen as chromosome abnormalities in shape, size and variable number of chromosomes.

Malignant tumors can be classified according to their origin from the three embryonic cell layers:

- carcinomas are solid tumors derived from endoderm or exoderm,
- sarcomas are solid tumors of mesodermal origin,
- leukemias (meaning "white blood") are regarded as a subdivision of sarcomas, but do not grow as solid tumors, but as individual cells in the blood.

Metastasis. Malignant tumors are able to invade neighboring tissues and organs, or can break free and enter the circulation of the blood stream or the lymphatic system and take up residence at distant parts of the body in a process known as metastasis. The spread of malignant tumor cells can generate not only one metastasis, but can be multifocal or multicentric. Multicentric tumors are formed separately and often in different areas of the same tissue (e.g. breast and liver cancer). Cancer formation and spread in different tissues and their treatment will be discussed separately. The best way to avoid cancer formation is prevention. Invasive tumor spread (metastasis) will be the topic of Chap. 5.

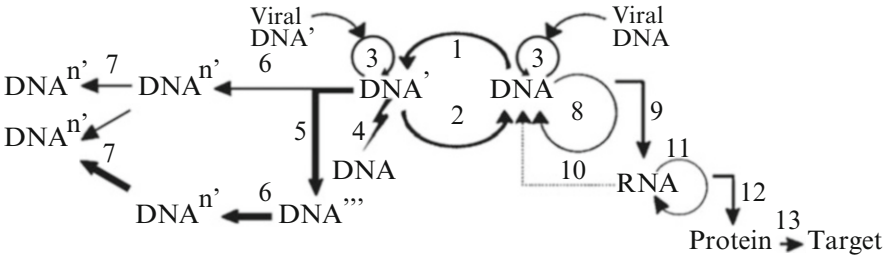

Fig. 4.2 Age related processes involved in the transfer of intracellular genetic information. *1–8* DNA <=> DNA transfer processes. *1* Mutation: DNA => DNA′. *2* Repair: DNA′ => DNA. *3* Viral infection. *4* Apoptosis or programmed cell death with high levels of DNA damage not worth to be repaired. *5* Aging with increasing number of mutations and persistent DNA damage: DNA′ => DNA″ => DNA‴. *6* Malignant transformation with multiple mutations: DNA => DNA$^{n'}$ (persistent DNA damage, accumulation of mutations). *7* Metastatic spread of tumor cells, the transfer of malignantly transformed information to distant locations of the organism. *8* DNA replication: DNA <=> DNA reduplication (high fidelity, HiFi process, 1:10^{10} misincorporated deoxyribonucleotide). *9–10* DNA <=> RNA transfer. *9* Transcription: DNA => RNA (medium fidelity, MeFi process, 1:10^5 misincorporated ribonucleotide). *10* Reverse transcription: RNA => DNA (only in retroviruses). *11* RNA replication: RNA <=> RNA (in RNA viruses). *12* Translation: RNA => protein (low fidelity, LoFi process, 1:10^4 misincorporated amino acid) *13* Protein targeting: information reaches intra- or extracellular destinations and becomes communication (Modified with permission Banfalvi 2009)

4.1.2.4 Modified Transfer of Genetic Information in Tumor Cells

To place tumor formation in a molecular perspective, core processes of the transfer of intracellular genetical information have been summarized in a comprehensive hierarchical arrangement in healthy cells, where malignant transformation is likely to occur only with aging (Fig. 4.2). Malignant transformation is not to be confused with the "transformation" used to describe non-viral DNA transfer in bacteria, non-animal eukaryotic and plant cells.

During malignant transformation the balance of several processes related to the transfer of genetic information is upset at younger age (Fig. 4.3). In transformed tumor cells DNA has been mutated and does not obey the regulatory processes of homeostasis. The division of transformed tumor cells is faster, repair is insufficient or non-existent, suicide signals are weak to trigger cell death.

As far as the malignant transformation is concerned recently three major theories tried to short-circuit the process. One of the theories is regarding cancer as an inflammatory process that is able to bypass the immune system. Another theory hypothesizes that transformed cells exploit the potency of indestructible embryonic stem cells that are present in small number in tissues and organs and by breaking free from their primary tumors migrate to distant places of the organism and grow into a new colony of cells known as metastasis. The third view considers malignant transformation as the growth of multistep mutated cells escaping through the disruptions of the primary tumor and growing at distant locations. Since these views are not necessarily antagonistic, they may share and contribute to the understanding of carcino- and metastatogenesis.

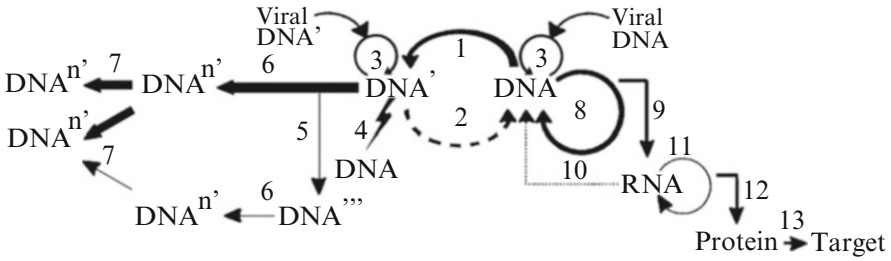

Fig. 4.3 Modified transfer of genetic information in cancerous cells. *1–8* DNA <=> DNA transfer processes. *1* Monoclonal mutation of a single cell: DNA => DNA′. *2*. Repair: DNA′ => DNA at low level or missing. *3* Viral infection can introduce tumor causing transforming agents. *4* DNA is heavily damaged but programmed cell death (apoptosis) does not function. *5* Aging is not the primary cause of persistent DNA damage: DNA′ => DNA″ => DNA‴. *6* Malignant transformation with multiple mutations: DNA => DNA$^{n'}$ (persistent DNA damage, accumulation of mutations, e.g. mutant p53). *7* Metastatic spread of tumor cells, the transfer of malignantly transformed information to distant locations of the organism. *8* DNA replication: DNA <=> DNA reduplication. As repair is not working, S phase and the whole cell cycle will be shorter. The rest of the processes (*9–13*) is the same as in Fig. 4.2. (Modified with permission Banfalvi 2009)

4.1.3 Cancer Prevention

Cancer prevention makes sense only when we know the risk factors leading to cancer and take preventive measures accordingly. Most of the risk factors can be avoided with the notable exception of genetic causes which are inbuilt in the genetic material as mutations of former generations.

1. One of the most significant risk factor is smoking that can be almost completely eliminated or drastically reduced by never starting or to quit smoking.
2. Approximately half of the cancer diagnoses come from UV ray exposure. The risk of skin cancer can be easily minimized by avoiding mid-day Sun, staying away from tanning, wearing sunscreen and protective outdoor clothing.
3. Eat to beat cancer by a well-balanced diet rich in green, orange and yellow fruits and vegetables. Fruits and vegetables contain antioxidants that contribute to the repair process and neutralize reactive oxygen species (ROS). Eating dark fruits (berries and grapes) and cruciferous vegetables also prevent cancer development. Red meet contains more fat and cholesterol than poultry and fish, consequently reduced consumption of red meat has a beneficiary effect against many types of cancer.
4. Eat less – live longer. Calorie restriction lowers the frequency of neoplastic diseases (Weindruch et al. 1986) and extends the period of life span (Weindruch and Walford 1988). Caloric restriction reduces the production of reactive oxygen species (Lambert and Merry 2004). Reactive oxygen species (ROS) are known to cause DNA damages and play a key role in human cancer development (Wiseman and Halliwell 1996).

5. Reduced alcohol consumption. Alcohol causes primarily lung cancer but can be a risk factor also in other types of cancer.
6. Regular exercise is known to reduce cancer risk.
7. Protection against viral infections caused by unsafe sex (human papilloma virus, HIV)
8. Regular cancer screen: colonoscopy, Pap smear (detection of papilloma virus), PSA test to detect early prostate cancer, mammogram to detect breast cancer in women.

4.2 Primary Tumor Models

Here only those experimental tumor models are mentioned, that have been useful in the study of primary of tumor formation.

Local transplantation of tumor cells. To study tumor growth *in vivo* there are different ways to introduce tumor cells in experimental animals. One of the major groups of transplantations is represented by those methods that place tumor cells in the tissues of rodents. Among these methods are subcutaneous (*s.c.*), intradermal (*i.d.*) and intramuscular (*i.m.*) transplantations. Local implantations result in local tumors and generate only occasionally metastasis. Subcutaneous (*s.c.*) tumor implantation normally does not metastasize. Based on own experiments local injections resulted in local tumor formation at the site of injection or if tumor cells moved elsewhere, it remained unpredictable where they adhered if there was a tumor formation at all.

Administration of tumor cells to the blood circulation. The most frequently applied method to introduce tumor cells into experimental animals used to be intravenous (*i.v.*) administration. Other forms of administration to the circulatory system are intra-arterial (*i.a.*) or intracardiac (*i.c.*) injections. The generation of liver metastasis turned out to be a relatively reliable method, when the tumor cell suspension was injected directly into the spleen or in the portal vein. When tumor cells are injected immediately into the cardiovascular system, important steps of the metastatic process are likely to be missing. A further danger of injecting aggregated tumor cells in the middle caudal vein of rodents carrying blood from the lower part of the body is that tumor cells are deposited in the next available capillaries. The tail vein leads directly to the *inferior vena cava* and the right atrium. From the right ventricle of the heart the pulmonary circulation carries tumor cells to the capillaries of lung where aggregated tumor cells may cause microemboli and initiate metastasis. Even more unpredictable is the fate of the tumor cells injected directly into the heart, as metastases can spread to different locations including bones. One of the few advantages of the progression of these tumors is that they can be used as models to study the seed and soil theory. This theory is related to the clonal expansion of metastasized cells as a consequence of modified gene expression in these cells, whereas in primary tumors the microenvironment of the tumor and host tissues play decisive roles in tumor progression (Fidler 2003). The seed and soil

theory was supported by the administration of those tumor cell lines that consistently disseminated to the liver, the adrenal gland, the bone marrow, but not the lungs upon tail vein injection (Bogenmann 1996). Although, the introduction of tumor cell lines to the circulatory system might be advantageous in the development of metastatic variants of increasing metastatic potential (Fidler and Kripke 1977; Fidler and Nicolson 1977), a serious drawback of these models is as mentioned that they will lack early steps of the metastatic cascade.

Transplantation of tumors. Originally transplantation means to graft healthy cells, tissues or organs to another organism, or to other location of the same organism so that they maintain their original function. Recently this definition has been used in a broader sense, with particular emphasis of its importance in developmental, immunological and endocrinological research, as well as in plant breeding and surgery.

4.2.1 Animal Tumor Models

Historically it was the end of the nineteenth century when researchers realized that spontaneous tumors in man and in animals did not provide deep enough and satisfactory knowledge regarding the development of these diseases. Veterinarians were the first to notice that the transplantation of spontaneous tumors to other species did not cause tumors. Transplantability of animal tumors was established by placing rat tumors in other rats (Hanau 1876). It was observed that mice of different localities responded differently to grafts (Jensen 1903). The transmissibility of murine cancer was confirmed by recognizing that implanted tumors behaved like human malignancies and that the new tumor did not develop from the host organism but from the cells of the original tumor, proving that real transplantation took place (Bashford and Murray 1904). A real breakthrough was achieved when the first dibenzanthracene induced tumors were produced in mice (Andervont 1934). On the basis of this discovery several tumor strains and cell lines have been described and maintained primarily in rodents that have been induced by chemical carcinogens. These tumors turned out to be applicable not only for biological investigations but also for testing new chemotherapeutic agents. It deserves mention that the tumor strains adapted to rats carry the names of their establishers e.g. Jensen rat sarcoma, Flexner-Jobling rat tumor, Guetin rat tumor, Walker rat tumor, Novikoff rat hepatoma, Yoshida ascites sarcoma, Shay chloroleukemia, etc. Further investigations clarified that tumor strains from the host cannot be transplanted to other closely related species and will be rejected even within races of the same species. It was found that various races of mice had different susceptibilities for cancer transplantations: Mice from Copenhagen “took” transplantations in a higher percentage than did mice from Berlin (Michaelis 1906). To find a solution against the rejection of transplants within murine races mating was made to construct an inbred mouse strain that contributed significantly to the understanding of genetics and tumor biology (Little 1941). A further advance in transplantation was the

elimination of transplantation resistance in thymus deficient nude mice (Rygaard and Poulsen 1969). This recognition led to the implantation of human tumor xenografts under the renal capsule of immunodeficient mice to test the potential of chemotherapeutic agents (Bogden et al. 1979). As animal experiments are known to be expensive the transplantation procedure was simplified by placing the tumors under the capsule of the kidney of immunocompetent healthy mice and the evaluation of results was limited to 6 days. These precautions helped to bypass disturbances caused by the degradation of implanted human tumor cells (Kangas and Perila 1985). The subrenal tumor capsule model seemed to pose some difficulties with respect to the evaluation of the chemosensitivity of antineoplastic agents (Tueni et al. 1987). In our experiments this model turned out to be the most useful one to investigate not only the growth of the primary tumor but also to follow the metastatic tumor spread. Further details of this rat model and metastatic tumor progression will be given in Chap. 5.

4.3 Classification of Tumors

Diseases have been categorized in the International Statistical Classification and Related Health Problems 10th Revision (ICD-10) classified by the World Health Organization (WHO). In this classification benign and malignant neoplasms belong to ICD-10 Chapter II: Neoplasms (blocks from C00 to D48). Malignant neoplasms of this categorization have been summarized in Table 4.1.

As will be shown by different methods, there is no single test that could be universally used for the accurate diagnosis or staging of cancer. Normally several test are performed to distinguish among different conditions (e.g. infection) that may

Table 4.1 Malignant neoplasms based on the ICD-10 Chapter II classification of WHO

Malignant neoplasms	Blocks
Lip, oral cavity and pharynx	C00–C14
Digestive organs (C16 stomach, C18 colon, C22 liver and bile ducts, C25 pancreas)	C15–C26
Respiratory system and intrathoracic organs (C34 lung, C37 thymus, C38 heart)	C20–C39
Bone and articular cartilage	C40–C41
Skin	C43–C44
Connective and soft tissue (C46 Kaposi sarcoma, C47 peripheral nervous system)	C45–C49
Breast and female genital organs (C50 breast, C51 vulva, C51 ovary, C58 placenta)	C50–C58
Male genital organs (C61 prostate, C62 testis)	C60–C63
Urinary organs (C64 kidney, C61 renal pelvis, C66 ureter, C67 bladder)	C64–C68
Eye (C69), brain (C71), central nervous system (C72)	C69–C72
Endocrine glands and related structures (C73 thyroid, C74 adrenal gland)	C73–C75
Secondary and ill defined malignancies	C76–C80
Lymphoid, heamatopoietic tissues (C81 Hodgkin's, C91 Lymphoid leukemia)	C81–C96
Multiple sites of malignancies	C97

mimic the symptoms of cancer. These methods involve diagnostic tools, monitor the progress of tumor development and contain plans for the effective treatment of cancer. Diagnostic procedures may include laboratory tests (blood tests, tumor markers) genetic tests, imaging techniques, tumor biopsy, endoscopic examinations and surgery.

Cancers in medical practice can be classified by the:

- primary site of origin (breast, lung, prostate, liver, kidney, oral, brain cancer, etc.),
- tissue types (carcinoma, sarcoma, myeloma, leukemia, lymphoma, mixed types),
- grade (1–4) – is related to the abnormality of cells with respect to the surrounding tissues:
  - grade 1 – slight abnormality, well differentiated cells
  - grade 2 – slightly more abnormality, moderately differentiated cells
  - grade 3 – abnormal, poorly differentiated cells
  - grade 4 – undifferentiated, immature and primitive cells
- classification by clinical staging.

4.3.1 Tumor Staging

The clinical staging of malignant tumors is different from ICD-10 classification. Of the several staging methods the most often used one is TNM staging. The *TNM classification* comes from the abbreviation of tumor, lymph nodes and metastasis. This staging method was developed in 1946 (Denoix 1946) and is related to the size and extension of the primary tumor and its metastatic progression. Without going into details the TNM classification can be applied for most of the tumors. Notable exceptions are the brain tumors. Major parameters of the TNM classification are:

- T is the size or the extent of the primary tumor. Among the subparameters Tx refers to tumor that cannot be evaluated, the meaning of T*is* is carcinoma *in situ,* T0 = no signs of tumor, T1–T4 represent size and/or extension of the tumor.
- N is the spread of tumor to regional lymph nodes: Nx, the lymph node cannot be characterized; N0 tumor cells are not present in regional lymph nodes; N1–N3 extent of tumor cells in lymph nodes.
- M indicates the presence of distant metastasis: M0, no metastasis; M1, metastasis to distant organs.
- G (1–4): grade of cancer, where G1 denotes low grade cancer cells similar to normal cells G4 are high grade, poorly differentiated cancer cells.
- S (0–3) elevated serum tumor markers.
- R (0–2) completeness of removal of cancer after resection.
- L (0–1) tumor invasion in lymphatic vessels.
- V (0–2) tumor invasion in vein.
- C (1–5) modifier of the quality.

The AJCC Cancer Staging Manual, 7th edition contains a similar classification system developed by the American Joint Committee for Cancer (AJCC) utilizing in part the TNM scoring system including stage grouping and anatomic drawings for major cancer sites and is used by physicians and health care professionals to facilitate the uniform description of neoplastic diseases (Edge et al. 2010).

Many tests have been developed that help to determine whether a person has cancer, or if another condition (such as an infection) is mimicking the symptoms of cancer. These tests are briefly overviewed below.

4.4 Tumor Tests

4.4.1 Tumor Biopsy

Biopsy is a sample of tissue removed from the body for closer examination. The suspicious space-occupying area is called a tissue lesion, a tumor or simply a mass. Space occupying areas can be formed by substantial physical lesions (e.g. neoplasm, hemorrhage, granuloma), or lesions within a space (thorax, abdomen, cranium, bone marrow cavity) of the body. External suspicious areas are noticed during the physical examination of the patient or by imaging techniques inside the body. Biopsies are most often taken to determine whether or not a tumor is benign or malignant. The doctor (surgeon or interventional radiologist) takes a sample of cells or tissues from the patient that will be examined by the pathologist under a microscope and analyzed chemically. Before taking biopsies antibiotics may be given to prevent infection. Biopsies are taken most often from, but not limited to:

– breast cancer – when mammography shows a lump or mass,
– melanoma – a mole on the skin that changes shape or color,
– hepatitis – cirrhosis is suspected.

Of the three major types of biopsies:

(a) local incisional biopsies are minimally invasive,
(b) needle and
(c) excisional biopsies are more invasive interventions.

(a) Incisional biopsy (core biopsy) – only a sample of the suspicious area is removed under minimally invasive conditions. A small local anesthetic injection can make the biopsy almost painless.

    • Skin biopsy (punch biopsy) – a circular blade is used to get a sample of the skin.

(b) Needle biopsy – most biopsies take a fluid or tissue sample with a needle. These are more invasive biopsies normally performed in hospitals and surgery centers by specialized experts after sedation and use pain relievers to reduce discomfort.

- Aspiration biopsy – a fine needle withdraws a sample from a mass.
- Computerized tomography-guided biopsy –a series of pictures of areas inside the body taken from different angles are made by a computer linked to an X-ray machine that is guiding the doctor to find the exact position of the targeted tissue where needle biopsy will be taken. A dye may be injected into a vein or swallowed to visualize organs or tissues more clearly.
- Ultrasound-guided biopsy – an ultrasound scanner directs to the site where needle biopsy will be performed.
- Bone marrow biopsy – a large needle is injected into the pelvic bone to collect bone marrow when leukemia or lymphoma is suspected.
- Liver biopsy – the needle enters the liver to capture a liver sample.
- Kidney biopsy – the needle is injected on the back into the kidney.
- Prostate gland biopsy – upon blood test showing a high-level of prostate-specific antigen (PSA) or digital rectal examination indicating an abnormal prostate, a thin needle is inserted by the urologist either through the rectum, through the urethra or through the perineum (area between anus and scrotum) to remove a small sample of the prostate tissue.

(c) Excisional (surgical) biopsy – the entire tumor or a suspicious area is removed either by an open or by a laparoscopy surgery from a hard-to-reach tissue.

After the intervention pathologists read the biopsy, they report back to the surgeon. This can vary from a preliminary report taking few minutes to a week or even longer to reach a final conclusion.

4.4.2 X-ray Imaging

Roentgen radiation was detected first by Wilhelm Konrad Roentgen who named this unknown type of radiation X-radiation in 1895 and received Nobel Prize for his seminal discovery in 1901 (Novelline 1997). In several countries Roentgen-ray remained the name instead of the term X-ray. X-rays have a short wavelength (0.01–10 nm) and high energy, ranging between 100 electrovolt (eV) and 100 kiloelectrovolt (keV), equal to 1.6×10^{-23}–1.6×10^{-20} megajoule (MJ). The wavelengths of X rays are longer than those of gamma rays but shorter than ultraviolet (UV) rays. X-rays with lower energy are called soft X-rays and those above 5–10 keV hard X-rays. As the wavelengths of hard X-rays are similar to the size of atoms X-rays are suitable to detect crystal structures by X-ray crystallography. Soon after Roentgen's discovery X-rays have been used to identify bone structures by medical imaging. X-ray imaging remained the most frequently used application of ionizing radiations. It turned out that for medical use the low energy so called soft X-rays are unsuitable since they are absorbed by the body and increase the unwanted radiation burden without contributing to the image formation. The soft X-rays are normally filtered out and the spectrum is hardened by a thin metal (normally aluminum) sheet. Beside radiographs of the skeletal system, chest X-ray, abdominal

X-ray, detection of pathological gallstones, kidney stones, dental radiography (e.g. cavities), angiography using an iodinated contrast agent, computed tomography (CT scanning), fluoroscopy with an X-ray image intensifier and charge-coupled device (CCD camera) and contrast agents are used. For the visualization of coronary arteries cardiac catheterization, for esophageal disorders barium sulfate contrast agent is being used. X-ray therapy is a treatment used for cancer therapy, keeping in mind that large diagnostic doses of X-ray especially CT scans themselves increase the risk of cancer development (Brenner and Hall 2007; De Santis et al. 2007; Hall and Brenner 2008). Children and pregnant women are at a higher risk of X-rays.

Other uses of X-rays are:

- X-ray crystallography produced by X-ray diffraction by macromolecules such as DNA (Nobutami and Kakudo 2005).
- X-ray emission from astronomical objects.
- X-ray analysis of microscopic objects.
- X-ray fluorescence – X-rays are generated within a specimen and outgoing X-ray identifies the composition.
- Industrial radiography for the inspection of industrial parts.
- Industrial CT to generate three-dimensional images.
- Pigment analysis (e.g. to test the originality of paintings).
- Airport security luggage scanners.
- Border control – inspecting the interior of trucks.
- X-ray photography for artistic purposes.
- X-ray hair removal used in the 1920s, but banned by Food and Drug Administration.
- Shoe-fitting fluoroscope (Pedoscope) – to show how much room is available for the toes inside the shoe. Banned after 1970 upon realizing its radiation hazard.
- Roentgen stereophotogrammetry – assessment of three-dimensional micromotion of a joint replacement prosthesis relative to the bone it is attached to.
- X-ray photoelectron spectroscopy – quantitative technique to measure the elemental composition of the elements that exist within a material.
- X-ray laser device – proposed for Strategic Defense Initiative by U.S. President Ronald Reagen to protect the United States from attack by strategic nuclear ballistic missiles powered by thermonulear explosion, but gave inconclusive results.
- Phase-contrast X-ray imaging of soft tissues.

4.4.3 Ultrasound Images

High-frequency ultrasound waves are used to create images of soft tissues that do not show up well by X-ray analysis. The ultrasound images are not as detailed as those obtained by CT or MRI scans. The ultrasound machine projects high-frequency sound waves into the body and the computer receives the reflected waves

and creates images called sonograms. The major advantage of the ultrasound test over X-ray and CT scans is that it costs much less, there is no ionizing radiation involved that would pose any risk or cause tissue damage. The test is done by applying a clear, water soluble conducting gel to the skin over the area that will be examined to help the transmission of ultrasound waves. The quality of ultrasound images is lower in obese people. Most frequently used ultrasound tests are:

- pregnancy ultrasound test is an imaging technique that uses sound waves to see the developing baby and to check the female pelvic organs during pregnancy. The fetal anatomy survey can also show fetal anomalies, not only the sex of the baby,
- imaging abdominal organs (liver, gallbladder, spleen, pancreas, kidneys, blood vessels leading to these organs),
- Doppler ultrasound of arm, leg, heart. Doppler flow machines give information of the blood flow through the vessels without using contrast agents. This may be a helpful information as the blood flow in tumors differs significantly from that in normal tissues,
- testicle ultrasound test examines the testicles and other parts inside the scrotum. The testicles produce sperm cells and the hormone testosterone,
- thyroid ultrasound – visualizes the thyroid gland in the neck that regulates the metabolism of the body,
- transvaginal ultrasound across the vagina – provides a pelvic ultrasound image of a woman's reproductive organs, including the uterus, ovaries, cervix, and vagina,
- breast ultrasound – can help to show noncancerous growth of cysts with fluid filled sacs, solid growth of noncancerous fibroadenomas, noncancerous fatty lumps (lipomas). Breast cancers can also be detected with ultrasound.

The major advantages of ultrasound can be summarized as:

(a) useful modality for tumor detection available to clinicians for evaluating patients for neoplastic processes or for tumor staging,
(b) providing a cross sectional anatomy without radiation in a noninvasive fashion,
(c) a relatively inexpensive method compared to other modalities,
(d) requires relatively little patient preparation,
(e) produces little patient discomfort (Mittelstaedt 1980).

Limitations of sonography:

- in large patients there is a poor penetration of the sound beam resulting in low resolution images,
- in patients with large amount of bowel gas the penetration of ultrasound is similarly poor,
- in some cases of liver metastases no lesions were detected,
- patients with surgical opening (ileostomy, colostomy) the incision may be difficult to examine,
- uncooperative patient may cause poor detection of tumors and their staging (Mittelstaedt 1980).

4.4.4 Cancer Blood Test

If cancer is suspected, laboratory tests (urine, fluid, tissue, biopsy analysis) and cancer blood tests will be performed. Such blood tests are decisive if cancer cells grow in the blood (e.g. leukemia), but give inconsistent results in other cancerous or noncancerous conditions. These tests give information with respect to the function of organs or if the metabolism of organs has been disturbed by cancer.

Typical examples of blood tests to diagnose cancer:

Cell count. The simplest method is to count the cells and estimate their size in a hemocytometer. This indispensible method visualizes cells and distinguishes among small and large cells as well as cell aggregates. Particle counters generally monitor changes in conductivity and help to automatize the counting procedure. Most of these devices provide information with respect to particle size and volume distribution. However, size distributions do not exclude errors generated by aggregation, coincidence and by nonspherical particles. Coincidence can be taken into consideration by counting the same cell population several times in a Burker chamber and by the particle counter. The ratio of cell count obtained by the cell number counted by the Burker chamber over the cell number obtained by the particle counter serves then as a factor which is used to multiply the counter number to get the real cell number (Banfalvi 2011).

Complete cell count (CCC). In the common blood test the amount of various types of blood cells are measured as complete blood count (CBC). Patients with blood cancer may have too many, too few or abnormal cells in their blood.

The three main types of blood cancers are:

- leukemia – rapid production of abnormal white blood cells,
- lymphoma – abnormal lymphocytes become lymphoma cells,
- myeloma cancer cells – specifically target plasma cells and prevent the normal production of antibodies.

To confirm blood cancer bone marrow biopsy is recommended.

In the Burker chamber the following equation is used to count cells in 1 ml:

$$\mathrm{CCC} = \mathrm{n}\, 10^4/\mathrm{z}$$

where CCC is the complete cell count/ml, n is the whole number of cells in all counted 1 mm^2 squares and z is the number of counted squares (1 mm^2). For the sake of clear distinction, cells in the visible field of the light microscope should be between 10 and 100. When the cell number is high suitable dilution is made and the cell number is multiplied by the dilution (Fig. 4.4). Whole blood samples may be used undiluted unless the cell count is high e.g. in leukemia. EDTA and heparin are preferred as anti-coagulants.

Flow cytometry has been mentioned as a technique that allows the counting and examination of small (0.2–150 μm) particles including cells, nuclei, chromosomes suspended in a stream of fluid passed through an electronic detection apparatus. Flow cytometry is carried out for various medical purposes including the counting

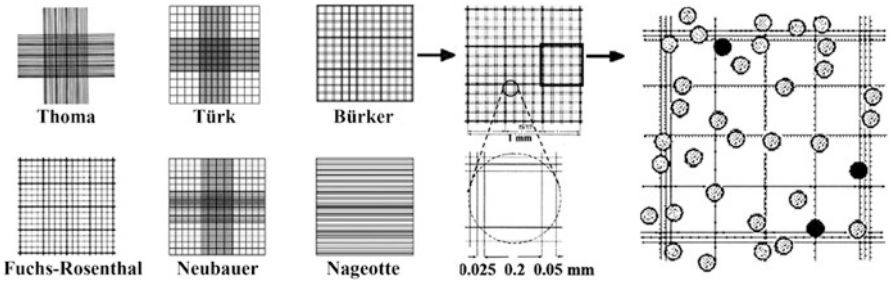

Fig. 4.4 Cell counting in different chambers under the microscope. It is suggested to perform counting using trypan blue staining to count viable cells that do not take up dye molecules and dark dead cells that are permeable to trypan blue. In the Burker chamber cells are normally counted within the whole 1 mm^2 area using the subdivisions bounded by three parallel lines and single grid lines to aid counting. To avoid the counting of the same cell twice those cells are counted that lie on the *top* and *left hand lines* of each *square*, but not the ones that are on the *bottom* or *right hand lines*

of blood cells, detection of pathogenic microbes, etc. However, the running of commercial flow cytometers is expensive and requires skilled operators. Microchip-based flow cytometers have been developed which require much lower volumes of reagents.

Blood protein test is also known as total serum protein test. This test measures the total amount of protein in blood and gives separate values for total protein and for major protein components, such as albumin, globulin and abnormal immunoglobulins that can be distinguished by electrophoresis in patients with multiple myeloma. Albumin level reflects liver and kidney function, dietary protein intake and helps to detect edematic swelling of the ankles, in the lung or in the abdomen as ascites. Higher globulin level may indicate infection, blood disease (e.g. multiple myeloma, macroglobulinemia, tumor formation).

Tumor marker tests. These markers are chemicals produced by tumor cells and detected in blood. However, tumor markers are also produced by normal cells. Elevated levels of tumor markers can be misleading as they do not necessarily indicate cancer; consequently they are of limited value regarding tumor diagnosis. More precise diagnostic tests have to be developed for tumor markers. Examples of tumor markers are:

- Prostate specific antigen (PSA) – blood levels are higher in prostate cancer, benign prostatic hyperplasia and prostatitis.
- Prostatic acid phosphatase (PAP) – normally present in small amounts, elevated in testicular cancer, leukemia, non-Hodgkin lymphoma, and occasionally under noncancerous conditions.
- Cancer antigen 125 (CA-125) for ovarian cancer, but cancers of cervix, uterus, pancreas, breast, lung, liver, pancreas, colon and digestive tract may also elevate the level of this cancer antigen.
- Calcitonin for medullary thyroid cancer – elevated levels of calcitonin after thyroid surgery and swollen lymph nodes may indicate metastasis or recurrence of cancer.

- Alpha-fetoprotein (AFP) is normally higher only in pregnant women produced by the fetus, but not present in adults. In patients elevated levels of AFP may indicate primarily liver cancer, and with lower incidence ovary or testicle cancer. Occasionally AFP levels are higher under noncancerous conditions.
- Carcinoembryonic antigen (CEA) is present in trace amounts in the blood, and elevated in colorectal cancer and can be present in other cancerous cases.
- Human chorionic gonadotropin normally present in pregnancy produced by the placenta is abnormally high in the cancers of germ cells (ovary, testis) and in other cancers (liver, stomach, pancreas, lung, etc.
- Cancer antigen tumor markers:
  - CA 15-3 marker helps the identification and treatment of women with advanced breast cancer.
  - CA 27-29 similarly to CA 15-3 is used to follow the treatment of advanced breast cancer
  - CA 125 is an antigen present with high incidence in nonmucinous ovarian carcinomas.
  - CA 19-9 is a monoclonal antibody produced against colon carcinoma.
- Lactic acid dehydrogenase (LDH) test – this general marker measures tissue damages (heart, liver, kidney, brain, blood cells, lungs). Its level is elevated in noncancerous diseases such as heart failure, cerebrospinal accident (e.g. stroke), hypothyroidism, hemolytic anemia, lung and liver diseases, muscle injury, muscle dystrophy, pancreatitis, tissue death. Several cancers can raise the LDH level.

Urine analysis. Blood in the urine (*hematuria)* can indicate infection but may be the consequence of a benign condition or a malignant process. Protein in the urine (*proteinuria*) can be the reflexion of kidney or cardiovascular problems.

4.4.5 Bone Marrow Test

Bone marrow is a sponge-like tissue inside the bones containing stem cells. While embryonic stem cells can develop into any cell type of the body, bone marrow (hematopoietic) stem cells can differentiate into three major types of cells:

- Red blood cells (erythrocytes) that carry oxygen to the tissues and protons (H^+ ions) back to the lung.
- White blood cells consist of granulocytes (neutrophils, basophils and eosinophils) and monocytes (macrophages and dendritic cells) (Table 1.7). Granulocytes and monocytes are collectively termed myeloid cells. Further white blood cells are B and T lymphocytes. B lymphocytes produce antibodies, T lymphocytes either kill directly or isolate inflammation cells that are recognized as foreign (virus infected, cancer cells) to the body.
- Platelets derived from megakaryocytes help prevent bleeding.

The hematopoietic system is known to be extremely sensitive to X-irradiation. After the atomic bombings of Hiroshima and Nagasaki it was suspected that victims of lower doses died over a prolonged period of time as their hematopoietic system could neither regenerate to produce sufficient white blood cells to fight infections nor enough platelets to stop bleeding. Murine experiments confirmed this notion, mice died after subjecting them to a minimal lethal dose of whole body X-irradiation from hematopoietic failure (Jacobsen et al. 1949). It was demonstrated that the life of whole-body-irradiated animals (mice and guinea pigs) could be rescued by bone marrow transplantation (Lorenz et al. 1951) and that the injected bone marrow cells directly regenerated the blood-forming capacity of the recipient animals (mice and rats) (Nowell et al. 1956; Gengozian et al. 1957). It is now a generally accepted view that after whole body X-irradiation bone marrow transplantation or hematopoietic stem cell transplantation is the most efficient therapy to restore the blood-forming system (Manz et al. 2004; Yu and Thomson 2008).

Sources of hematopoietic stem cells. As most of the stem cells are located in the bone marrow of adults under steady state conditions, the hematopoietic cell transplantation became synonymous with bone marrow transplantation. Direct bone marrow transplantation is infrequently used as a source of hematopoietic stem cells. These stem cells are released into the blood by cytokine mobilization and mobilized peripheral blood rather than bone marrow is being harvested from the donor. Mobilized peripheral blood can be further enriched by cells expressing CD34 marker resulting in a mixed population of cells containing hematopoietic progenitor cells as the major component and other cells such as T cells. Autologous graft (autotransplant) from a cancer patient contains mobilized peripheral blood of the same patient containing a mixed population of hematopoietic progenitor cells and cytotoxic T cells. It was doubted whether or not the induction and use of autologous grafting with the occurrence of auto-cytotoxic lymphocytes could result in an anti-tumor effect (van der Wall et al. 2000) or could pose a risk and contribute to the ongoing debate about its oncological safety (Krastev et al. 2013).

Performing bone marrow tests. Bone marrow tests are done when blood cell (red, white blood cell or platelet) disorders are suspected, conditions could have changed (thrombocytopenia, anemia) or cancer (lymphoma, leukemia, myeloma, *polycythemia vera*) could have occurred or if cancer (Hodgkin's or non-Hodgkin's lymphoma) has spread to the bone marrow. The metastatic spread of different cancers (lymphoma, prostate, breast, lung) to bone marrow requires tumor grading, and to describe how tumor cells are differentiated and how they look under the microscope.

The two major types of bone marrow tests are:

(a) A small sample of bone marrow cells is taken either by aspiration from the breast bone (*sternum*) or from the hip bone by using a needle and a syringe. Bone marrow cells can be used for stem cell transplantation, chromosome analysis or cultured and looked for infections.
(b) A small piece of bone marrow is taken from the inner side of the hip bone by trephine biopsy. This test shows the structure of the bone marrow inside the bone.

Both tests can be performed by the aspiration test done first, followed by the biopsy test. Each test is preceded by the administration of a sedative drug that makes the patient drowsy. A local anesthetic anaesthetic injection is given into the skin next to the breast or hip bone to reduce the pain of the test. Beside the painful prick of the needle the patient will notice a pulling sensation when the aspiration test is performed or feel the turning of the needle when biopsy is taken. Mild painkiller is given after the test for a few days to ease the pain of the aching bones. The bone marrow sample will be examined in a cell biology laboratory under a microscope. This procedure may need differential staining of cells (hematoxylin-eosin, Giemsa, methanol-Giemsa, May-Grünwald, Grünwald-Giemsa, sudan-B, myeloperoxidase reaction, alpha-naphthyl-acetate esterase, acid phosphatase), immunohistochemical staining for the diagnosis of abnormal cells and for typing tumors and the analysis of different cell types.

4.4.6 Positron Emission Tomography (PET Analysis) Methods

These methods have gained success in tumor detection and cancer staging. PET imaging with ^{18} F labeled fluorodeoxyglucose (^{18}FDG) has been a widely accepted tool in molecular imaging and is regarded as one of the main paradigms of the twenty-first century biology (Luker and Piwnica-Worms 2001; Dobrucki and Sinusas 2005; Weissleder 2006). PET models can be subdivided to:

(a) *PET scan.* The patient receives an injection of the radioactive tracer (usually [^{18} F]fluorodeoxyglucose abbreviated as ^{18}FDG) that will be absorbed by the cells. The PET camera takes images of the body or areas, where more active cells will light up as hot spots. PET scan creates an image of the cellular activity, which is expressed as standardized uptake value (SUV). SUV is representing the activity of a particular area in comparison to another area of the body. The normal cellular activity corresponds to baseline 1 value of SUV 1. Standardized uptake value is often used in PET imaging for the semiquantitative analysis of ^{18}FDG images for cancer therapy monitoring (Lucignani et al. 2004). The advantage of PET scan over other imaging techniques (X-ray, computed tomography, magnetic imaging resonance) is that it identifies both chemical and metabolic changes. Among the limitations of SUV measurements are changes that can take place during the uptake of the tracer such as extreme body weight (overweight, leanness of the body), glucose levels (diabetes), fasting prior to the test, unrest and motion of the patient during the scan, inflammation and infection, etc. PET scans may indicate: tumor formation, spread of cancer, particularly lymphomas, cancers of lung, colon, prostate, head and neck.

(b) *Compartment model* is a basic concept to quantitatively evaluate PET data. After the *i.v.* introduction of the radioactive tracer, PET data are obtained in a sequential fashion representing the tracer concentration (Bq/ml) at a certain time. To interpret PET kinetic data physiologically, four separate pools named compartments have been distinguished (Watabe et al. 2006). The first

compartment is the arterial blood where the radiotracer is distributed. From the arterial blood the radioligand passes into the second or so called free tissue compartment. The third compartment is the region of specific binding to be observed, also referred to as region of interest (ROI). In the fourth compartment there is no specific binding, but the exchange with the free compartment takes place. The single tissue compartment model has only one tissue, normally blood. Correspondingly its most important application is blood flow measurement by ^{15}O labeled water and PET. This model is often sufficient to describe the kinetics of several radioligands. The disadvantage of the compartmental analysis with the arterial input function is that arterial sampling is invasive and technically difficult (Watabe et al. 2006). Alternatively, several other compartmental techniques have been developed. The two tissue model fits many radioligand tracers, e.g. ^{18}FDG is one of the most often used two tissue compartmental models. Generally the three or four tissue compartment models consists of components of plasma, free ligand in tissue, specific binding, non-specific binding and the rate constants measuring the transport and binding.

(c) *Reference region models* are non-invasive, but in these techniques more assumptions are involved and their use needs more caution. The selection of a reference region may result in under- or overestimation of specific binding in the targeted region (Kropholler et al. 2006).

(d) *Spectral analysis*. Data of this dynamic positron emission tomography consist of time courses of label in tissue regions of interest and in arterial blood following the administration of radiotracers. This method generates a simple spectrum of the kinetic components that relate the tissue's response to the blood activity curve. This technique has been developed to facilitate the interpretation of dynamic PET data and to simplify comparisons between regions (Cunningham and Jones 1993). Spectral analysis provides an alternative approach to compartment analysis and can be applied beside PET to other dynamic data acquired by planar scintigraphy or single photon emission computed tomography (SPECT) (Murase 2003).

(e) *Ratio analysis*. After the equilibration of the radiotracer, the ratios of the time-averaged static signals in regions of interest (ROI) and graphical approaches proved to be useful tools. To overcome blurred anatomic boundaries inherent to PET analysis, the PET image is fused with the magnetic resonance image (MRI). Nuclear magnetic resonance imaging (NMRI) uses magnets, radio waves and a computer to take a series of pictures of areas inside the body. The fusion image then shows the PET as an overlay on the underlying MR images. The improved localization of anatomic boundaries allow for more accurate ROIs, and subsequently better diagnostic performance. Simple ratios of radiotracer uptake by subregions are useful to separate healthy controls from patients suffering from Parkinson disease (Jokinen et al. 2009). MR fusion to ^{18} F-DOPA PET improved the accuracy of the diagnosis of Parkinson's disease (Struck et al. 2012).

(f) *Multiple–time graphical analysis.* This graphical method of analysis is applicable to ligands that bind reversibly to receptors or enzymes requiring the

simultaneous measurement of plasma and tissue radioactivities many times after the injection of a radiolabeled tracer (Logan et al. 1990).

(g) *Retention index* (earlier used as fractional uptake rate). The retention index is a term also used in chromatography, but not dealt with here. In clinical studies the retention index is representing the fractional (percentual) change in standardized uptake value (SUV) between PET scans taken at different time points. To estimate the retention index the parametric slope images are obtained from dynamic PET data and fitted as a straight line to the SUV curve. (Herzog et al. 2008; Apostolopoulos et al. 2011).

(h) *Combination of biology and technology* is expected to explore new ways of diagnosing tumors, monitoring progression and delivering drugs to fight them off. One such approach is to inject nano-size particles that find and home in tumors, trace tumor development that can be visualized by imaging techniques.

4.5 Carcinogens

Carcinogenic agents are directly involved in causing cancer by damaging the genetic material or altering cellular processes. As a result of the cellular damage cells do not obey the homeostatic control. Balanced cell death and mitosis turn to uncontrolled, malignant division, leading to tumor formation. Unrepairable DNA damage usually leads to programmed cell death, but if the apoptotic pathway is damaged, cancerous cell division is likely to prevail.

4.5.1 Classification of Carcinogens

There are several known chemical carcinogens and many other carcinogenic effects that have not been recognized, yet. This book does not keep detailed record on each type of carcinogenic exposures. Only the most important chemical carcinogens are mentioned, such as inorganic asbestos, industrial smoke and tobacco containing polycyclic aromatic compounds, tobacco specific nitrosamines, aflatoxin produced by the fungus *Aspergillus flavus,* dioxins and dioxin-like compounds, benzene, etc. Known human carcinogens are listed by the International Agency for Research on Cancer (IARC, Web site: www.iarc.fr) as part of the World Health Organization (WHO) and the National Toxicological Program (NTP) formed by different US governmental agencies including the National Institutes of Health (NIH, Web site: http://ntp.niehs.nih.gov), the Centers of Disease Control and Prevention (CDC) and the Food and Drug Administration (FDA Web site: www.fda.gov). Reports on carcinogens by NTP are regularly updated.

The International Agency for Research on Cancer has placed human carcinogens into four major groups:

Group 1: carcinogenic (∼ 100 of the 900 likely candidates tested)
Group 2: likely to be carcinogenic

2A: probably carcinogenic
2B: possibly carcinogenic

Group 3: unclassifiable with respect to carcinogenicity
Group 4: probably not carcinogenic

The *Report on Carcinogens* by NTP distinguishes only two groups:

Group 1: Known to be human carcinogen
Group 2: Anticipated to be human carcinogen

The electronic database of US Environmental Protection Agency (EPA Web site: www.epa.gov) uses a rating system with groups similar to that of IARC:

(a) carcinogenic to humans
(b) likely to be carcinogenic
(c) evidence suggesting carcinogenicity
(d) inadequate evidence to asses carcinogenicity
(e) unlikely to be carcinogenic

Among the agencies dealing with carcinogenicity the institutes forming the National Toxicological Program (NTP) and other institutes and cancer societies can independently report or comment on whether or not a substance or any other exposure should be regarded carcinogenic. As a result different carcinogenic lists exist and an agent may appear on one list but may be absent on the other list. The confusion can be contributed by the different names of carcinogens.

4.5.2 Irradiation

Ionizing and non-ionizing radiation. An estimated 10 % of cancers are thought to be caused by radiation including ionizing (cosmic rays, alpha and beta particles, gamma and X-rays) and non-ionizing ultraviolet radiation (Anand et al. 2008). Ionizing radiation also includes some of the ultraviolet spectrum. Ionizing radiation is also emitted by radon gas. Radiation can cause cancer anywhere in the body at any age. This does not mean that the susceptibility to radiation is independent of age. The embryonal development, childhood and adolescence are more sensitive to mutagenic and carcinogenic radiation exposures. Radiation induced solid tumors grow relatively slowly and take about 10–15 years to become clinically manifest, while the development of leukemias induced by radiation is faster and takes place within 2–10 years. Radiation combined with other carcinogens has a cumulative effect (Little 2000). Ionizing radiation can hit biomolecules, particularly DNA randomly and can cause chromosomal breaks, abnormal number of chromosomes, chromosomal translocations, inactivate genes, delete DNA sequences, etc. Upon

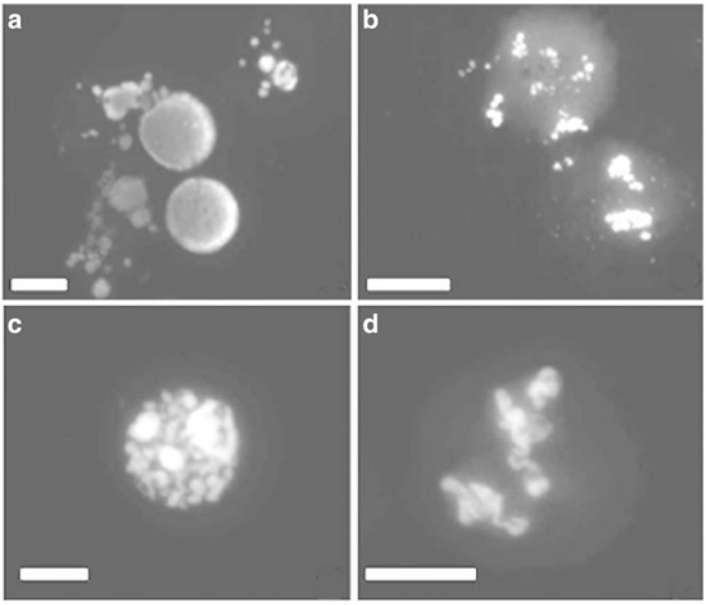

Fig. 4.5 Chromatin damages in γ-irradiated murine preB cells. Cells were irradiated with 400 R for 2 h and synchronized by centrifugal elutriation. Apoptotic chromatin structures were isolated from nuclei representing early S phase (**a**, **b**), and late S phase (**c**, **d**), respectively. Bars 5 μm each (Modified with permission Nagy et al. 2004)

higher exposures to ionizing radiation cells dye, small doses leave cells functional, but may proliferate and develop into cancer. Ionizing radiations unlike chemical carcinogens are not strong mutagens, trigger cellular responses that indirectly induce mutations (Little 2000).

Ionizing radiation is used for the identification and medical imaging of some kinds of tumors and to treat cancer. The ultraviolet irradiation of Sun can cause melanoma and other skin cancers. The non-ionizing medium wave UVB is also of growing concern, that can cause non-melanoma skin cancer, the most common form of cancer (Cleaver and Mitchell 2000). To the question whether one could make distinction between chromosomal aberrations generated by ionizing or non-ionizing irradiation the chromatotoxic effects of the ionizing gamma irradiation (Fig. 4.5) and the non-ionizing UVB irradiation (Fig. 4.6) have been compared. Upon gamma irradiation the number and size of apoptotic bodies were inversely related to the progression of the cell cycle. Many small apoptotic bodies were formed in early S phase (Fig. 4.5a, b), and less but larger apoptotic bodies in late S phase (Fig. 4.5c, d).

Cell cycle dependent chromatin changes after UVB irradiation were seen as a fine fibrillary network covering the chromatin structures and incompletely folded primitive chromosomes (Fig. 4.6). Based on observations after UVB and gamma irradiation as well as upon heavy metal treatment it was confirmed that typical

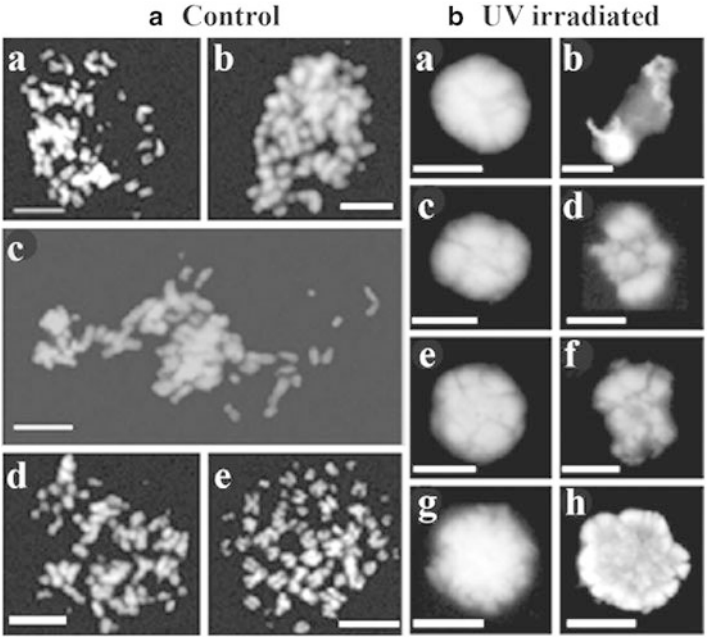

Fig. 4.6 Chromatin condensation in non-irradiated and UVB irradiated human erythroleukemia K562 cells. (**a**) Complete chromosome formation in non-irradiated cells. These late S phase control cells (elutriation fraction 18) were reversibly permeabilized, regenerated and subjected to colcemid treatment and chromatin structures were isolated. (**b**) Segregation of nuclear material without chromosome formation of irradiated cells. Bars 5 μm each (With permission Ujvarosi et al. 2007)

chromosomal changes characteristic to genotoxic agents can be recognized and classified (Ujvarosi et al. 2007).

It was suspected earlier that other non-ionizing radiations (radio frequency radiation of mobile phones, electric power transformers) could have some moderate carcinogenic effect. The International Agency for Research on Cancer (IARC) has classified the radiation of the mobile phones into group 2B referring to its carcinogenic potential.

Diagnostic X-rays. Diagnostic X-rays are regarded as one of the major medical advances of the twentieth century and the largest man-made source of radiation exposure to the general population, contributing to about 14 % of the total annual exposure worldwide from all sources. As there is no threshold of radiation dose, cancer risk is attributable even to very low doses of ionizing irradiation (Brenner et al. 2003). The cumulative effect of small doses of irradiation poses a small (0.5–3 %) but not negligible global risk in cancer development (Berrington de González and Darby 2004). However, the benefits of X-ray procedures, e.g. the early detection of cancers by radiological examinations and their early treatment exceed by far the low risk. The cost-benefit approach is expected to facilitate decision-making and could result in avoiding unnecessary X-ray procedures. Responsible thinking

can help to reduce the number of chest X-rays by an estimated 30 %, (McCreath et al. 1999). Unnecessary CT examination could shorten hospital stay (Fleszler et al. 2003). Unless the number of CT scans will decrease, the estimated 0.4 % of cancers caused by CT imaging can go up as high as 1.5–2 % in the United States (Brenner and Hall 2007).

4.5.3 Hormones as Tumor Causing Agents

The overproduction of hormones promoting cell proliferation can induce tumor formation. These hormones are involved in metabolism causing thyroid and bone cancer, sex-related cancers (breast, endometrium, ovary, prostate, testis) (Henderson et al. 2000).

4.5.4 Chemical Agents

Three major groups of chemical carcinogens have been distinguished:

(a) Polycyclic polyaromatic hydrocarbons
(b) Aromatic amines
(c) Nitrosamines

(a) *Polycyclic polyaromatic hydrocarbons*. The experiments of rabbit ears painted with coal tar, developed skin tumors (Yamagiwa and Ichikawa 1918) and led to the isolation of carcinogenic polycyclic aromatic hydrocarbons (benzanthracene and benzopyrene). These compounds are composed of fused benzene rings that are generated during the incomplete combustion of fossil fuels from coal tar and vegetables and are released as the most common environmental contaminants. British chemists not only identified these polycyclic aromatic hydrocarbons of coal tar (Kennaway 1930), but have also proved their carcinogenic effect (e.g. benzpyrene) in mouse skin (Cook et al. 1933). Polycyclic aromatic hydrocarbons that are inert chemicals, are activated in a multistep enzymatic process known as biotransformation taking place in the smooth endoplasmic reticulum of liver cells. In biotransformation polycyclic aromatic hydrocarbons are converted to the ultimate carcinogenic metabolite diol-epoxide, that binds to DNA (Fig. 4.7). Intercalation through the guanine base at N2 amino group distorts the secondary DNA structure (Volk et al. 2003), interferes with strand separation processes (DNA replication, transcription and recombination) and induces mutations leading to cancer.
Such conversions led to the postulation that polyaromatic hydrocarbons may activate electrophiles to form covalent adducts with informational macromolecules (Miller and Miller 1947) and to the identification of microsomal enzymes of the smooth endoplasmic reticulum (cytochrome P450, epoxide

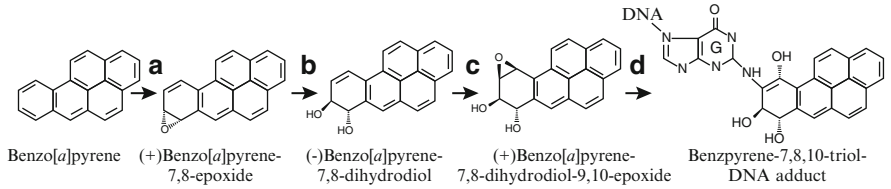

Fig. 4.7 Conversion of procarcinogenic benzo[*a*]pyrene to its carcinogenic DNA adduct. Steps of conversion: (*a*) benzo[*a*]pyrene is oxidized by the enzymes cytochrome P4501A1 and 1B1 (CYP1A1, CYP1B1) in the presence of molecular oxygen by the removal of water, (*b*) (+)benzo[*a*]pyrene-7,8-epoxide is hydrolysed by epoxide-hydrolase reacting with water, (*c*) another oxidation with cytochrome P450 1A1 and oxygen removes water and yields (+)benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide, (*d*) intercalation in DNA by covalently binding epoxide diol to the guanine (G) base of DNA

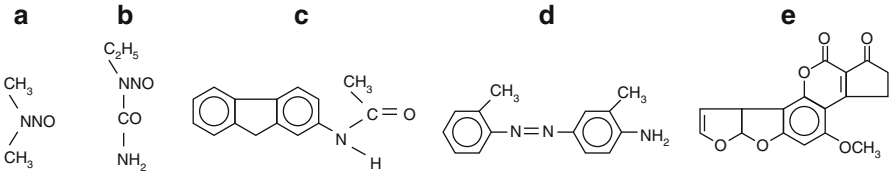

Fig. 4.8 Structures of nitrosamines, aromatic amines and aflatoxin. (**a**) N-Nitroso-dimethylamine, (**b**) 1-Ethyl-1-nitrosourea, (**c**) 2-Acetamino-fluorene, (**d**) O-Aminoazotoluene, (**e**) Aflatoxine B_1

hydrolase), responsible for the initial epoxidation and for the formation of the ultimate carcinogenic epoxide-diol (Conney et al. 1956). Epoxide formation is likely to induce guanine – thymine transversions that inactivate the protective tumor suppression gene p53 in smoking associated cancers (Pfeiffer et al. 2002).

(b) *Aromatic amines.* Aromatic amines are monocyclic, polycyclic or heterocyclic chemicals utilized by the plastic and chemical industries as dyes, polyurethane foams, pesticides, semiconductors, pharmaceuticals, rubber tires. But these carcinogenic compounds are also formed as inhalable aerosols during grilling meats or fish. The accumulation of monocyclic amines in the breast milk of lactating women (DeBruin et al. 1999), particularly o-toluidine caused mammary tumors in rodents (Layton et al. 1995) increased the risk of breast cancer (De Votcht et al. 2009). Most of the studies of aromatic amine metabolism were performed with 2-acetylaminofluorene (Fig. 4.8c). Human exposure to aromatic amines has long been associated with an elevated risk of urinary bladder cancer (Talaska 2003). The bladder cancer mortality of dyestuff workers ended after terminating the exposure and ending dyestuff production (Rubino et al. 1982; Decarli et al. 1985; Piolatto et al. 1991). Aromatic amines are also components of the tobacco smoke. Increasing evidence supports the notion that the higher incidence of bladder cancer in smokers is attributable to aromatic amines rather than to other contaminants of tobacco smoke such as nicotine or polycyclic aromatic hydrocarbons (Vineis and Pirastu 1997). The metabolites of most

arylamines react both *in vitro* and *in vivo* with the C8 atom of guanine. This adduct is known to be responsible for the point mutations and the cancerogenic effect. Other metabolic pathways of aromatic amines may lead to the formation of reactive oxygen species (Ohnishi et al. 2002; Makena and Chung 2007).

Aflatoxin (not to be confused with alfa toxin of bacteria). The naturally occurring fungal aflatoxin (Fig. 4.8e) is produced by different *Aspergillus* species and present in minute quantities in food. Upon entering the body, aflatoxins are oxidized in the liver forming reactive epoxide intermediates and are further hydroxylated to the less toxic aflatoxin M_1. The mechanism of aflatoxin carcinogenesis is similar to that of benzo[*a*]pyrene adduct formation with DNA (Fig. 4.7), with the exception that aflatoxin binds to the N7 nitrogen of guanine (Eaton and Galagher 1994). Aflatoxins are among the most carcinogenic compounds (Hudler 1998).

(c) *Nitrosamines.* Nitrosamines are used in certain chemical manufacturing, metal industries, the production of cosmetics, pesticides and rubber products (Altkofer et al. 2005). Tobacco smoke is another source of nitrosamines. Of the ~300 nitrosamines tested most of them turned out to be carcinogenic in a variety of experimental animals. One of the most dangerous nitrosamine is dimethylnitrosamine (Fig. 4.8a), that was reported to produce liver tumors in rats (Magee and Barnes 1956) and deemed to be carcinogenic. Dimethylnitrosamine is formed in the meal as a result of a chemical reaction between dimethylamine, a commonly occurring amine in meal, particularly in fish and nitrosating agent primarily sodium nitrite. In formaldehyde and sodium benzoate preserved fish meal there was also noticed considerable dimethylnitrosamine formation (Koppang 1974). The question arose whether dimethylnitrosamine formed from amine and sodium nitrite in fish meal, could also be present in human foods. Indeed, nitrosamines are generated not only by nitrite pickling and preservation of meat and cheese products, but also upon high temperatures caused by frying bacon, curing meats, brewing beer. Another question is concerning the removal of sodium nitrite from foods to prevent nitrosamine formation. The answer to this question is that the concentration of sodium nitrite should be minimized, but not go under the necessary level that could increase the risk of botulism poisoning. Nitrosamines can also be formed from nitrites and amines including amino acids under strong acidic conditions by endogenous nitrosation in the gastric juice. Similar considerations apply to the assessment of the long-term exposures to minute amounts of polycyclic aromatic hydrocarbons, heterocyclic amines or aflatoxin in various foods and beverages.

4.5.5 Physical Agents

Asbestos has been mentioned among the chemical agents, but its tumorogenicity is often regarded as a physical, rather than a chemical effect. Asbestos as a naturally occurring mineral consist of fine fibers, transforms the protective lining of internal

organs named mesothelium and turns them to mesothelioma, a cancer of the serous membrane. Other less frequently occurring asbestos-like fibers with similar effects are wollastonite, attapulgit, glass wool, rock wool (Maltoni et al. 2000). Repeated, long-term subjection of hot objects to the body causes burns and the trauma can cause tumor to the same part of the skin (charcoal hand warmers, hot tea to the *esophagus*) (Gaeta 2000).

4.5.6 Genetic Factors

The majority of cancers are sporadic ones, not related to inherited genetic defects. Genetic mutation is responsible for less than 3–10 % of cancers (Roukos 2009). Such inherited mutations occur in breast cancer 1 and 2 (BRCA1, BRCA2), in ovarian cancer and nonpolyposis colorectal cancer genes (Roukos 2009; Cunningham et al. 2010).

4.5.6.1 Viral Oncogenes

The discovery of the chicken sarcoma-inducing virus named after its discoverer Rous sarcoma virus was the first infectious agent that could be extracted from tumor cells and after injecting tumor cells into other chicken developed into sarcoma (Rous 1911). The integration of DNA tumor viruses or RNA retroviruses into the genomic DNA can disrupt genes and result in the expression of viral oncogenes of infected cells. The foreign genes of the tumor virus entering the host cell result in new properties primarily through anchorage-independent growth leading to the immortalization of the infected cell. Although, the viral oncoproteins expressed by human tumor viruses are different, but they often inactivate the same or similar tumor suppressors and/or signal pathways and immortalize infected cells. Known oncogenes are the viral E6 and E7 of papilloma virus, E1A and E1B oncogenes of adenovirus, the large and small t antigens of polyomavirus, the Tax oncogene of human T-lymphotropic virus type 1 (HTLV-1). Epstein. Barr virus (EBV) encodes the viral oncoprotein, latent membrane protein 1 (LMP1). The HBx gene is likely the viral oncogene of hepatitis B virus (Kim et al. 1991). The regulation of the expression of oncogenes may take different pathways that are not fully understood.

The classification of human tumor viruses is based on the genetic material (DNA, RNA) and follows the recent guidelines of Zheng (2010):

– DNA tumor viruses

  • Human papilloma viruses (HPVs). HPV16 and HPV18 are the major factors causing cervical cancer and other tumors (zur Hausen 2002). Most of the human papilloma viruses cause no symptoms, while others may cause warts (*verruca*). The minority of cases are transmitted typically through sexual contact and infect the anogenital region (cervix, vulva, vagina, penis and

anus), or are associated with cardiovascular diseases among women (Kuo and Fujise 2011), or with oropharyngeal (throat), head and neck cancer (Gillison 2004).

- Polyomaviruses. These are circular, double-stranded DNA viruses, ~5,000 base pairs long, icosahedral in shape and have no lipoprotein envelope. Their genome possesses early and late genes, contributing to a complex transcription program. Their oncogenic potential may persist as latent infections but may also produce tumors in a host with ineffective immune system or in other species. The earlier major genetically-related clades (Pérez-Losada et al. 2006), have been reclassified by the International Committee on Taxonomy of Viruses and recommended to divide the family of Polyomaviridae into three genera (Johne et al. 2011) known as:

  Genus Orthopolyomavirus (type species SV40)
  Genus Wukipolyomavirus (type species KI polyomavirus)
  Genus Avipolyomavirus (type species Avian polyomavirus)

  The human polyomavirus, Merkel cell polyomavirus (MCV), induces human carcinomas (MCCs) (Feng et al. 2008). The best known member of this family is the simian virus 40 (SV40) that does not infect humans, but its large T antigen induces malignant transformation in rhesus monkeys.
- Epstein-Barr virus (EBV) – infects B lymphocytes and turns them to lymphoblasts causing infectious mononucleosis, Burkitt lymphoma (primarily in Africa), B-cell lymphoma in immune-suppressed individuals, nasopharyngeal sarcoma, Hodgkin-type disease.
- Kaposi sarcoma or human herpesvirus 8 (KSHV, HHV8) – encodes viral G-protein-coupled receptor. This viral oncogene is thought to be responsible for the immortalization of endothelial cells (Montaner et al. 2003; Bais et al. 2003).
- Adenoviruses. Human adenoviruses cause human respiratory infections, but do not induce human tumors, only rodent cell transformations in rats and hamsters and have been useful tools to study tumor progression in these model animals (Berk 2005).
- Hepatitis B virus (HBV) is epidemic in the Southeast Asia and the sub-Saharan belt of Africa, the infection of which is associated with liver cancer. The deregulation of hepatocyte function is probably related to the lack of degradation of misfolded proteins by proteasomes contributing to the enhanced rate of HBV replication (Zhang et al. 2010).

– RNA tumor viruses

- Human T-lymphotropic virus type 1 (HTLV-1) – endemic in Japan, South Africa, Caribbean islands. The transmission of infected T cells takes place by breast feeding, blood transfusion, sexual intercourse and develops slowly into an adult T-cell leukemia or later to other asymptotic infections (Takatsuki 2005).

- Xenotropic murine leukemia related virus (XMRV) – is a human retrovirus associated with prostate cancer and could be associated with chronic fatigue syndrome (Lombardi et al. 2009; Mikovits et al. 2010; Lo et al. 2010).
- Hepatitis C virus (HCV) – consists of a plus stranded RNA genome with a long open reading frame and as a polycystronic messenger encodes ten different proteins causing the development of liver cancer (Liang and Heller 2004).

4.6 Techniques of Tumor Screening

Tumor screening is a means of detecting cancer early in people who have no symptoms. These tests can help to find cancer at early stage and make cancer treatment easier and more effective (Kramer 2004). By the time the symptoms are manifested in larger tumors, the cells may have left the primary tumor and spread to neighboring tissues or to distant places of the organism. Larger primary tumors have a higher incidence to metastasize in regional lymph nodes and distant sites. There are no general tests that could be applied uniformly. Unfortunately some cancer types have no screening tests at all. Moreover, finding the cancer does not necessarily mean successful treatment, increasing life expectancy or saving the life of the patient, especially at stages of late diagnosis. The four major screening tests include:

4.6.1 Physical Examination and Patient History

Beside the routine checkup, signs of unusual cell growth (e.g. lumps) and the history of past illnesses and their treatments are examined. Direct or assisted visual observation helps to identify suspicious lesions of the skin, retina, lip, mouth, larynx, external genitalia and cervix. Second most important physical examination is palpation to detect nodules, tumors and lumps (breast, mouth, salivary glands, thyroid, subcutaneous tissues, neck, axilla, groin, testes, ovaries, uterus). Digital rectal examination is often part of the physical examination (prostate, rectum).

4.6.2 Laboratory Methods

Samples are taken from the patient's tissues, blood, urine or other substances and subjected to diagnostic procedures.

4.6.3 Imaging Techniques

Internal cancers require pictures taken of different areas, tissues and organs of the body (endoscopy, gastroscopy, X-ray, PET, ultrasound scanning).

4.6.4 Genetic Tests

Gene mutations linked to special types of cancer are tested. Strong family history in first-degree relatives is indicating genetic mutations, polymorphism associated with specific cancers in these high-risk individuals.

4.6.5 Screening Tests Developed for Different Cancers

- *Mammogram* is a picture taken every 1 or 2 years by x-ray imaging to find breast cancer early in women.
- *Pap test (Pap smear)* sample is taken 3 years after the first sexual intercourse, but not later than age 21 from the cervix. The test reveals changes that may lead to cancer caused primarily by human papilloma virus. The test is repeated every 3 years.
- *Colon and rectum tests. Detection of polyps.* This test detects cancer or polyps in the colon and rectum in people aged 50 or older.
  - *Bleeding polyp test (fecal occult blood test)* detects blood in the stool.
  - *Colonoscopy and sigmoidoscopy.* Inside examination of rectum, colon with a lighted tube called colonoscopy or a device called sigmoidoscope. Polyps can be removed through the tubes of these instruments.
  - *Barium sulfate contrast agent.* Its suspension in water is conventionally the universal contrast medium used for examination of the upper gastrointestinal tract. When pumped into the rectum and colon, X-ray images of the lower gastrointestinal tract can be made.
  - *Lung cancer screening.* Such screening studies have been performed only in high risk individuals (smokers, workers subjected to occupational exposure to substances suspected to cause lung cancer).

Routine screening is not performed for skin, oral, bladder, testicular, ovarian, pancreatic cancers and for prostate cancer in men over age 75.

Misinterpretation of screening tests. Most of the screening tests are noninvasive or represent only a minimal threat some of the screening tests have some risks:

(a) the test may appear normal even if the patient has cancer (false negative test) (Nishizawa et al. 2009),

(b) the test may appear abnormal even if there is no cancer (false positive tests) (Croswell et al. 2009),
(c) the test may cause additional problems (e.g. bleeding),
(d) finding the cancer may not necessarily improve the status of the patient,
(e) treatment of cancer may have side effects,
(f) it can be difficult to make decisions about the tests,
(g) statistics of screening can be misleading, improved survival rates and increased early detection do not prove that cancer screening tests save lives (Wegwarth et al. 2012).

The benefits of screening are opposed by the statistics that takes into account how long the patient lives after diagnosis. The lead-time bias comes from the screening that finds cancer earlier than the cancer would have diagnosed after the symptoms. Lead-time bias has been exemplified by a cancer diagnosed because of symptoms without screening at age 67 and died at age of 70. The 5-year survival is 0 %. If this man was diagnosed earlier (e.g. at age 60) with screening without symptoms but also died at age 70, his life was not extended, although the statistics shows a 5-year survival equivalent to 100 % (Wegwarth et al. 2012). The highest level of evidence for cancer screening is taken as mortality reduction in controlled, randomized clinical trials. This means that the useful screening contributes to longer and healthier life of the patient.

4.7 Antitumor Agents

Earlier chemotherapy encompassed any chemical treatment, not only cancer therapy but also other diseases and conditions (spondylitis, multiple sclerosis, psoriasis, arthritis, lupus erythematosus, scleroderma, Chron's disease, etc.). The classic usage of chemotherapy changed to targeted chemotherapy referring to chemotherapeutic agents applied in antineoplastic treatment. Chemotherapy is often combined with other cancer treatments, such as radiation therapy and surgery. Chemotherapeutic agents can act by:

(a) preventing cell division and killing fast growing cells including cancer cells, bone marrow cells, cells of the digestive tract and hair follicles by chemicals known as cytotoxic drugs,
(b) targeting enzymes, hormones, food sources of cancer cells,
(c) preventing the formation of new blood vessels of tumors (angiogenesis inhibitors),
(d) the induction of programmed cell death (apoptosis).

The potential short-, and long-term side effects of chemotherapy not comprising a full list are: common symptoms such as confusion, dizziness, numbness, headache, jaw pain, short-term joint pain, drowsiness, nausea, vomiting and/or diarrhea, rash, labored breathing, bronchospasm, ringing in ears, hearing loss, abdominal pain, abdominal cramping, constipation, allergic reactions, dry skin, change in

taste, decreased appetite, flu-like symptoms. More characteristic symptoms are immunosuppressive decrease of blood cell counts, inflammation of the digestive tract (*mucositis*) and loss of hair (*alopecia*). The side effects help oncologists to decide which drug should be chosen and which drug combinations would have the lowest side effects.

Antitumor agents can be categorized by their mechanisms of action. Here only the major groups are mentioned without listing their names:

- inhibitors of angiogenesis
- intercalating/cross linking agents
- inhibitors of DNA synthesis
- regulators of transcription
- enzyme inhibitors
- regulators of gene expression
- inhibitors of microtubule formation.

4.7.1 Tumor Therapy

Tumor therapy overlaps with chemotherapeutic drugs that are classified based on their chemical structure and function:

- ***Alkylating agents*** – cause direct damage to DNA and prevent the growth of cancer cells (nitrogen mustards, nitrosoureas, alkyl sulfonates, triazines, ethyleneimines).
- ***Platinum drugs*** – often grouped with alkylating agents as they also damage DNA.
- ***Antimetabolites*** – this is the largest group of chemotherapeutic agents that interferes with DNA and RNA synthesis.
- ***Anti-tumor antibiotics*** – interfere with the enzymes of DNA synthesis, but have strong membrane damaging side effects including the anthracyclines (dauno-, doxo-, epi-, idarubicin) and other antibiotics (bleomycin, mitomycin C, actinomycin D).
- ***Restoration of p53 function*** – developing compounds that restore the transcriptional transactivation function to mutant p53 (Leone et al. 2013).
- ***Topoisomerase inhibitors*** – prevent the separation of DNA strands and thus interfere with strand separation processes (DNA replication, transcription, recombination):
  - topoisomerase IB inhibitors: irinotecan, topotecan, camptothecin and amellarin D
  - topoisomerase II inhibitors: etoposide, teniposide, doxorubicin, daunorubicin, mitoxantrone, amsacrine, ellipticines, aurintricarboxylic acid, quinolone HU-331.
- ***Mitotic inhibitors*** – many of them are nitrogen containing organic bases of plant origin with strong physiological effects, named alkaloids that can damage cells

at different stages of the cell cycle (paclitaxel, docetaxel, the vinca alkaloids vinblastine, vincristine and other mitotic inhibitors).
- ***Hormone therapy*** – either as hormones or hormone-like substances may kill cancer cells and considered chemotherapeutic drugs, or are used against side effects such as nausea and vomiting (anti-emetics) or prevent allergic reactions. Hormone therapy slows down the growth of breast, prostate, uterine cancers (antiestrogens, estrogens, aromatase inhibitors preventing estrogen formation, progestins, anti-androgens, agonists of gonadotropin-releasing hormone).
- ***Cancer immunotherapy***: monoclonal antibody, non-specific immunotherapy and adjuvants boosting the immune response, immunomodulating agents, cancer vaccines.

Dendritic cells induce strong immunological responses in cancer treated patients and have been proposed to be adjuvants in cancer immunotherapy (Banchereau and Palucka 2005), but clinical investigations clarified that dendritic cells have proteasomes that are different from tumor cells explaining the modest effectiveness of dendritic cells.

T-cell activation. The antimicrobial response present in tumor cells has been exploited by activating cytotoxic CD8 T cells specific for tumor-generated peptides that could directly recognize and kill tumor cells (Saccheri et al. 2010).

T-cell-mediated tumor-protection can be induced against poorly immunogenic malignancies and applied for cancer immunotherapy. Posttranscriptional modification of DNA vaccine induced complete protection against hepatic metastases in a murine model of neuroblastoma (Pertl et al. 2003).

Administration of monoclonal antibody. Specific targeting of tumour-initiating minority population cancer stem cells could be a strategy to eradicate cancers currently resistant to systemic therapy (Schatton et al. 2008).

Bacterial live vaccine encompassing type I secretions systems in combination with cholera toxin subunit B have been successfully used for delivery of prostate specific antigen to induce cytotoxic CD8+ T-cell responses resulting in an efficient prevention of tumor growth in mice (Fensterle et al. 2008). *Salmonella typhimurium* induced immune response was directed against distal tumors (Avogadri et al. 2005; 2008). Efficient tumor targeting has been reported by anaerobic butyrate-producing bacteria (Tirandaz and Mohammadi 2013).

Bacteria as antitumor agents. Mechanisms of bacterial anticancer agents have been intensively studied and reviewed (Forbes 2010). The two major mechanisms are:

- expression of proteins possessing physiological activities against tumors,
- transfer of eukaryotic expression vectors into infected cancer cells.

Both mechanisms utilize three categories of anticancer agents produced by bacteria:

1. bacterial toxins such as cytotoxic agents to kill directly tumor cells,
2. cytokines to stimulate immune cells that will attack cancer cells,
3. tumor antigens to sensitize the immune system against cancer cells.

Human trials have confirmed that bacteria can be utilized for cancer therapy: the oncolysis by *Clostridium butyricum* was exploited against squamous cell carcinoma, malignoma, neuroma, leiomyosarcoma, sinus sarcoma (Carey et al. 1967) and glioblastoma (Heppner and Möse 1978). Bacterial gene therapy represents a potential new modality for tumor treatment. The tumor-specific transfer of *Clostridium* genes (Theys et al. 2001) and clostridium spores as live 'Trojan horse' vectors (Wei et al. 2008) were also reported as potential means for cancer gene therapy. The other preferentially used bacterial strain *Salmonella typhimurium* provided efficient treatment against metastatic melanoma, renal cell carcinoma, squamous cell carcinoma and adenocarcinoma (Pawelek et al. 1997; Jain 1998; Theys et al. 1999). It likely that the proper combination of bacteria with other cancer therapies can bring a real breakthrough in the complete eradication of tumors and metastases (Forbes 2010).

Other types of anticancer drugs may serve targeted therapy, cell maturating agents (retinoids, arsenic trioxide).

Without providing a complete list, frequently used chemotherapeutic agents are given in alphabetical order:

- abiraterone acetate – against prostate cancer,
- alemtuzumab – against refractory B-cell chronic lymphocytic leukemia,
- alteramine – ovarian, lung, breast, cervical cancers,
- asparaginase – acute and chronic myeloid leukemia, acute lymphoblastic leukemia,
- bendamustine – B-cell non-Hodgkin lymphoma, chronic, lymphocytic leukemia,
- bevacizumab – breast, colorectal cancer, renal cell carcinoma, glioblastoma,
- bleomycin – testicular, squamous cell, head, neck, skin, cervix, vulva, penis cancer,
- busulfan – chronic myeloid leukemia, lymphomas, bone marrow transplantation for leukemia,
- carboplatin – for ovary, head, neck and lung cancer,
- chlorambucil – chronic myeloid leukemia, Hodgkin lymphoma, non-Hodgkin lymphoma, choriosarcoma, lymphosarcoma,
- cisplatin – against bladder, ovarian, testicular, bladder, uterine, cervical, lung, head, neck cancers,
- cyclophosphamide – lymphoma, breast, ovarian, lung, testicular, bladder cancers, mycosis, neuroblastoma,
- cytarabine (ara-C) – acute and chronic myeloid leukemia, acute lymphoblastic leukemia, non-Hodgkin lymphoma, intrathecal leukemia of the central nervous system,
- dacarbazine – melanoma, Hodgkin lymphoma, sarcomas, neuroblastoma,
- dactinomycin – testicular cancer, Wilms' tumor, rhabdosarcoma, Ewing sarcoma,
- daunorubicin – acute myeloid leukemia, acute lymphoblastic leukemia, Kaposi sarcoma,
- docetaxel – breast, prostate, ovarian, pancreas, head, neck, esophagus, stomach, cervical, uterine, bladder cancers, Kaposi sarcoma,

– doxorubicin (lymphoma, breast cancer, myeloma multiplex),
– etoposide (leukemia, lymphoma, lung, testicle cancer),
– 5-fluorouracil (colon, breast, stomach, and head and neck cancer),
– gemcitabine (breast, ovary, lung, pancreas cancer),
– irinotecan (colon, rectum),
– methotrexate (cancers of lymph system, breast, lung, blood, bone),
– paclitaxel (relapsing breast, ovary, lung),
– topotecan (ovary, lung),
– vincristine (leukemia, lymphoma),
– vinblastin (testis, head, neck).

4.8 Benign Tumor Formation in Different Tissues and Their Treatment

Tumor has been defined as a swelling or a lump of cells that does not necessarily pose a health threat especially if its growth is slow. Different types of tumors usually reflect the tissue they appear in and their shape. Nodules of tumors are smaller than 20 mm, while the mass normally refers to a lump that exceeds 20 mm at its widest point. The tumor formation is a slow process, especially at the beginning of tumor formation. It is generally believed that tumor formation starts with the genetic alteration of a rare cell that acquires the ability to grow into a primary tumor and progresses in a sequential manner and can spread rarely to distant locations (Fidler 2003; Chambers et al. 2002). Virtually all human tumor development appears to be monoclonal. Less frequently more cells undergo different genetic alterations and the resulting tumor consists of the descendants of these mutant cells. This tumor is polyclonal in nature (Vamus and Weinberg 1993). It would be difficult to acquire direct evidence to prove the monoclonal origin of tumor development. As most of the tumors are regarded monoclonal, polyclonal tumor development will be disregarded.

4.9 General Steps in Tumor Formation

The process of tumor formation is equally applicable to solid tumors and to tumor cells that float freely in blood but are not limited to a well defined location in the body. Solid tumor formation is schematically depicted in Fig. 4.9. The initiation of tumor formation starts with the mutation of a single cell (Fig. 4.9a). This mutation can lead to a faster growth of the mutated cell (Fig. 4.9b). The tissue containing the tumorous cells becomes disorganized due to the increasingly abnormal growth (Fig. 4.9c). The primary tumor may grow into a local (*in situ*) cancer, that upon invading neighboring tissues becomes an invasive cancer (Fig. 4.9d). The invasion

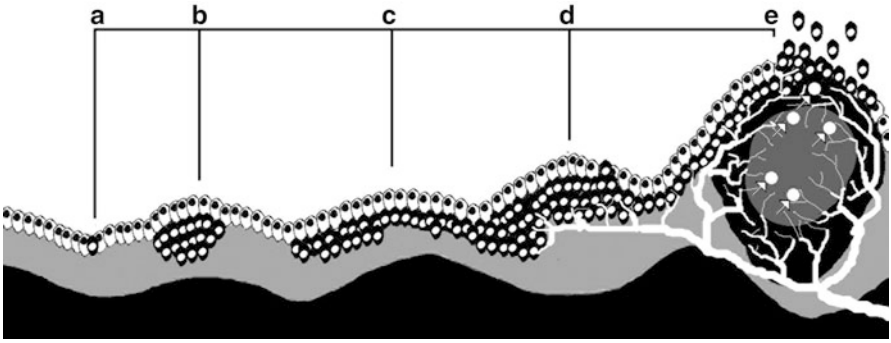

Fig. 4.9 Multistep process of tumor development. (*a*) Mutation of an ancestral tumor cell (*b*) division of ancestral cell displaying faster proliferation than normal cells, (*c*) benign tumor formation of abnormal cells, (*d*) invasion of neighboring tissues inducing angioneogenesis, (*e*) malignant invasion of tumor cells from the interstitial space and through the tumor disruptions of the primary tumor to the neighboring tissues or tissue fluid. *Black cells* with *small*, *white nuclei* are tumor cells. *White arrows* pointing to *larger circles* represent tissue disruptions. Angioneogenesis in the metastatic tumor is shown by the *white blood vessels* at the *right* (*e*). The *dark grey* area inside the enlarged tumor is reflecting necrotic cell death

may take place not only by invading neighboring tissues, but under hypoxic conditions that are characteristic to solid tumors lagging angiogenesis will cause tissue disruptions at the outer part of the tumor (Fig. 4.9e).

Angiogenesis is a fundamental step during the transition of tumors from benign to malignant state. It was hypothesized that tumor blood vessel formation is based on humoral induction (Chalkley 1948; Warren and Shubik 1966). That angiogenesis in tumor growth is a "hot and bloody" process (Folkman et al. 1971) is demonstrated in Fig. 4.10. It is assumed that when the slow primary tumor development switches to a fast track growth the formation of capillaries between the arterial and venous vessels is delayed or missing. Due to the delayed angiogenesis tumor and blood cells are discharged and enter the interstitial space. During the extravasation the arterial pressure sheds blood cells along with the tumor cells from the new but dead ended capillaries through the tissue disruptions into neighboring tissues and out of the tumor-bearing organ into the body fluids.

Tumor and blood cells expelled from the primary tumor are collected by the neighboring lymphatic capillaries and accumulate in the nearest lymphatic node, known as sentinel lymph node. Due to the multistep nature of metastatic cascade the vast majority of circulating tumor cells is unable to grow at distant places in the organism (Tarin et al. 1984; Fidler 1970; Cameron et al. 2000), confirming the notion that the tight junction of epithelial cells of blood vessels prevent the escape of tumor cells from the blood vessels and the colonization inside the arteries and veins, unless the vessels are damaged. The complex and multistep process of metastatic tumor development includes proliferation, local invasion, angiogenesis, intravasation, circulation, adhesion, extravasation (Fig. 4.9).

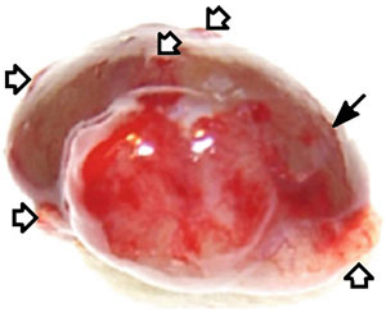

Fig. 4.10 Angiogenesis and disruptions in the primary kidney tumor of rat. Large hepatocellular (HeDe) primary tumor formed on the kidney of rat 7 days after hepatocarcinoma cell implantation. Small disruptions are indicated by the *white arrows*. *Black arrow* shows the site of angiogenesis seen under the surface of the kidney (Photo taken by G. Trencsenyi)

4.10 Benign Tumors of Major Organs

Primary tumors can develop in virtually any body tissue, the initial steps of which are similar, yet the features of the primary tumors are characteristic to the tissue. To deal with them individually would exceed the limits of this book. Only the most frequently occurring benign tumors of major organs will be reviewed.

4.10.1 Benign Liver Tumors

The most often occurring primary tumors of the liver are:

- hemangiomas,
- focal nodular hyperplasia
- hepatic adenomas

Hemangiomas are the most common benign liver tumors. As they normally do not metastasize, and cause no symptoms, they need no treatment. Tumor growth starts in blood vessels and occasionally can bleed indicating that the tumor formation is related to the disruption of damaged blood vessels or to the immature endothelial lining as in certain types of infantile hemangioendotheliomas. Patients with hemangiomas have a good prognosis. Children suffering in hemagioendothelioma have a chance for spontaneous regression after the 1st year, but may also have severe heart failures during the first 6 months of their life (Horton et al. 1999; Keslar et al. 1993). Asymptomatic lesions of hemangioendotheliomas may cause anemia or thrombocytopenia that can be compensated by the administration of blood products. Clinical cases of cardiac failure may need treatment with diuretics, digoxin, corticosteroids, interferon-α-2a. Tumor growth can be slowed down by

chemotherapy and radiation therapy. Life threatening tumor formation may need surgical resection, partial hepatectomy and the embolization of blood vessels (Banks and Podraza 2010).

Focal nodular hyperplasia. Congenital arteriovenous malformation may initiate hepatocytic tumor growth, but the liver function remains within the physiological range. In arteriovenous malformation the arterial and venous connection bypasses the capillary system and occurs most often in the central nervous system. Cell growth initiated by arteriovenous malformation appears in the liver as the second most frequent benign tumor of the liver. Focal nodular hyperplasia is often discovered incidentally during imaging for non-specific symptoms of the abdomen requiring no active treatment (Foster et al. 1977). Unusually rapid tumor growth can be an indication for resection (Hsee et al. 2005). This type of tumor is characterized by the presence of red-brown or tan mass with nodules laced with fibrous septa (Foster et al. 1977; Nguyen et al. 1999) more common in females, especially in premenopausal women than in men (Herman et al. 2000; Cherqui et al. 1995).

Hepatic adenomas. These are epithelial tumors with large lesions in the right lobe. Hepatic adenomas are rare ($1{:}10^6$), non-cancerous liver tumors most frequently develop in the liver of those women, between the ages of 20 and 45, who have used contraceptives or steroids. These adenomas cause abdominal pain and occasionally can turn to hemorrhage, rupture and further to malignant transformation. As we do not have sufficient data, the prognosis of hepatic adenoma is not quite clarified.

Important conclusion can be drawn from benign cases of liver tumors:

- hemangiomas: unless the blood vessels or their endothelial lining are damaged the vascular spread of tumor cells is unlikely to occur,
- focal nodular hyperplasia: unless arteriovenous malformation takes a serious form, blood vessels resist malignant tumor formation,
- hepatic adenomas: only serious lesions, hemorrhages and tissue disruptions result in malignant tumor transformations.

4.10.1.1 Symptoms of Liver Tumors

Hepatic tumors start to grow as an abdominal mass and sensed as abdominal pain, jaundice or liver dysfunctions. Liver tumors are normally not diagnosed until they start to produce symptoms, which may represent an already advanced stage as the symptoms can be caused by other conditions such as:

- loss of weight, loss of appetite,
- feeling of fullness even after a small meal,
- nausea, vomiting, fever, itching, pain and swelling in the abdomen, enlarged veins on the belly,
- enlarged liver felt as a palpable mass under the right side of the ribcage,
- enlarged spleen felt as a palpable mass under the ribs on the left side.

4.10.1.2 Diagnostic Tests of Liver Tumors

While skin tumors are obvious, to determine the location of tumors inside the liver needs different tests:

- complete blood count,
- blood tests based on tumor markers,
- bone marrow biopsy (but more suitable for the diagnosis of leukemia and lymphoma),
- biopsy of liver tumor – a sample of tissue from the liver tumor is obtained and examined by a pathologist to determine if it is noncancerous, slow growing (benign) or fast growing and cancerous (malignant). Final diagnosis cannot be made until liver biopsy is performed.
- radiological tests of the liver:
  - computer tomography (CT) (abdomen/pelvis CT scan including liver),
  - abdominal magnetic resonance imaging (MRI, also known as magnetic resonance imaging or nuclear magnetic resonance = NMR imaging), to create a picture of the liver tumor.
- nuclear medicine tests are more organ and tissue specific producing images of liver tumors and provide functional information:
  - 2D scintigraphy uses internal short-lived radionuclides to generate two dimensional images of the liver or

    perfusion scan for noninvasive evaluation of liver function (hepatobiliary scan).
  - 3D nuclear medicine imaging techniques have to be kept under As Low As Reasonably Practicable (ALARP) conditions:

SPECT (single photon emission computed tomography) scan reconstructs liver structures in 3D from many projections using a gamma camera and ^{99m}Tc label.
PET (positron emission tomography) uses coincidence detection to remove background after *i.v.* injection of ^{18} F-FDG. PET scans became the most popular tests to find different tumor types including liver tumors,
Hybrid scanning techniques combine imaging methods by superimposing scans referred to as image fusion such as SPECT/CT and PET/CT.

Nuclear medicine is assuming an increasing role in the detection and therapy of patients with cancer. In tumor diagnosis scintigraphy starts with the intravenous administration of a radioisotope that has been coupled to a carrier molecule directing the radiotracer to the tissue that is subjected to subsequent imaging. Liver tumor scintigraphy helps the identification and evaluation of primary, metastatic and recurrent tumors. The specificity of scintigraphy depends on the radiopharmacon being used. One of the most popular radioactive tracer is ^{111}Indium-pentetreotide suitable

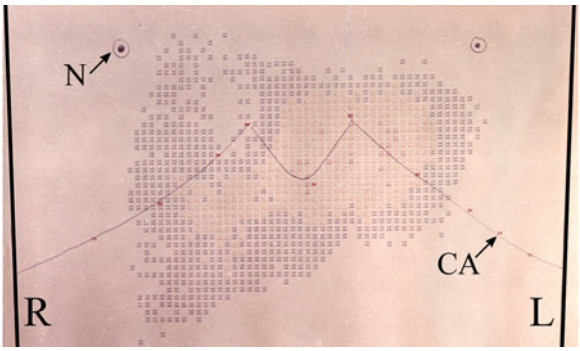

Fig. 4.11 Human liver tumor scintigraphy using ^{113}In-colloid. In the upper right lobe of the liver the lack of colloid accumulation indicates a suspicious space-occupying area that turned out to be a tumor. Scintigraphy of the control liver was shown in Fig. 3.2a. *R* right side, *L* left side of the patient, *CA* costal arch, *N* nipple (With permission Banfalvi 1971)

for the diagnosis and staging of primary and metastatic tumors bearing somatostatin receptors such as liver tumors, carcinoid tumors, gastric tumors, pancreatic tumors, thyroid carcinomas, pituitary adenomas and leukemias (Bohdiewicz et al. 1995; Olsen et al. 1995; Raderer et al. 1996; Kahn et al. 1998; Myssiorek and Palestro 1998; Petronis et al. 1998; Raj et al. 2002). By measuring the distribution of ^{113m}Indium colloidal particles upon intravenous administration in rats it was found that 98 % of the colloid was extracted by the monocytes of the liver known as Kupffer cells, 1.5 % by the spleen and only 0.2 % by the lung (Banfalvi et al. 1972). The human ^{113m}In- colloid scintigraphy scan (Fig. 4.11) shows the liver scintigraphy of a patient with tumor in the upper right corner of the liver seen as an empty area where only tumor cells were present but Kupffer cells that would accumulate colloid were missing.

4.10.1.3 Therapy of Liver Tumors

Most important aspects of tumor care are to control pain and bleeding, to treat obstructions of flow of urine or bile (catheters, stents) and treating blood clots. In liver tumor when the bile ducts become blocked the bile cannot drain from the liver. In such cases the interventional radiologist places a catheter into the bile ducts or a small metal cylinder, called a stent to hold the blocked area open. To prevent portal hypertension a catheter is introduced through a small incision of the skin near the neck and guided to the blocked blood vessel in the liver. The introduction takes place under ultrasound or magnetic resonance guidance creating a tunnel in blocked blood flow. The tunnel can be kept open by the insertion of a small metal stent. Radiation of liver tumor can be useful to help to relieve pain and other symptoms. Radiation therapy is commonly used to cancerous tumors due to its potential to control cell growth. Ionizing radiation damages DNA of the exposed tissue and induces cell death.

4.10.1.3.1 Surgical Removal of Liver Tumor

Hepatocarcinoma of hepatocytes is the most common form of liver tumors. The reason of hepatocarcinoma occurring twice as frequently in men than in women is not exactly known but could be explained by the higher food intake, oxidation rate, production of reactive oxygen species, and higher detoxification rate of liver in men. A recent study suggests the importance of smoking and heme iron from animal meat in colorectal cancer risk (Kato et al. 2013), as heme is known to exert cytotoxic effects via catalyzing lipid peroxidation, impairing lipid membranes and organelles and promoting damages to macromolecules (Vincent 1989; Nath et al. 2000).

In cholangiocarcinoma the tumor has mixed components consisting of hepatocarcinoma and tumor cells of the bile duct. Cancers of the blood vessels of the liver are known as hemangioendotheliomas. Rare forms of mixed liver tumors are: mesenchymal tumors, sarcomas, hepatoblastomas of the right lobe (primarily in children), cholagiocarcinomas (bile duct cancers), angio-, and hemangiosarcomas (in the blood vessels of the liver), lymphomas (infiltrating into the liver).

The elevated repair and chronic regeneration of the liver is likely to lead to tumor formation. Due to the central metabolic role of liver, tumor cells can spread from any part of the body to the liver, but most often come from abdominal tumors, primarily from colon and rectum. Consequently, other types of uncontrolled cancers carry the risk of secondary liver tumors known as metastases. Although, surgical removal would be the best therapy, the location of the tumor (e.g. in major blood vessels), the large size of the primary tumor and the distribution as many small secondary tumors throughout the liver are likely to prevents operation in most of the liver tumors.

4.10.1.3.2 Interventional Therapy

This therapy has been devised to attack the liver tumor from inside without medicating or affecting other parts of the body. The major strategies of interventional therapy apply embolization to cut off the blood supply to the tumor (embolization), to deliver radiation to a tumor (radioembolization), to combine radioembolization with chemotherapy to deliver the cancer drug directly to the tumor (chemoembolization), to administer radiofrequency heat to kill cancer cells (radiofrequency ablation) or freeze the tumor through cryoablation.

4.10.1.3.3 Magnetic Chemotherapy

Magnetic therapy is a new technique being in experimental phase that aims to use magnets that are pulling chemotherapy drugs into tumors. We do not have sufficient data to evaluate properly this treatment.

4.10.1.3.4 Gene Therapy

In gene therapy, only somatic cells are targeted for treatment. Consequently changes to the genes of an individual will have impact only on the somatic cells of the person without passing genetic changes to his/her children. Gene therapy is still in its early, experimental stage to:

(a) alter the patient's cancer-fighting genes and strengthen the natural immune system to attack cancer cells,
(b) alter cancer cells so that the immune system can attack them,
(c) replace mutated genes responsible for cancerous growth with healthy copies of genes that function under normal growth control,
(d) inactivate or "knock out" mutated genes that do not function properly,
(e) introduce new genes to help fight diseases,
(f) make tumors more susceptible to chemotherapy,
(g) make bone marrow and other fast growing cells resistant to chemotherapy.

The two major options to smuggle working human genes into the organism are:

1. *Viral vectors* are capable of carrying the healthy gene into the tumor cell and insert the gene into the genetic make-up of the tumor cell. After the gene is transplanted, the healthy gene has to be switched on and the tumor gene inactivated to produce the protein that was previously missing or altered. In cancer cells a further problem is imposed by the fact that metastatic genes (e.g. Twist, VEGFA and ITGB1) do not obey regulatory signals.
2. *Stem cells* offer another technique to deliver gene therapy. These immature cells can be manipulated *in vitro* by introducing new genes that can change their properties, e.g. increase their tolerance to chemotherapy for the benefit of patients following stem cell transplantation.

Gene therapy could be a promising option for some types of tumors, but so far did not bring the expected breakthrough in cancer treatment. Such interventions remained risky and studies in preliminary stage. The technique of gene therapy has to be made safe and effective before the current human tests for the treatment of tumors that have no other cures can be reliably applied.

4.10.2 Benign Gastrointestinal Tumors

Benign or malignant tumors of the gastrointestinal tract (stomach, small intestine, colon) can be found anywhere in the tract and begin to grow in interstitial cells of Cajal and in the wall of the gastrointestinal tract. Small tumors are common and normally diagnosed for other reasons during X-ray analysis of surgery. Other diagnostic tools of gastrointestinal tumors are: physical examination and history, computerized tomography, magnetic resonance imaging, endoscopic ultrasound and biopsy. The endoscope is a long tube-like instrument supplied with a light and a

lens that is inserted through the mouth of the patient into the esophagus, to the stomach and the upper part of the small intestine. High energy ultrasound waves are projected from the end of the endoscope to the tissues or organs. The echoes of the waves forming a picture named sonogram are guiding the doctor and helping to take a biopsy sample from the gastrointestinal tumor using a thin needle. The tissue sample will then be viewed under a microscope to decide if the tumor was benign or malignant. To confirm malignancy immunohistochemistry and mitotic tests will be performed. As most of the small and benign gastrointestinal tumors do not grow it is doubted whether or not they should be removed. The chance of recovery depends on: the size and location of the tumor, how fast the tumor cells are growing, whether or not the tumor has spread to other tissues or can be completely removed by surgery.

4.10.3 Benign Lung Tumors

Similarly to other noncancerous conditions benign lung tumors are indicative of abnormal growth with no particular purpose and are not found to be cancerous. In their initial stages lung nodules and tumors are caused by infectious fungi, tuberculosis, lung abscess, pneumonia. Non-infectious inflammations of lungs are caused by rheumatoid arthritis, granulomatosis, sarcoidosis, birth defects and lung malformation. Most of the lung tumors are benign and only 2–5 % of the primary lung tumors are malignant. At their initial stage benign tumors have no symptoms.

Types of benign lung tumors:

- hamartomas,
- bronchial adenomas,
- other rare benign tumors.

Hamartomas. Pulmonary hamartomas occur most often in older men. These tumors are usually small, solitary, grow slowly, and exhibit the disorganized growth of different tissue types present in the lung (fat, epithelial, fibrous, cartilage) with little or no malignant potential. Multiple tumors of the Carney syndrome include chondroma, leiomyosarcoma and ganglioma (Lancha et al. 1994; Valverde et al. 2001; Wales et al. 2002; Rodriguez et al. 2007). The differential diagnosis of hamartomas is important in order to distinguish them from other bronchogenic carcinomas (Jacob et al. 2008). Lung hamartomas showing a popcorn-like distribution on chest X-ray are more common in men than in women. Hamartomas may occur in many other parts of the organism similarly to the closely related diseases known as choristomas that are found in unusual locations. Hamartomas are normally not treated; the complications of surgical resection carry more risk than the lack of treatment.

Bronchial adenomas are responsible for about 50 % of benign lung tumors belonging to a diverse group generated by the mucous glands, ducts of the windpipe and major airways. Most of these tumors are of low-grade malignancies with respect to their slow growth rate and spread. Mucous gland adenomas are the most harmless benign tumors lacking malignant potential. Due to their small size

bronchial adenomas remain undiagnosed or other bronchial conditions (asthma, bronchitis, etc.) may mask their symptoms. Such symptoms are: shortness of breath (dyspnea), abnormal breathing sound when adenoma is in larger bronchi or in the windpipe, high-pitched wheezing sound produced in narrower airways, cough, fever, sputum production. Coughing up blood caused by ulceration is the most dangerous sign indicative of the severity of the disease.

Other rare benign tumors of the lung. This heterogeneous group of benign tumors includes bronchial adenomas, hamartomas, and uncommon benign neoplasms (chondromas, fibromas, lipomas, leiomyomas, hemangiomas, teratomas, pseudolymphomas, endometriosis, and other bronchial tumors) made up of the connective or fatty tissue of the lung.

Most of the *bronchial adenomas* belonging to a diverse group of tumors spread slowly and are low-grade malignancies. Mucous gland adenomas arising from mucous glands and ducts of the windpipe (trachea) and large airway (bronchi) are noncancerous lacking the potential to become malignant.

Lung hamartomas composed of the same elements as their surrounding connective tissues grow slowly, but in a disorganized manner. About 3/4th of all benign lung tumors are hamartomas. These pose real threat only when situated deep in the linings of the bronchi. Treatment, if necessary at all, is by surgical resection with excellent outlook. More dangerous are the complications generated by surgery itself.

Pulmonary chondroma composed of hyaline cartilage is part of the Carney's triad. The Carney triad describes the coexistence of three neoplasms, including: gastrointestinal stromal tumors, pulmonary chondroma, and extra-adrenal paraganglioma. Most asymptotic cases of pulmonary chondroma occur in young women forming "popcorn like" peripheral lesions in the lung (Carney 1999; Rodriguez et al. 2007). Calcification in chondromas is less common than in hamartomas. Although, chondromas are normally benign but occasionally can turn to carcinomas.

Pulmonary fibromas. While fibroid tumors on the surface also known as skin tags are visible, pulmonary fibromas may even not be known or diagnosed. Angiofibroma is a benign tumor of the nasal passage primarily in adolescent males causing nose bleeding, congestion or swelling. Pulmonary fibromas require removal only if they interfere with lung function or rarely turn to be malignant.

Pulmonary lipomas. Intrathoracic lipomas are rare and peripheral lung lipomas exceptionally rare and even if diagnosed (chest radiograph, barium enema film) surgical excision can be avoided (Wood and Henderson 2004). The low incidence of other rare benign pulmonary tumors does not necessitate their individual discussion.

4.10.4 Benign Renal Tumors

Benign tumors of the kidney are:

- Renal oncocytoma
- Cystic nephroma

- Angiomyolipoma
- Metanephric adenoma
- Renomedullary interstitial cell tumor

Renal oncocytoma. This benign tumor of the kidney is composed of large epithelial oncocytes with eosinophilic (acidophilic), and granular cytoplasm and an increased number of mitochondria. Uni-, or multifocal renal oncocytomas are incidentalomas as they are rarely and only incidentally diagnosed by abdominal computerized tomography or ultrasound imaging as they are asymptomatic. Symptomatic diagnostic signs are hematuria, pain, abdominal mass that may need nephrectomy.

Cystic nephroma (Renal epithelial stromal tumor). As the name is indicating, it is an epithelial type of kidney tumor with low incidence and benign character. It is diagnosed by chance during medical imaging. Direct diagnosis can be obtained by kidney biopsy or excision, to differential-diagnose cystic nephroma and cystic renal cell carcinoma (Israel and Bosniak 2005; Turbiner et al. 2007).

Angiomyolipoma. These are the most common benign tumors of the kidney composed of a mixture of vascular (angio-), smooth muscle (-myo-) and fat (-lipoma) tissue elements. Potential catastrophic hemorrhage may develop only after their size becomes larger than 4 cm in diameter. Angiomyolipomas are associated with a rare genetic disease known as tuberous sclerosis, where the tumor metastasizes to other organs (brain, heart, eyes, lung, skin, etc.). The rare genetic disorder tuberous sclerosis causes noncancerous tumors to grow mainly in the brain, but also in kidney.

Metanephric adenoma. This kidney tumor should neither be confused with the similarly sounding mesonephric (nephrogenic) adenoma, another benign tumor typically grown in the urinary bladder, nor with the similar symptoms and microscopic appearance of the papillary renal carcinoma (nephroblastoma, or Wilms' tumor) (Bastos Nettó et al. 2007). Symptoms of metanephric adenoma are characterized by increased red blood cells (polycythemia), abdominal pain, hematuria, palpable increase of cell mass and higher incidence of calcification than in other kidney tumors (Davis et al. 1995). Beside surgery in serious cases other treatments have not been used.

Renomedullary interstitial cell tumor (earlier known as renal medullary fibroma) is the most common kidney tumors of adults with an incidence from 16 to 42 % increasing with age (Petersen 1992). Under healthy conditions renal medullary cells regulate blood flow, water and salt absorption and blood pressure through the secretion of prostanoids. Renomedullary interstitial fibroblast cell tumors overexpress cycloxygenase-2 (COX-2) gene (Gatalica et al. 2008). The tumor is normally less than 0.5 cm in diameter composed of stellate or polygonal cells localized in the innermost part of the kidney, where the medulla is divided into several sections known as the renal pyramids. As this tumor does not produce clinical symptoms it does not require treatment. Its differential diagnosis is characterized by concentric rings of stromal cells around entrapped renal tubuli and serves to distinguish this tumor from the metanephric stromal tumor.

4.10.5 Benign Brain Tumors

Similarly to small tumors of other organs, benign tumors of the brain grow slowly, do not invade other tissues and have well distinguished borders or edges that can be diagnosed by computerized tomography or magnetic resonance scans. The causes of brain tumors are not perfectly understood and only a few of the risk factors are known such as family history, radiation, some chemicals (e.g. vinyl chloride, formaldehyde). Small, benign brain tumors do not relapse after surgical removal. Larger tumors can cause life-threatening compression of the brain tissue and other structures inside the skull associated with general, nonspecific symptoms such as: vision, hearing, balance, mental problems, headaches, nausea, vomiting, seizures, muscle jerking, facial paralysis, etc. The names of benign brain tumors are reflected by their brain-associated tissues:

- meningiomas developing in the membranes of the brain and spinal cord,
- schwannomas arise from Schwann cells insulating the 8th cranial (vestibulocochlear or auditory vestibular) nerve,
- pituitary gland tumor is a benign adenoma of the pituitary, the master gland of other glands controlling numerous hormonal functions of the body. As the anterior pituitary also controls growth and reproduction, even its benign tumor may affect the pituitary function and needs the surgical removal of the tumor,
- hemangioblastoma produces a relatively rare vascular tissue mass accounting for $\sim$2 % of brain tumors,
- craniopharyngiomas develop from embryonic tissues those are partly responsible for the formation of the pituitary gland. Pressure of the tumor exerted on the pituitary gland reduces the storage capacity of neurohypophysial hormones (oxytocin, vasopressin) in the posterior pituitary gland, especially that of vasopressin and raises the cranial pressure. The treatment of craniopharyngioma necessitates cranial surgery.
- papilloma of the choroid plexus. The name of the choroid plexus comes from the Greek "khorion" (afterbirth) membrane enclosing the fetus and the word "plexus" meaning network. The choroid plexus is responsible for the production of the cerebrospinal fluid that fills the ventricles and the spinal cord, provides a protective cushion for the brain and regulates the intraventricular pressure by the secretion and absorption of the cerebrospinal fluid. Choroid plexus papilloma is uncommon $\sim$1 % of brain tumors, classified as grade I if typical and grade II if atypical by the World Health Organization. Grades I and II tumors are slowly growing benign brain tumors. Only a small fraction of brain tumors belonging to higher grades (III–V) are malignant tumors with a high proliferation rate.

4.10.6 Benign Heart Tumors

Primary heart tumors occur in the population in 1:2,000 and are among the rarest tumors. Of the non cancerous heart tumors *atrial myxoma* deserves mention that

develops in the upper left chamber with higher incidence in women (Knepper et al. 1988). The name myxoma is related to the Latin *muxa* (mucus) and not to be confused with myxomatosis (*Myxoma virus* infection). Myxoma is diagnosed by echocardiography upon listening heart sounds (*auscultation*) as it gives an audible extra heart sound. Differential diagnosis with magnetic resonance imaging helps to distinguish it from other cardiac tumors such as lipomas, benign tumors of the striated muscle (rhabdomyomas) and teratomas. Cardiac rhabdomyomas occur more often in infants and children (fetal type), the incidence of adult type and genital type of rhabdomyomas is lower (Bader et al. 2003).

4.10.7 Benign Spleen Tumors

Tumors of this "forgotten organ" are rare. The tumoral features of spleen are important to distinguish between benign and malignant masses diagnosed mostly incidentally. Benign tumors of spleen are cysts, hemangiomas, angiomas and lymphangiomas. Principal malignant spleen tumors occur as lymphomas and metastasizing hemangiosarcomas (Giovagnoni et al. 2005).

4.10.8 Benign Bone Tumors

Benign tumors of the bone are not necessarily neoplasms, but can be focal maltransformations such as hamartomas, particularly osteochondromas. Benign bone tumors are osteoma, osteoid osteoma, osteochondroma, osteoblastoma, enchondroma, giant cell tumor of bone, aneurismal bone cyst, and fibrous dysplasia. Benign bone tumor are to be distinguished from malignant primary tumors of the bone e.g. osteosarcoma, chondrosarcoma, Ewing's sarcoma, fibrosarcoma, etc.

4.10.9 Other Benign Tumors

Benign tumors can develop anywhere in the body at any age. The low incidence of benign tumors in other tissues and organs does not necessitate their discussion.

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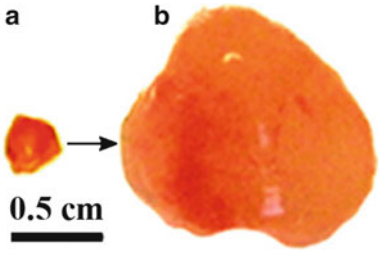
Chapter 5
Metastasis



Abstract This chapter deals with the transition from primary cancerous tumors to metastatic tumor progression. Earlier theories suggested that tumor spread from the primary tumors starts with the intravasation of tumor cells that are carried to distant places of the organism by the blood circulation. The chapter describes a new tumor model named renal capsule – parathymic lymph node model where tumor spread starts with extravasation. The new model and provides evidence that in poorly vascularized primary tumors (liver, kidney) neoangiogenesis takes place at the outer part of the tumor and tumor is necrotized inside. Extravasated tumor cells enter the interstitial fluid and are expulsed along with blood cells into the abdominal cavity through the peripheral tumor disruptions. Intraperitoneally administered colloidal particles, among them tumor cells, blood cells, and colloidal carbon, traverse the diaphragm, are collected by the thoracal lymphatic vessels and enter the thoracal lymph nodes, primarily the parathymic lymph nodes as the first site of metastasis. The steps of the metastatic cascade have been deduced from the new tumor model.

Keywords Historical view • Etiology of cancer • Spread of tumor cells • Metastatic views • Multistep metastasis • Complexity of metastasis • Cancer models • Syngenic model • Xenograft model • Renal capsule model • Parathymic lymph nodes • Mediastinal tumors • Implantation of tumor cells • Major steps of metastasis • Lung cancer • Breast cancer • Breast cancer theories • Liver cancer • Brain cancer • Kidney cancer

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Contents

5.1 Historical View of Metastasis

That metastasis resistant to therapy is the major cause of death from cancer (Talmadge and Fidler 2010) has been described in the Edwin Smith Surgical Papyrus, the first medical book, that deals with tumors, among them several breast cancer cases known by Egyptian physicians some 3,500 years ago. Already these ancient physicians distinguished between benign and malignant tumors and removed primary surface tumors surgically, but acknowledged that there is no efficient treatment for the bulging, malignant breast tumor (Allen 2005). Hippocrates (460–370 A.D.) named the malignant tumor "*karkinos*" (*Carcinus*).

Carcinus According to the mythology, the giant crab (*Carcinus*) had come to assist the nine-headed serpent named *Hydra*. *Carcinus* was not only battling with Herakles but snapped his foot. The hero Heracles defeated the *Hydra* and killed the crab by crushing its shell with his foot.

Hippocrates agreed with the metastatic view of Egyptians that the black bile is dangerous and advised palliative treatment: the removal of black bile by venesection, cupping, purgation and enemas. Galen (∼129–200), echoed the view of

Hippocrates that the "black bile" poisoned the entire body and could not be healed by tumor removal, but would create new tumors. Already these ancient observations distinguished between curable (benign) and incurable (malignant) tumorous diseases. Corresponding to the ancient Egyptian and Hippocratean metastatic view only palliative therapy of malignant tumors was advised. The humoral theory of incurable malignancies prevailed until the blood circulation was discovered in the seventeenth century.

It is now generally believed that systemic metastases arise from tumor cells that spread *via* the blood and lymphatic system. Tumor spread to the lymph nodes is not a random process, distinct patterns of metastasis vary depending on tumor type. A common pattern of metastasis, particularly for carcinomas is the dissemination of tumor cells to the lymph nodes as a bridgehead of metastasis (Sleeman 2000).

Despite the unending war against cancer the process of tumor metastasis remained controversial. The "War on Cancer" declared by president Nixon in 1971 also indicated that despite all earlier efforts the victory remained elusive as metastasis turned out to be responsible directly or indirectly for more than 90 % of all cancer deaths (Sporn 1996). Although, a metastatic lymph node *per se* is not lethal and can be removed surgically, but for different types of carcinomas derived from putative epithelial cells, the presence of metastatic tumor cells in a regional lymph node is an indicator of poor prognosis. Moreover, in solid human tumors it is the lymph node status that is the most important indicator for clinical tumor staging and the basis of rational therapy against cancer (Leong et al. 2006).

5.1.1 Etiology of Cancer

Tumor causing agents have been discussed under the Sect. 4.5 "Carcinogens" in Chap. 4. The etiology of cancer is multifactorial, dealing with genetic, environmental, medical, and lifestyle factors that often interact to produce a specific malignancy. Cancer genetics is among them a rapidly expanding field that contributes to the:

(a) understanding of cancer biology, risk and malignancies,
(b) establishment of treatment based on molecular fingerprinting of malignant diseases,
(c) development of new DNA-based tests of mutations,
(d) tailoring of preventive, screening and therapeutic modalities.

Different mechanisms of cancer development have been claimed to be universal, and indeed most of such ideas turned out to be correct. We now know that several factors contribute to the human cancer development. The history of the chemical etiology for cancer goes back to Theophrastus Bombastus von Hohenheim (1493–1541), who became known and "immortalized" as Paracelsus, the alchemical genius of the middle ages. Paracelsus was the first to suspect that the "wasting disease" of

miners could be attributed to the exposure of arsenic sulfide. The etiology of cancer is best exemplified by the history of tobacco smoking (reviewed by Proctor 2001):

- First century BC – Mayans were smoking the leafs of tobacco.
- 1723 – The city of Berlin banned smoking (Bejach 1927).
- 1761 – John Hill in London described that excessive inhaling of tobacco through the nostril was linked to the formation of nasal polyps (Proctor 2001).
- 1854 – Friedrich Tiedemann in Germany recognized that tobacco induced cancers of the tongue (Proctor 1995).
- 1787 – Percival Pott noted the correlation between cancer and environmental toxicants such as smoking. He also demonstrated that 'soot wart' of chimney sweeps was an epithelial carcinogen for the scrotum (Dobson 1972).
- 1858 – Etienne-Frédéric Bouisson reported that tobacco smoking caused mouth cancer of pipe smokers (Bouisson 1858).
- 1898 – Tobacco dust (not smoke) is linked to lung cancer among German tobacco workers (Proctor 2001).
- 1943 – Schairer and Schöniger established a conclusive tobacco-lung cancer link (Davey Smith et al. 1994; Proctor 1997).
- 1964 – The Surgeon General of the US reported that cigarette smoking is related to lung cancer, the magnitude of which is far outweighing all other factors (USPHS 1964).
- 1981 – 80–90 % of all lung cancers are attributed to smoking (Doll and Peto 1981).
- 1996 – benzopyrene, the constituent of tobacco smoke causes mutations of the gene encoding p53 (Denissenko et al. 1996).
- 1999 – Tobacco industry acknowledges that tobacco smoking is a human health risk (Proctor 2001).
- 1982 – The Surgeon General of the USA announced that cigarette smoking is dangerous to health.

Cancer cases can be associated with other rare diseases and aggravate in the order listed in Table 5.1.

The loss of genetic variation that occurs when a new population is established by a very small number of individuals from a larger population, known as founder effect may reveal further mutations characteristic to certain ethnic groups with higher prevalence. These mutations of founder effects can be clinically useful in genetic testing of tumors but also potentially stigmatizing.

Global Cancer Cases

quoted from (GLOBOCAN 2008 http://globocan.iarc.fr/factsheet.asp):

Nearly 12.7 million new cancer cases and 7.6 million cancer deaths occurred in 2008 worldwide. The number of new cancer cases ranges from 3.7 million in Eastern Asia to about 1,800 in Micronesia/Polynesia. In men, the incidence of cancer is high in Northern America (ASR 334 per 100,000),

(continued)

Table 5.1 Cancer aggravation including hereditary cases

Single malignancy in one organ
↓
Multiple cancer formation in one organ
↓
Younger than usual age of cancer development
↓
Multiple malignancies in different organs
↓
Bilateral cancer in paired organs
↓
Formation of rare cancer
↓
Rare cancer associated with another rare disease
↓
Malignancy normally not occurring in this sex (e.g. breast cancer in men)
↓
Cancer caused by congenital failure
↓
Congenital cancer associated with another rare disease
↓
First degree relative with the same or related cancer
↓
More first-degree relatives with cancer at the same location
↓
More first-degree relatives in two generations, cancer at the same location

(continued)
Australia/New Zealand (ASR 356.8) and in Northern and Western Europe (ASRs 288.9 and 335.3 respectively) as a consequence of high rates of prostate cancer in these regions (ASRs greater than 80 per 100,000 in all). As in males, the regions with highest incidence rates in females are Northern America (ASR 276.4 per 100,000) and Northern and Western Europe (ASRs 257.8 and 250.5, respectively) as a consequence of the highest rates of breast cancer in these regions (ASRs greater than 75 per 100,000). The lowest cancer incidence rates are in middle and Western Africa and in South-Central Asia for men and Middle and Northern Africa for women (ASRs less than 100 per 100,000). The ratios of ASRs of incidence between developed

(continued)

(continued)
and developing regions are 1.8 in men and 1.6 in women, while the same ratios for mortality are much lower, 1.2 for men and almost 1.0 for women. Women living in sub-Saharan Africa have the same high risk of dying from cancer as women living in Central and Eastern Europe (ASRs greater than 90 per 100,000 in all). A number of common cancers in developed countries are associated with reasonably high survival (prostate, breast and colorectal cancers), whereas several common cancers with poorer prognosis (liver, stomach and oesophageal cancers) are more common in less developed regions.

5.1.2 Cancer Statistics

The global picture of cancer statistics shows an increasing rate both in the incidence and mortality rates of cancer related to the growth, aging of the human population and improved methods of cancer diagnostics. The data presented in Tables 5.1 and 5.2 are based on GLOBOCAN 2008 of Agency for Research for Cancer (IARC). An explanation to the global cancer statistic has been given and suggestions made to reduce the proportion of the worldwide burden of cancer by implementing programs (tobacco control, vaccination), early detection and treatment, promoting public health campaigns (physical activity, sport) and healthier food intake (Jemal et al. 2011).

In 1985 of the estimated five million deaths from cancer around 56 % occurred in developing countries and 44 % in developed countries. The most frequent cause of death was lung cancer, accounting for 22 % of cancer death in men. Among women the leading malignancy was breast cancer accounting for 16 and 11 % of all cancer deaths in developed and developing countries, respectively. The second most frequent cancer death in both sexes was stomach cancer followed by liver cancer in men and colorectal cancer in women. Coloreactal and prostate cancers were also high ranking in men of developed countries, while among women lung, ovary and pancreas occupied a similar rank. Esophagus, mouth and pharyngeal cancers followed death cases in developing countries (Pisani et al. 1993) (Table 5.2).

It was predicted that between 1985 and 2000 the number of deaths cases in developed countries could increase by about 20.4 % causing about 2.65 million deaths and in developing countries by 18.1 % amounting to 3.3 million and a total of about six million cancer deaths per year (Pisani et al. 1993; Parkin et al. 1993). By extrapolating this death rate increase to 2000–2015 an estimated 7.4 million cancer deaths could have been expected by 2015. That the death rate increase was underestimated is reflected by the GLOBOCAN 2008 statistics were the elevation of cancer deaths was much higher and amounted to 7.6 million cancer death already in 2008 (Jemal et al. 2011). The GLOBOCAN 2008 statistics shows that 64 % of

Table 5.2 Top ten cancer cases worldwide for leading cancer sites

	Estimated new cases		Estimated cases of death	
Rank	Male	Female	Male	Female
1	Lung and bronchus	Breast	Lung and bronchus	Breast cancer
2	Prostate	Colon and rectum	Liver	Lung and bronchus
3	Colon and rectum	Cervix and uterus	Stomach	Colon and rectum
4	Stomach	Lung and bronchus	Colon and rectum	Cervix and uterus
5	Liver	Stomach	Esophagus	Stomach
6	Esophagus	Corpus uteri	Prostate	Liver
7	Urinary bladder	Liver	Leukemia	Ovary
8	Non-Hodgkin lymphoma	Ovary	Pancreas	Esophagus
9	Leukemia	Thyroid	Urinary bladder	Pancreas
10	Oral cavity	Non-Hodgkin lymphoma	Non-Hodgkin lymphoma	Leukemia

Source: GLOBOCAN 2008

cancer death occurred in the developing world indicating a faster increase in death incidence and mortality in these areas. Breast cancer remained the leading cause of cancer in women accounting for 23 % of total cases and lung cancer in men (23 %). The second most deadly stomach cancer in men changed to liver cancer primarily in developing countries caused by viral infections (hepatitis B and C). The mortality rate of lung cancer for females is now as high as the burden of cervical cancer. The ranking of estimated new cases and estimated deaths of top ten cancers worldwide shows that there are significant differences between males and females (Table 5.2).

Significant differences among incidence and mortality rates of cancer were found not only between males and females, but also between developed and developing countries (Table 5.3). The list of mortality risk data of different cancer types reflects a gradual shift depending on the geographical location, sex and age.

5.2 Spread of Tumor Cells from Local Tissue to Distant Organs

5.2.1 Metastatic Views

The spread of tumor cells involves a series of events leading to a metastatic cascade, starting with the detachment of cancer cells from the primary site through to attachment and tumor growth at a distant site. It is generally believed that a critical step of tumor spread to distant sites is the arrest of circulating tumor cells within the host organ (Nicolson 1988a; Feldman and Eisenbach 1988). Regarding the

Table 5.3 Top 12 incidence and mortality rates and probability to develop cancer by age 75

	Developed countries		Developing countries	
	Incidence	Mortality	Incidence	Mortality
	Cumulative risk (%)		Cumulative risk (%)	
Rank	Age 0–74		Age 0–74	
Males				
All cancers (ASR 300.1)	30.1	15.0	17.0	12.7
1 Lung (C33–34)	5.7	**4.7**	3.3	2.9
2 Liver (C22)	1.0	0.9	2.2	**2.0**
3 Stomach (C16)	2.0	1.2	2.5	**1.9**
4 Colorectum (C18–21)	4.4	**1.7**	1.4	0.8
5 Esophagus (C15)	0.8	0.6	1.4	**1.2**
6 Prostate (C61)	7.8	**0.9**	1.4	0.5
7 Pancreas (C25)	1.0	**0.9**	0.3	0.3
8 Leukemia (C91–95)	0.9	**0.5**	0.4	0.3
9 Bladder (C87)	1.9	**0.5**	0.6	0.3
10 Kidney (C64–66)	1.4	**0.5**	0.3	0.1
11 Brain (C70–72)	0.6	**0.4**	0.3	0.3
12 Non-Hodgkin lymphoma (C82–86, C96)	1.1	**0.4**	0.5	0.3
Females				
All cancers (ASR 225.5)	22.0	9.1	14.0	9.0
1 Breast (C50)	7.1	**1.7**	2.8	1.2
2 Lung (C33–34)	2.3	**1.6**	1.3	1.1
3 Cervix uteri (C53)	0.9	0.3	1.9	**1.1**
4 Colorectum (C18–21)	2.7	**1.0**	1.1	0.6
5 Stomach (C16)	0.8	0.5	1.1	**0.9**
6 Liver (C22)	0.3	0.3	0.9	**0.8**
7 Ovary (C56)	1.0	**0.6**	0.5	0.4
8 Pancreas (C25)	0.6	**0.6**	0.3	0.2
9 Esophagus (C15)	0.1	0.1	0.7	**0.5**
10 Corpus uteri (C54)	1.6	**0.3**	0.7	0.2
11 Leukemia (C91–95)	0.6	**0.3**	0.3	0.3
12 Brain (C70–72)	0.4	**0.3**	0.3	0.2

Ranking (bold numbers) is based primarily on mortality rates
Source: GLOBOCAN 2008
ASR age standardized ratio per 100,000 persons-year

mechanism of metastatic cascade major hypotheses became known as: seed and soil model, random mechanical colonization, clonal evolution model and cancer stem cell hypothesis.

1. *Seed and soil hypothesis*. This model suggested that the seeds (tumor cells) will find the best soil (microenvironment of the body) and colonize there (Paget 1889). Related to the seed and soil hypothesis it caused a dilemma to explain why only a very small fraction (∼0.01 %) of the many primary tumors turn to a metastasis, a secondary tumor at distant places (Wong and Hynes 2006).

The inefficiency of metastatic tumor spread is likely to be overcome by the vast number of tumor cells that enter the systemic circulation, estimated to be $\sim 4 \times 10^6$ cells/g primary tumors (Butler and Gullino 1975).

2. *Random mechanical colonization*. The seed and soil theory was contrasted by the random mechanical colonization theory of circulating tumor cells assuming that the specific anatomical structures of the vascular system predict the location of the secondary tumor known as metastasis (Ewing 1928). Both models are still debated (Chambers et al. 1992; Naumov et al. 1999; Koop et al. 1995; Ito et al. 2001; Al-Mehdi et al. 2000), especially since interactions among adhesion molecules and their ligands can be influenced by several factors (Takahashi et al. 2007; Bastida et al. 1989; Kitayama et al. 1999). These factors may modulate adhesion, invasion, growth and other properties important to develop into highly malignant multisite metastases and each metastatic tumor spread may achieve its organ specificity due to its own set of unique properties and responses to the host microenvironment (Nicolson 1988b).
3. *Clonal evolution*. This model suggests that a premalignant or malignant subpopulation that accumulates mutations over time provides certain advantages in the future for the natural selection of tumors. Carcinogenesis can take place in a single cell that accumulated several mutations the genetic and epigenetic alterations of which generate increasingly aggressive, invasive and drug resistant phenotypes. In this model hereditary changes are the driving force of heterogeneity.
4. *Cancer stem cell hypothesis*. The model of clonal evolution, has been challenged by the cancer stem cell hypothesis. This theory proposes that a small subpopulation of cells similar to stem cells drives tumor initiation and progression (Ichim and Wells 2006; Reya et al. 2001; Al-Hajj and Clarke 2004; Bjerkvig et al. 2005; Bapat 2007). Differentiation of this subset of cells generates all cell types in the tumor and is responsible for intratumor heterogeneity. The generation of heterogeneity in both cancer stem cell and clonal evolution metastatic progression is based on the self-renewal potential of cancer cells that persists over time (Michor and Polyak 2010).
5. *Lymphatic spread*. Tumor cells are released through the disruptions of the primary tumor and drained to the lymph nodes. The new version of the lymphatic hypothesis will be discussed in more details in the Sect. 5.3.1.2 (Banfalvi 2012b).

5.2.2 Multistep Metastasis

It may take a 20–50 year lag from carcinogen exposure to the clinical detection of tumors. The exponential relationship in cancer incidence as a function of age suggested that tumor progression takes place is a series of steps (de Visser et al. 2006). The multistep process has been demonstrated first by the classical genetic model of colon tumorogenesis starting with the progression of hyperplastic epithelial developing to adenoma, carcinoma and finally to metastasis. The sequential order

of mutagenic steps in colon involved mutations, DNA hypomethylation, activation of k-*ras* oncogene, loss of heterozygosity and loss of p53 (Fearon and Vogelstein 1990). As each cancer cell harbors a pool of thousands of mutations, it is likely that these random mutations provide selective advantage for tumorigenesis (Smith et al. 2002), explaining the high frequency of nonclonal mutations in human cancers relative to normal tissues and the genetic variability of cancer cells.

As far as the steps of tumorigenesis are concerned, the simplest model of carcinogenesis that used croton oil to generate murine skin tumor supposed that the malignant process consisted of two basic steps: initiation and promotion (Berenblum and Shubik 1941). This model was followed by the three step carcinogenesis model (Rous and Kidd 1941; Mottram 1944) that distinguished: (a) initiation, (b) promotion and (c) deregulation of cellular growth.

(a) In the initiation step a single somatic cell undergoes heritable mutation. In contrast to neighboring cells, the initiated cell can escape the cellular regulatory mechanisms. This initiating mutation may give the initiated cell the growth advantage needed for tumor development.
(b) In the second stage of promotion the initiated cell is exposed to a tumor promoter which causes these cells to undergo phenotypical clonal expansion. Only initiated cells are stimulated to grow (Cerutti 1988).
(c) Further genetic alterations take place. Here cellular growth becomes more deregulated.

However, tumor development does not stop at this third stage, thus a further one has been included. As the tumor progresses, sensitivity to dietary compounds, inhibitors of growth, and enhancers of differentiation gradually disappears until the tumor becomes progressively more autonomous and uncontrollable (Guyton and Kensler 1993). The fourth stage is known as the metastatic development of cancer, namely the spread of tumor cells from the primary site to distant parts of the body. This fourth step, namely the formation of secondary tumor remains the most significant and unsolved problem of cancer research. Despite the successful removal or therapy of the primary tumor, the prognosis of patients with metastases is generally grave. Due to the complexity of metastatic development it is difficult to predict fluctuations of the cascade, consequently not much is known how the tumor formation turns to a metastatic process. It is now generally believed that the transformation of a single cell can lead to a complex process of metastasis involving different molecules governing invasion (enzymes, motility factors), adhesion (integrins, selectins, cadherins, annexins immunoglobulin-like superfamilies) and growth (paracrine, autocrine, endocrine) factors, that act through multiple steps in an organized, non-random, sequential order. The concerted action of these factors is responsible whether homeostasis is maintained or the tumor cell will develop from a single malignant cell to an organ selective, or multiorgan, metastatic disease (Yeatman and Nicolson 1993).

Existing tumor models agree that primary tumors turn to a secondary metastatic tumor through the detachment of tumor cells, migration and proliferation at distant

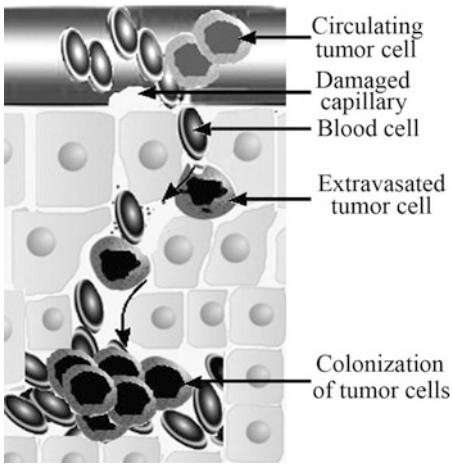

Fig. 5.1 Demonstration of extravasation of tumor and blood cells during metastasis. Tumor cells escape the circulation and migrate to mesenchymal tissues where they are arrested and start to colonize

sites. In this multistep process metastasis alone has been divided into four further subphases (Nguyen et al. 2009):

(a) Intravasation into the blood vessels means local invasion of tumor cells through the basal membrane into the blood or lymphatic vessels. During the epithelial-mesenchymal transition tumor cells change their epithelial characteristics by loosing cell polarity and cell-cell interactions and by increasing their motility (Berx et al. 2007; Hugo et al. 2007).

(b) Survival of circulating tumor cells. As circulation takes place in a hostile environment, only those cells that can efficiently exit the blood stream have a distinct advantage in the metastatic events (Bos et al. 2009; Gupta et al. 2007).

(c) Extravasation from the blood stream – cancer cells leave the capillaries. Extravasation refers to cancer and blood cells exiting the damaged capillaries (Fig. 5.1).

Extravasation of tumor cells was monitored in chick embryo chorioallantoic membrane model system (Luzzi et al. 1998; Podsypanina et al. 2008; Sahai 2007; Townson and Chambers 2006). This model system allowed to conclude that tumor cell extravasations could involve passive or active tumor cell movement within the vessel lumen, adhesion of tumor cells to the vascular wall and transendothelial passage of tumor cells by unknown mechanisms (Colmone et al. 2008; Kienast et al. 2010; Luzzi et al. 1998; Sipkins et al. 2005; Voura et al. 2004; Yamaguchi et al. 2006). It was demonstrated that the extravasation of tumor cells is a highly dynamic process influenced by metastatic genes that target adhesion and intravascular migration of tumor cells, and induce endothelial remodeling of the vasculature. These findings contradict the earlier belief that tumor cell extravasation is a passive process and not a crucial determinant in the metastatic spread (Stoletov et al. 2010). Nevertheless, extravasation regarded as a late step of the metastatic cascade remained poorly characterized.

(d) Colonization of tumor cells at distant metastatic sites. The metastatic spread of malignant cancers is a common feature, but the disparities in the progression of different cancers changed the general view from dissemination to organ-specific colonization (Nguyen et al. 2009). The expression of metastatic genes Twist, VEGFA and ITGB1 affect intravascular migration of arrested tumor cells, and their capability to remodel the vasculature and exit the circulation. These findings suggest that genetic determinants and epigenetic factors could provide the metastatic advantage that leads to the selection of metastatic cells (Nguyen et al. 2009; Stoletov et al. 2010).

5.2.3 Complexity of Metastasis

The multistep nature of the metastatic inefficiency was tested in a melanoma murine model where melanoma cells were transplanted intraportally into the liver. Two aspects of the insufficiency of the metastatic spread were mentioned: the failure of solitary cells to initiate growth and the failure of early micrometastases to grow into macroscopic metastases (Luzzi et al. 1998). Tumor spread is not only a multistep process, but also a complex process governed by different classes of molecules, through multiple steps in an organized, nonrandom and organ-selective fashion (Takahashi et al. 2007; Bastida et al. 1989; Kitayama et al. 1999). The entities that contribute to the complexity of the metastasis are accounted for by proteinases, motility factors that govern the invasion, promote the adhesion (integrins, selectins, cadherins, annexins) and/or the growth (growth factors) of tumor cells (Yeatman and Nicolson 1993). The flexibility of interactions of tumor cells is reflected by the expression levels of adhesion molecules, proteases, protease inhibitors, motility factors and growth factors (Bussemakers and Schalken 1996). The colonization of particular tumor cells at certain organ sites is supposed to be determined, in part, by the responses to growth factors and to the extracellular matrix of the targeted organ (Nicolson 1991).

5.2.3.1 Gene Expression Patterns of Metastasis

The aim of these studies was to decide whether the gene expression of distant metastases is the same or different from those of primary tumors. Inconclusiveness of these experiments was indicated by controversial results, suggesting that the gene expression pattern of metastases was similar to those of the primary tumors (Ramaswamy et al. 2003; Veer et al. 2002). Further investigations showed that the overall gene expression patterns were remarkably similar between primary tumors and their metastases as well as between metastases in different organs of the same host, although acknowledging a few specific differences between individual metastases (Suzuki and Tarin 2007; Montel et al. 2005). The information gathered from different laboratories has also indicated that the gene expression pattern of

several thousand genes were similar between metastases in different organs (Suzuki and Tarin 2007). Data also confirmed that clinically obtained and xenogeneic metastases complemented each other and should be considered when it comes to the discussion of heterogeneity among metastases (Tarin 2008). Others have found that the expression of specific genes has been changed during metastasis (Clark et al. 2000; Kang et al. 2003; Yang et al. 2004). A compromistic model suggested that the primary and metastatic tumor cells share a common, basic gene expression pattern that is necessary, but not sufficient to complete all the steps of metastasis (Kang et al. 2003; Hynes 2003). The expression of additional genes can render sub-populations competent for metastasis (Wong and Hynes 2006). Growing evidence supports the notion that malignant tumor cells express a flexible, multipotent phenotype resembling embryonic stem cells growing without the regulatory control of major checkpoints. This phenotype allows the activation of embryonic, particularly the Nodal and Notch signaling pathways (Strizzi et al. 2009). Nodal is a secretory protein belonging to the transforming growth factor (TGF) superfamily, encoded by the NODAL gene and is expressed during the early embryogenesis of mice (Zhou et al. 1993) and its human homolog (Gebbia et al. 1997). Notch signaling regulates left-right asymmetry determination by inducing Nodal expression (Krebs et al. 2003).

5.3 Animal Cancer Models

Earlier in vivo models overviewed in Chap. 4 are suitable to follow tumor growth. The recent upsurge of cancer models is indicated by the multitude of new publications many of them mentioned in the references. Representative animal cancer models are important for understanding the underlying molecular pathogenesis of these cancers, for prioritization of cancer treatments and the development of novel targeted anticancer therapeutics (Decker and Sausville 2011; Gober et al. 2013). Executing studies on metastasis face conceptual and practical difficulties that must be overcome to conduct clinical investigations and experimental studies. The conceptual difficulty of metastatic study is related to the kinetic nature of metastasis that occurs in the living body, consequently metastasis has to be studied *in vivo* and cannot be modeled *in vitro*. The kinetic character of metastasis makes it difficult if not impossible to predict when it will occur and recur (metastasis from metastasis) especially in human patients (Tarin 2008). The dissemination is a necessary but not satisfactory requirement for metastasis (Goodison et al. 2003; Tarin 2008).

The practical aspects causing difficulties in metastatic studies are:

1. Human metastasis samples are often studied with a few probes and by subjective semiquantitative methods. Studies are supposed to apply impartial statistical analysis of data from high-throughput screening techniques.
2. The number of patients is normally small and may cause overinterpretation both in diagnosis and therapy.

3. Primary and secondary human tumor samples are essentially end-stage assays which can give useful information regarding pathologic and molecular aspects of the lesions at the time of sampling without knowing the previous properties or future consequences.
4. Long tumor history complicates the collection of samples at appropriate stages, especially from human subjects.
5. The long time span of experiments may cause spontaneous metastasis in animals and disturb the comparison and coordination of interpretation of results.

To study the complex process of metastatic tumor formation necessitates the selection of optimal models. To meet most of the criteria of biological complexity, the selection of a complex experimental system, namely the use of *in vivo* animal cancer models is unavoidable. Experimental metastasis has been normally referred to the injection of tumor cells directly to the systemic circulation the site of which is expected to define the site of metastasis (Khanna and Hunter 2005). The most common site of injection is the lateral vein in mice. Tumor cell injection directly to the systemic circulation through the lateral tail vein of mice results primarily in pulmonary metastases. Tumor cells introduced by intrasplenic or portal vein injection are employed for developing metastasis in the liver. Metastasis targeting to lung and liver can be explained by the first capillary bed principle. Upon *i.v.* injection, particles larger than red blood cells will be trapped in the first capillary bed that they encounter. This is the principle of lung perfusion imaging in nuclear medicine. The intracardial injection of tumor cells will direct metastases to several end-organ targets, conforming to the explanation of the seed and soil hypothesis.

The adaptation of the seed and soil hypothesis to transplantation models resulted in the orthotopic transplantation of tumor cells into mice (Tan et al. 1977; Fidler et al. 1990; An et al. 1998; Nagamachi et al. 1998). Orthotopic transplantation refers to the delivery of tumor cells to the same anatomic location or tissue from which the tumor was derived. Orthotopic models have been applied to predict which therapy should be used for transplantable tumors (Killion et al. 1998; Hoffman 1999; Bibby 2004). As far as the latest cancer models are concerned, brain, breast, colorectal, prostate, liver, lung, leukemia models deserve special mention:

Brain cancer models. Human brain tumors comprise a multitude of various different tumors that can be distinguished at both a histological and a molecular level. Models have been reviewed by summarizing the use of genetically engineered mice to model glioma and the embryonal tumors medulloblastoma (MB) and primitive neuroectodermal tumors (PNETs) (Swartling et al. 2013).

Murine breast cancer models. More than 20 mutant mice carrying germline mutations of human breast cancer-associated gene 1 (Brca1) have been created to study functions of Brca1 in mammary development and tumorigenesis. Different gene targeting approaches have been applied, ranging from null, hypomorphic, isoform, functional, missense, and tissue-specific knockouts (Dine and Deng 2013).

Colorectal cancer models. Comprehensive review on the molecular genetics of colorectal cancer has been recently given by Fearon (2011). Three major categories of colorectal cancer models have been reviewed: spontaneous intestinal cancers of

different animal species, chemically or environmentally induced cancers in rodents and cancers induced by genetic manipulation of mice (Johnson and Fleet 2013).

Prostate cancer models. Distinguishing prostate tumors that will progress to cancer from those that will remain benign is one of the major clinical challenges, besides understanding the role of androgen signaling in prostate tumorigenesis and the propensity of prostate cancer to take up residence in bone. A novel model was introduced as knock-in prostate cancer model that demonstrated tumor architecture of heterogeneity similar to that of human prostate cancer and was turned out to be suitable for preclinical studies (Gabril et al. 2005). Murine prostate cancer models have been recently reviewed by Irshad and Abate-Shen (2013).

Animal models of liver cancer. Hepatocarcinoma, the fifth most frequent cancer worldwide, has been studied preferentially. Tumor models of hepatocarcinogenesis have been developed in animals primarily in rodents. Although, murine, rabbit, woodchuk, tree shrew, dog, pig and primate have been reviewed, available data are not satisfactory to elucidate the sequence of events that lead to hepatocellular carcinoma (Quiao et al. 2012; Walker et al. 2012).

Lung cancer models. The four major types of lung cancer are adenocarcinoma, squamous cell carcinoma, small cell carcinoma and large cell carcinoma. Mouse lung adenocarcinoma models have been used extensively to evaluate lung cancer chemopreventive agents (Herzog et al. 1997; Malkinson 1992). Murine lung squamous cell carcinoma (SSC) models have been developed in the 1990s (Wang et al. 2004, 2009, 2010) and recently reviewed (You et al. 2013).

Animal models of leukemia. Major techniques for generating murine models include carcinogen-induced, transposon and viral-induced models, transgenic models, mosaic models by to taking hematopoietic stem cells from mice, manipulate them *ex vivo*, and transplant them into autologous or syngeneic recipients, xenograft models, none-mouse models primarily rats, animal models of ALL (children's common leukemias) (Cook and Pardee 2013).

5.3.1 Metastatic Tumor Models

To provide experimental tools for the study of metastasis, *in vivo* animal cancer models turned out to be indispensible, especially since the development of animal tumors follows the same common pathway as those of human tumors. The natural multitude of animal tumors, their complexity makes it impossible to use a single model to describe the whole process and necessitates a careful selection and application among models. The chicken chorioallantois-membrane model is one of the oldest metastasis models to study metastasis (Gorden and Quigley 1986). Its simplicity is contrasted by suitability questioning whether the evolutionary gap between birds and mammalian can be bridged by comparing their tumors.

Although, experimental metastasis was meant to be the injection of tumor cells directly into the circulation, it did not bring a breakthrough in metastasis research. The modeling of metastasis *in vivo* has then be performed by transplanting cancers

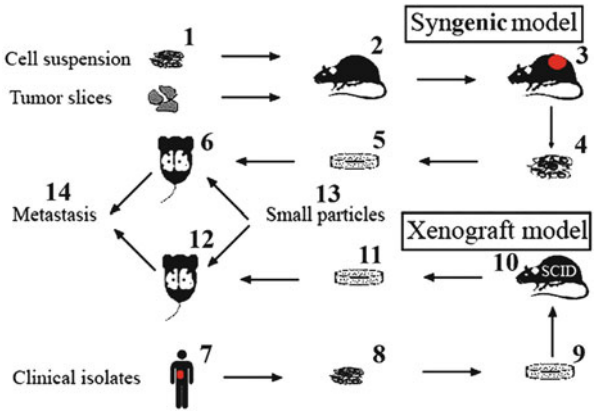

Fig. 5.2 Syngenic and xenograft murine tumor models. *Syngenic model*: *1* isolation of tumor, cutting to slices or preparing cell suspension, *2* implantation, *3* tumor formation, *4* isolation of tumor cells, *5* establishment of tumor cell line, *6* administration. *Xenograft model: 7* clinical tumor, *8* cell suspension, *9* tumor cell line, *10* implantation in SCID mice, *11* isolation of tumor cells, *12* administration of tumor cells, *13* administration of other antigens into rodents, *14* tracing spread of tumor cells in the body

that metastasize in small animals (mice or rats) (Khanna and Hunter 2005). The two best known transplantable models use either syngenic cells or xenografts (Fig. 5.2). Syngenic cell lines have been derived from carcinogen-induced tumors or from tumors that develop in a particular strain of rats or mice (Double et al. 1975; Takayama 1968; Brown et al. 1990). Human-mouse xenografts refer to human cancer cell lines or tissues that can be transplanted into immunocompromised animals to grow tumors. The most widely studied tissues have been xenografts of established human breast cancer cell lines into athymic nude mice. The nude mouse is not the only immunocompromised host to study xenografts. Additional mutant strains have been used successfully, including mice homozygous for the severe combined immune deficiency mutation (SCID mice) (Clarke 1996). The significant disadvantage of genetically engineered nude and SCID mice models for metastatic studies is their expense.

To mimic metastatic tumor formation orthotopic implantation was recommended by several authors (Takayama 1968; Double et al. 1975; Brown et al. 1990; Herzig and Christofori 2002; Huang et al. 2002; Jenkins et al. 2003). Orthotopic transplantation of tumor cells, compared with ectopic implantation, resembles more closely the tumor microenvironment and allows accurate expression of the clinical features of human cancer in mice. Moreover, models applying surgical implantation of tumor fragments improve reproducibility and the metastatic outcome within the orthotopic model (Hoffman 1999; Bibby 2004; Khanna et al. 2000) and allow to control morbidity (Bibby 2004; Khanna et al. 2000).

Based on the seed and soil hypothesis the orthotopic implantation of tumors to the same tissue of host mice (Nagamachi et al. 1998) mimicked more closely human

tumor development (Khanna et al. 2000). Consequently, orthotopic implantations were expected to be more useful to develop into metastasis than ectopic ones. Indeed limited efficiency of ectopic implants placed in non-identical tissues was demonstrated by injecting colorectal tumor cells into the lymphoid follicle of the cecum (Schackert and Fidler 1989), carcinoma cells in the kidney or under capsule of kidney (Naito et al. 1987), or osteosarcoma cell into bone (Berlin et al. 1993). Latest murine models mimicked human pancreatic tumorigenesis and metastasis (Qiu and Su 2013), skin cancers (Gober et al. 2013) and bone metastasis (Holzapfel et al. 2013).

5.3.1.1 Syngenic and Xenograft Metastatic Models

The advantage of implanting exact number of tumor cells, rather than tumor slices or tumor suspension is that the temporal aspects of tumor formation and metastatic cascade can be followed reproducibly. The syngenic and the xenograft models are viewed schematically in Fig. 5.2. Syngenic models preferentially use rodent (murine, rat) tumor cell lines or tissues that are genetically identical with the inbread host animals. Syngenic cell lines have been developed from chemically induced murine and rat tumors. The advantage of these models is that the transplanted tissue, the microenvironment of the tumor and the host organism are not only of the same genetic origin, but come from the same animal strain. At the same time these cell lines suffer from their homozygotic inbread origin that is not able to reflect the genetic complexity of human tumors (Gonzalez and Kimura 2001).

The other major group of useful metastatic models is represented by xenograft models. This procedure can apply human tumorous cell lines that are implanted in rodents made partially or completely immunodeficient and allow the growth of the foreign tumor. The developing cancer will show a mosaic character with cellular interactions between the human tumorous and stromal elements of the host (Schmidt-Hansen et al. 2004). Our attention was drawn to the growth of tumor xenografts implanted under the renal capsule of mice (Bogden et al. 1979, 1984).

5.3.1.2 Renal Capsule-PTN Metastatic Model

Cancer cells possess a broad spectrum of migration and invasion mechanisms including both individual and collective cell-migration strategies. Different migration/invasion programs bring us closer to the understanding how cancer cells disseminate and lead to new treatment strategies (Friedl and Wolf 2003). Most models of metastasis include both intravasation and extravasation steps in the process. It was suggested that in the lung, metastasis is initiated by the attachment of tumor cells to the vascular endothelium and that hematogenous metastasis originates from the proliferation of attached intravascular tumor cells rather than from extravasated ones (Al-Mehdi et al. 2000). Our metastatic view regarding tumor progression resembles other tumor models, with some notable steps being different.

The rat metastatic tumor model described as renal capsule-parathymic lymph node tumor model favors the idea of early extravasation, rather than the early intravasation of tumor cells.

The name of the rat renal capsule model is related to the implantation of tumor cells under the kidney capsule and on the involvement of parathymic lymph nodes (PTNs) in the metastatic spread. Two rat cancer cell lines (nephroblastoma, NeDe and hepatocarcinoma, HeDe) with similar metastatic potentials have been established and grown under the kidney capsule either as orthotopic or ectopic tumor models in rats. The advantage of using rats rather than mice is that parathymic lymph nodes (PTNs) are outside the thymus, while murine parathymic lymph nodes similarly to human PTNs are inside the thymus capsule and indistinguishable from the thymus. Similarly to benign tumors, that usually do not metastasize (discussed in Chap. 4), local (*s.c., i.d., i.m.*) administration of malignant cells regularly cause local tumors, but from these sites the growing tumors fail to metastasize (Liotta 1986). When cancerous tumor cells were injected into the vasculature, they bypassed the lymphatic pathway and interacted in tissues near the site of injection (Potter et al. 1983). The major disadvantage of administering tumor cells into the circulation (*i.v.*, *i.a.*, *i.c.*) is that even if metastasis would occur, important events including the escape from the primary tumor, invasion into adjacent tissue and extravasation into the hematopoetic system are likely to be missing (Khanna and Hunter 2005). Orthotopic implantation by grafting tumor cells into the same tissue of rodents was mentioned to be more useful than ectopic ones to study metastasis (Fidler 1991; Kerbel et al. 1991).

The following major steps of tumor formation and metastatic cascade have been distinguished by the renal-capsule tumor model:

(a) primary kidney or liver tumor formation upon chemical carcinogenesis,
(b) peripheral angiogenesis in primary tumor,
(c) necrosis inside the primary tumor,
(d) appearance of blood and tumor cells in the interstitial space,
(e) peripheral disruptions in the primary tumor, escape of tumor and blood cells,
(f) lymphatic spread of tumor cells from peritoneum and retroperitoneum to parathymic lymph nodes (metastasis).

5.3.1.3 Primary Kidney Tumor Formation

To be able to follow the temporal order of tumor development in a reliable and reproducible manner malignant tumor cell lines were developed. Experimental hepatocarcinoma and nephroblastoma tumors were generated in newborn Fisher 344 rats by chemical mutagenesis using nitrosodimethylamine (Dezso et al. 1991). The structural formula of nitrosodimethylamine is shown in Fig. 4.8a. From these tumors hepatocarcinoma (HeDe) and nephroblastoma (NeDe) cell lines have been established (Fig. 5.3).

Experimental surgery served to transplant exact number (10^6) of HeDe cells on a gelatine disc placed under the capsule of the kidney. Tumor development

Fig. 5.3 Establishment of tumor cell lines from primary tumors of rats. (**a** and **b**) successive implantation of tumor slices of hepatocarcinoma or nephroblastoma under the renal capsule of rat, (**c**) primary tumors treated with elastase and hyaluronidase to obtain hepatocarcinoma (HeDe) or nephroblastoma (NeDe) tumor cell lines. (**d**) Tumor cell (HeDe or NeDe) implantation under the renal capsule to obtain primary hepatocarcinoma or nephroblastoma tumor

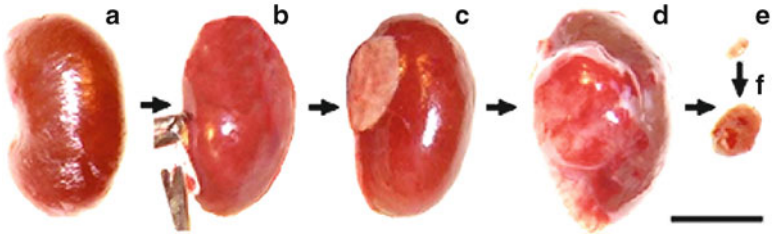

Fig. 5.4 Ectopic implantation of hepatocarcinoma tumor cells under the kidney capsule. (**a**) Normal rat kidney, (**b**) surgical opening of kidney capsule, (**c**) HeDe cells (10^6) placed on gelatin disc (Gelspon^R 4 mm in diameter) and implanted under the kidney capsule, (**d**) tumor development 6 days after implantation, (**e**) normal parathymic lymph node, (**f**) tumor formation and enlargement of parathymic lymph nodes 2 weeks after tumor cell implantation. Bar 0.5 cm

Table 5.4 Timing of metastasis formation in rats

Implanted HeDe cells	Metastatic death (in weeks)
10^6 cells	3
10^5 cells	4
10^4 cells	5
10^3 cells	~6

With permission Banfalvi (2012a)

was followed after euthanizing rats 1, 3 and 6 days after tumor cell implantation and removal of the tumorous kidney. After 2 weeks the parathymic lymph nodes became enormously (70–80x) enlarged. Other lymph nodes did not show significant morphological changes (Trencsenyi et al. 2009). Figure 5.4 shows tumor cell implantation and HeDe tumor development.

The complexity of the metastatic development makes it difficult to judge at which step and exactly when the tumor development turns to a deadly process. To avoid such uncertainity the timing of metastasis formation was standardized by the implantation of different number of tumor cells. Although, there are individual differences among rats regarding the deaths of animals upon hepatocarcinoma (HeDe) administration, nevertheless the occurrence of death could be determined after the implantation of cells ranging from 10^6 to 10^3 cells/rats (Table 5.4).

The timing of metastatic death caused by nephroblastoma (NeDe) cells gave similar results. For the surgical implantation 10^6 HeDe or NeDe cells have been

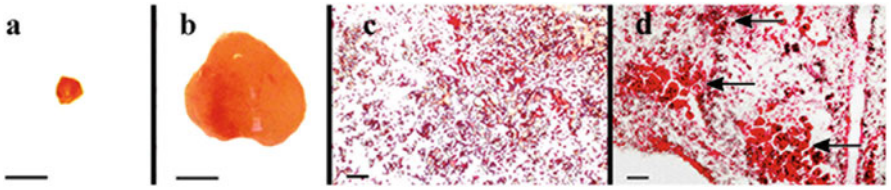

Fig. 5.5 Enlargement of rat parathymic lymph nodes after orthotopic implantation of nephroblastoma (NeDe) cells under the kidney capsule. (**a**) control PTN, (**b**) enlarged PTN 2 weeks after renal subcapsular placement of NeDe tumor cells, (**c**) hematoxylin-eosin stained control tissue section of PTN, (**d**) hematoxylin-eosin stained tumorous tissue section of PTN 12 days after NeDe cell implantation. Clusters of red blood cells are indicated by *black arrows*. Bars, 0.5 cm (**a**, **b**), 100 μm (**c**, **d**) (Modified with permission from Rozsa et al. 2009)

used that allowed to follow closely the steps of tumor and metastasis formation more closely. The reliability and reproducibility of the subrenal metastasis model offered a selective advantage of this metastatic model.

The enlargement of parathymic lymph nodes was similar when nephroblastoma cells (NeDe) were implanted under the kidney capsule of the rat (Fig. 5.5). The hematoxylin and eosin tissue staining has shown the presence of clusters of red blood cells in the enlarged PTNs indicating tumor development.

To prove that the increased size of PTNs after HeDe implantation is due to tumor formation, cells were isolated from enlarged PTNs and reimplanted (10^6) under the kidney capsule of tumor-free rats. Tumor formation was tested 3, 6 and 12 days after implantation. Tumors of gradually increasing size were observed under the kidney and in PTNs after 6 and 12 days of implantation (Trencsenyi et al. 2009). This experiment confirmed that tumor cells were transmitted from the primary tumor to the thoracal PTNs.

The kidney capsule is a vascular rich-region suitable for tumor transplantation and to induce blood-born metastasis. To prove that tumor formation is independent of the vascular effect of kidney, control experiments were carried out and HeDe tumor cells (10^6) isolated from enlarged PTNs were also implanted under the liver capsule (Fig. 5.6). Primary tumor formation was observed at the site of implantation and in PTN 2 weeks after implantation. Although, this experiment did not eliminate completely the vascular theory of tumor metastasis of PTNs, nevertheless it confirmed the establishment of a lymph node metastasis model of rat hepatocellular carcinoma. These experiments were sufficient to doubt the vascular and to favor the lymphatic spread of tumor cells.

5.3.1.4 Peripheral Vasculature Formation in Primary Tumors

The formation of tumor-supporting blood vessels during primary tumor formation is demonstrated in Fig. 5.7, 6 days after HeDe cell transplantation under the rat kidney.

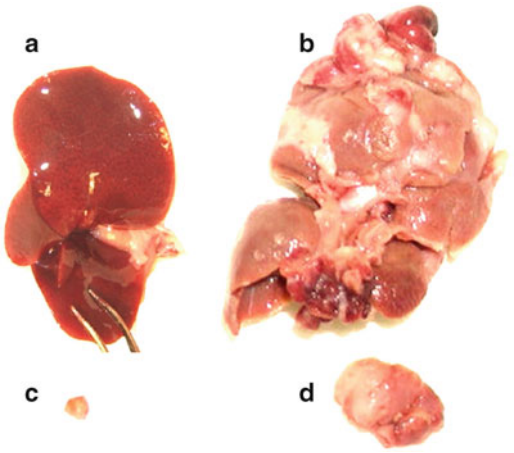

Fig. 5.6 Induction of primary liver and secondary PTN tumor with HeDe hepatocellular tumor cells. HeDe cells (10^6 in 40 μl) were dropped on gelatin sponge that was placed under the liver capsule of Fisher 344 rats. Rats (150 g) were sacrificed after 2 weeks and HeDe cells were isolated from liver tumors. (**a**) normal rat liver, (**b**) HeDe-infiltrated tumor, (**c**) normal parathymic lymph node, (**d**) tumor-infiltrated parathymic lymph node (With permission Trencsenyi et al. 2007)

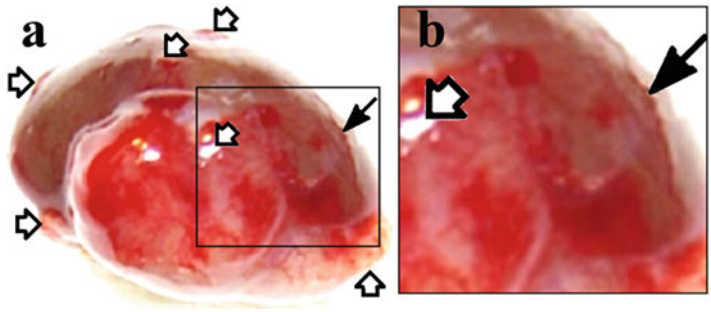

Fig. 5.7 Angiogenesis during hepatocarcinoma (HeDe) primary tumor formation in rat kidney. (**a**) Primary tumor 6 days after HeDe tumor cell implantation. *Black arrow* shows peripheral vessel where angiogenesis started. *Empty arrows* indicate peripheral disruptions. *White areas* are large disruptions. (**b**) *Magnified boxed* area containing several small new vessels, disrupted *light red* hemorrhagic clouds and *large white* disruptions

Angiogenesis starts from the peripheral blood vessels of the kidney speeding to the primary tumor indicated by the black arrow (boxed area of Fig. 5.7a). Disruption of the small new blood vessels at the surface of the primary tumor is seen as red hemorrhagic clouds. Large disruptions are seen as white spots (Fig. 5.7b). Similar observations were made when nephroblastoma (NeDe) cells were implanted under the kidney capsule of the rat (Rozsa et al. 2009).

5.3.1.5 Necrosis Inside the Primary Tumor

The *in vivo* distribution of ^{18}FDG was studied in the tissues of Fisher 344 rats. Rats were anesthetized, their lumbal areas were shaven prior to operation. A Gelaspon disc containing 10^6 HeDe cells was placed under the left renal capsule. Gelaspon disc without tumor cells was placed under the left renal capsule of control rats. On day 7 after implantation, rats were anesthetized and radioligand (14.8 mBq of ^{18}FDG in 1 ml saline) was injected into the left femoral vein of each rat. Animals were euthanized 60 min after the administration of radioligand, dissected and various organs/tissues were removed. Plasma was obtained from blood by centrifugation (3,000 g, 2 min). The radioactivity of parallel samples was measured in a gamma counter. The tissue uptake was expressed as differential absorption ratio (DAR) and calculated as:

$$\mathrm{DAR} = \frac{(\text{accumulated radioactivity/g tissue})}{(\text{total injected radioactivity/body weight})}$$

The metastatic potential of HeDe and NeDe tumor cells was compared in different tissues (Fig. 5.8). DAR values of plasma, muscle, kidney, liver and thymus were similar in the control and in tumor bearing animals. The DAR value was the highest in the tumor. The second highest DAR value was measured in PTNs. The difference between the DAR values of parathymic lymph nodes in the control and tumor bearing rat was significant ($p = 0.008$). The tumors grown under the kidney capsule were dissected. Their ^{18}FDG content was as expected significantly higher than in other tissues. The metastatic potential of plasma and resting muscle was low, while the PTNs showed an unexpectedly high metabolic rate in the tumor bearing animals relative to the PTNs of control animals (Fig. 5.8a). These DAR values are in correlation with our measurements of whole body autoradiography. The tumor formed under the subrenal capsule was surgically separable to the inner necrotic and other living parts. The living part of the HeDe primary tumor took up approximately 27 times more ^{18}FDG than its necrotic part while the living part of the NeDe tumor took up $\sim$18 times more ^{18}FDG than the necrotic part of the primary tumor estimated by their DAR values (Fig. 5.8b).

5.3.1.6 Appearance of Tumor Cells in the Interstitial Fluid of Primary Tumor

As a consequence of the insufficient angiogenesis in the primary tumor, the pink stream of interstitial fluid carried red blood cells and tumor cells to disruptions inside and outside the tumor (Rozsa et al. 2009) (Fig. 5.9).

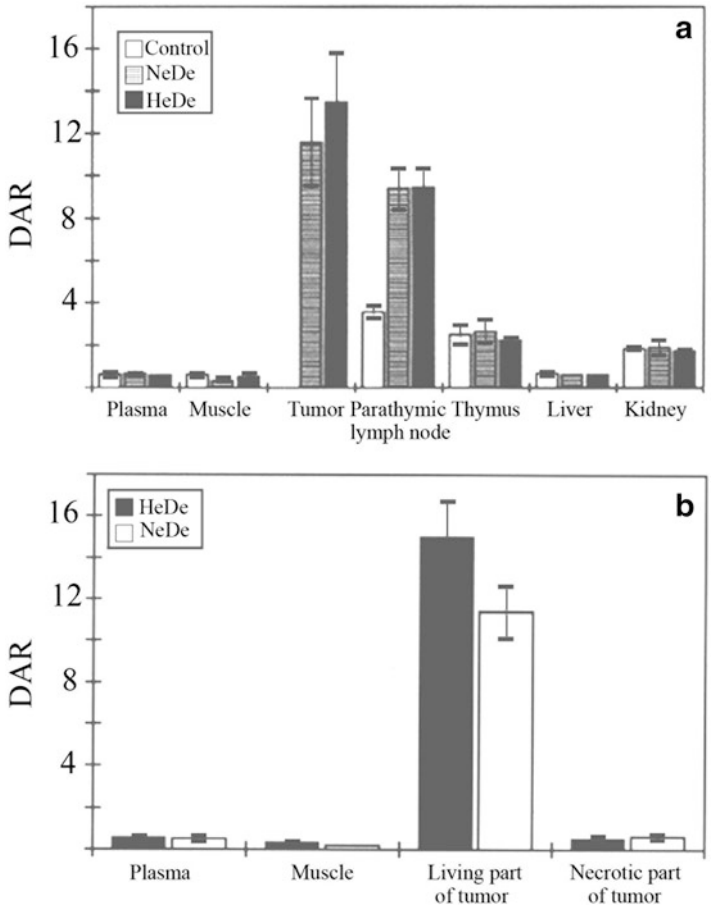

Fig. 5.8 Tissue distribution of ^{18}FDG radioactivity in NeDe or HeDe tumor-bearing rats. Tissue distribution was expressed as differential absorption ratio (DAR) after intravenous administration of ^{18}FDG (fluoro-deoxyglucose). (**a**) Metastatic potential of HeDe and NeDe cells in plasma, muscle, tumor, parathymic lymph nodes, thymus, liver and kidney expressed as DAR. (**b**) Distribution of ^{18}FDG in blood plasma, muscle and in the tumors after DeDe or NeDe implantation. The tissue uptake of ^{18}FDG was expressed as DAR (With permission Trencsenyi et al. 2010)

5.3.1.7 Lymphatic Spread of Tumor Cells to the Abdominal Cavity

The lymphatic spread of tumor cells through the disruptions of the primary tumor has been supported by several authors summarized in this subsection. The parietal peritoneum and the organs located in the abdominal cavity are also known as the visceral peritoneum. Retroperitoneum is the portion of the abdomen posterior to the peritoneal cavity from the diaphragm to the pelvic inlet. It is separated from the peritoneum anteriorly by the posterior peritoneal fascia and is bounded posteriorly

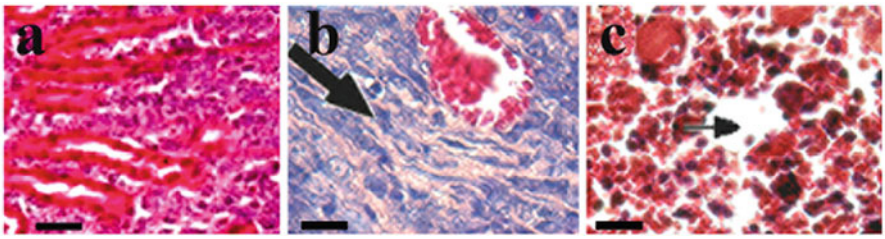

Fig. 5.9 Renal tumor formation and appearance of extravasated tumor cells in interstitial fluid. (**a**) Tissue section of NeDe tumor after hematoxylin-eosin staining showing disruptions filled with blood. Bar, 100 μm. (**b**) Detached tumor cells of the primary tumor entering the interstitial fluid, seen as channels indicated by the *black arrow*. The lack of angiogenesis and the presence of cancer cells is seen as stained interstitial fluid and accumulation of red blood cells (*upper right corner*) around tissue disruptions. Bar, 200 μm. (**c**) Tumor and blood cells breaking away through the disrupted tumor indicated by the *black arrow* in the middle. Bar, 50 μm (Modified with permission Rozsa et al. 2009)

by the transversalis fascia. It contains portions of the colon and duodenum as well as the pancreas, kidneys, adrenal glands, abdominal aorta, and *inferior vena cava* (Killeen et al. 1999).

Angiogenesis in tumor growth is a bloody process (Folkman et al. 1971). Bleeding, primarily hemorrhaging occurs in approximately 6–10 % of patients with advanced cancer (Pereira et al. 2000). It can be preceded by a longer period of occult bleeding and by the time bleeding becomes visible it has a poor prognosis. Upper gastrointestinal bleeding is one of the most common complications in the metastatic spread of hepatocellular carcinoma. Most tumors that cause severe upper gastrointestinal bleeding are of a malignant histologic type (Savides et al. 1996). Extraluminal bleeding to the abdominal cavity occurs less frequently and results in haemoperitoneum or acute peritonitis that may require urgent treatment. Hepatitis C virus-related hepatocellular carcinoma causes spontaneous ruptures also in the peripancreatic lymph nodes (Terada et al. 2003). Spontaneous rupture of hepatocellular carcinoma was also reported by others (Chou et al. 2002; Inoue et al. 1992). As a consequence of variceal ruptures copious amount of ascites fluid is likely to follow. Clinically relevant, current developments on hepatocellular carcinoma and its highly complex metastasis involving the generation of new blood and lymphatic vessels, growth, transport to other sites the molecular mechanism of liver cancer metastasis have been recently described (Balmer and Dufour 2012).

Despite a number of earlier ultrastructural observations on lymphatics (Fraley and Weiss 1961; Casley-Smith and Florey 1961) a consistent interpretation on how the lymphatic system is promoting metastasis has not been clarified. An important observation was made upon interstitial injection of tracer substances, among them colloidal carbon particles and latex spheres. Immediately after their injection, endothelial cells separated and passageways were provided between the interstitium and lymphatic lumen. All tracer particles accumulated within autophagic-like vacuoles (Leak 1971). The importance of lymphoid system in neoplastic spread has

been underestimated probably because the lymph node metastasis in itself is rarely life-threatening. Tumor cells are supposed to interact with the lymphatic vasculature in a number of ways such as vessel co-option, chemotactic migration, invasion into lymphatic vessels, and induction of lymphangiogenesis. Although, the blockade of tumor-induced lymphangiogenesis inhibits metastasis formation of certain lymph nodes and related organs, but lymph node metastases act only as indicators and do not govern the further spread of metastatic cells (Sleeman and Thiele 2009). The observation that after the blockade of lymphangiogenesis the spread of tumor cells is channeled to other the lymphatic vessels rather than to blood vessels has been explained by the high permeability of lymphatic capillaries (Leak 1971). Another reason that lymphatic spread is more likely than vascular uptake of tumor cells could be that lymphatic vessels have a mean capillary diameter of 56.3 ± 9.0 μm and can be further dilated upon ectasia to nearly 100 μm (Pfister et al. 1990) *versus* the 5–10 μm diameter of arterioles and venules. Intratumoral lymphatic vessels are considered to be rare (Weiss 2000) and nonfunctional due to the mechanical compression (Leu et al. 2000). Remote induction of intranodal lymphangiogenesis by prolymphangiogenic factors and receptors as well as by tumor derived growth factors (VEGF-A, VEGF-C, VEGFR-2, VEGFR-3) is supposed to promote the lymph flow from primary tumors to lymph nodes and fostering the formation of lymph node metastases (Harrell et al. 2007). Chemokines, particularly CCL-21 also promote the entry of tumor cells into the lymphatics (Shields et al. 2007). Immune suppression in regional lymph nodes was evidenced by tumor-induced reduction of dendritic, $CD4^+$, $CD8^+$ T-cells (Huang et al. 2000; Cochran et al. 2006), by increased levels of immune suppressive cytokines in sentinel lymph nodes, the relevance of which remains unclear for lymph node metastasis (Kohrt et al. 2005). In the hypothetical model of lymphangiogenesis tumor-induced pro-lymphangiogenic factors have both local and systemic effects, by generating lymphangiogenesis locally, promote metastasis to lymph nodes and in distant vital organs (Sleeman and Thiele 2009).

5.3.1.8 Transdiaphragmatic Traffic of Metastatic Cells

The movement of tumor cells broken away from the primary tumor through its disruptions is supposed to be similar to the direct transdiaphragmatic traffic of peritoneal macrophages to thoracal lymph nodes (Pitt and Anderson 1988). These channels drain into larger intrathoracic lymphatics that enter anterior mammary and parathymic lymph nodes before emptying into the bloodstream (Tilney 1971; Tsilibary and Wissig 1983). Particulates injected into the peritoneal cavity of hamsters are most likely translocated principally *via* the transdiaphragmatic lymphatic channels situated under the diaphragmatic mesothelium (Fritz and Waag 1999). It was reported that during acute gastrointestinal inflammation of rats peripheral exudate cells migrated from the peritoneal cavity through the diaphragm into the mediastinal lymphatics *en route* to the parathymic lymph nodes (Steer and Foot 1987).

The lymphatic drainage from the peritoneal to the thoracal cavity has been investigated in mice by intraperitoneal inoculation of an intracellular bacterium (*Listeria monocytogenes*) and an inert marker (India ink). Both agents were transported, either after phagocytosis by intraperitoneal macrophages or in suspension in the lymph, towards the cranial sternal lymph nodes (*Lymphonodi sternales craniales*) of the ventral thoracic lymphocentrum (*Lymphocentrum thoracicum ventrale*) and to the lymph nodes of the mediastinal lymphocentrum (*Lymphocentrum mediastinale*), prior to systemic dissemination (Marco et al. 1992). These authors were the first to mention that peritoneal lymph drainage could have relevance on experimental studies, particularly on the investigation of metastatic diffusion of neoplasms from the peritoneum (Marco et al. 1992). When aluminum phosphate–adsorbed tetanus toxoid was injected into the peritoneal cavity of young adult mice it was drained predominantly *via* the PTNs. These intraperitoneally injected small particles translocated into PTNs and surrounding lymphatics, and into the cortical parenchyma of the thymus (Mueller et al. 1987). It is repeatedly mentioned that murine parathymic lymph nodes cannot be distinguished from the thymus. PTNs have been identified in several species, but have not been characterized in humans (Tanegashima et al. 1999). It is assumed that a similar transdiaphragmatic movement of particulates can take place between the retroperitoneum and the thoracic lymph nodes. Subcapsular implantation of India ink and the appearance of dye molecules 6 h later in the PTNs of rats are demonstrated in Fig. 5.10.

We have confirmed the movement of retroperitoneal particles to parathymic lymph nodes not only by the implantation of Indian ink under the renal capsule (Fig. 5.10), but also by the injection of India ink directly into the peritoneum of rats. Both administrations resulted in the migration of ink particles to the parathymic lymph nodes. The metastatic spread of tumor cells was mimicked by the administration of India ink injected into the rats either *i.v*. or *i.p.* Only the intraperitoneally injected India ink accumulated in the parathymic lymph nodes (Fig. 5.11). The *i.v.* injected colloidal dye was extracted primarily by the liver. The accumulation of *i.v.* administered colloidal particles is described in the Sect. 5.5.1 "Spread of particles after *i.v.* administration". The experiments with India ink administered *i.p*. confirmed the notion that the colloidal particles migrated from peritoneal cavity to parathymical lymph nodes and suggest similar transdiaphragmatic traffic of tumor cells from the peritoneal cavity to PTNs.

5.3.1.9 Spread of Cancer Cells to Thoracal Lymph Nodes

The architecture of lymphatic vessels has been evolved and determined by its functional need during evolution. That the efficiency of the function depends on the architecture of the existing lymphatic systems was demonstrated by comparing the thoracal lymph nodes of marsupials and mammals (Fig. 2.5). Differences between rodent (e.g. rat and murine) lymphatic systems have already been mentioned. Even in closely related mammals (cat, dog) no relationship could be demonstrated between the number and size of lymph nodes (Patsikas and Dessiris 1996).

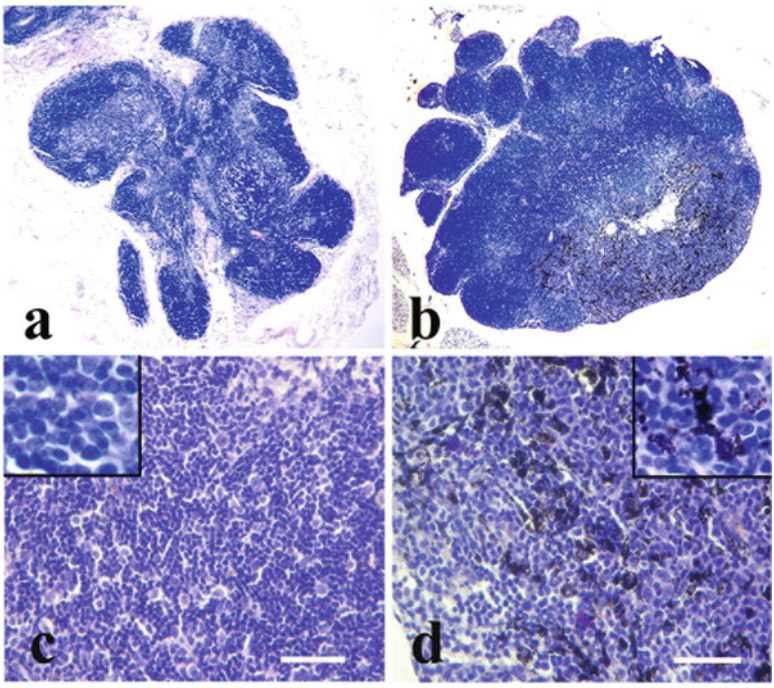

Fig. 5.10 Spread of India ink to parathymic lymph nodes upon implantation under the kidney capsule of rat. In control animals the administration of Pelikan ink was omitted. (**a**) Control parathymic lymph node at lower (4x) magnification after hematoxylin staining. (**b**) India ink was placed under the kidney's capsule. The appearance of India ink traced 6 h after administration in the cortical region of the left parathymic lymph node at low (4x) magnification. Ink particles occupy large areas of parathymic lymph nodes into which neutrophils and other inflammatory cells have infiltrated. (**c**) Control parathymic lymph nodes at higher (20x) magnification. (**d**) Pelikan ink-packed macrophages in parathymic lymph nodes at higher (20x) magnification. Bars, 50 μm each

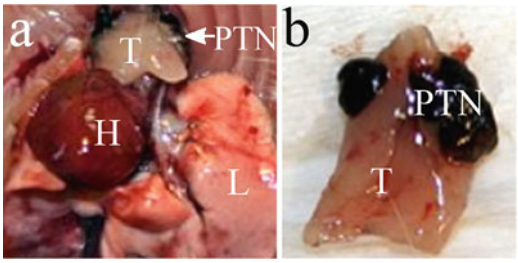

Fig. 5.11 Accumulation of India ink in parathymic lymph nodes upon *i.p.* injection into the peritoneal cavity. India ink (0.5 ml) (Gunther Wagner, Pelikan Werke, Hannover) was injected *i.p.* into rats. (**a**) Absence of India ink in heart (*H*), liver (*L*) and thymus (*T*) and its presence in parathymic lymph nodes (*PTN*). (**b**) Removal of thymus and PTN showing the presence of India ink in parathymic lymph nodes and its absence in thymus (With permission Kahlik 2013)

Variability is signified by hemolymph nodes in ruminants, not found in man, but present in some mammals. Hemal nodes represent an intermediate stage between lymph node and the spleen. In man, the spleen is a large hemal node containing lymph nodes with sinuses filled with blood rather than lymph.

5.4 Role of Parathymic Lymph Nodes in Metastasis

5.4.1 Mediastinal Tumors

Tumors in the anterior portion of the mediastinum include thymoma, lymphoma, pheochromocytoma and germ cell tumors including teratoma. Tumors in this area are more likely to be malignant than those in other compartments (Macchiarini and Ostertag 2004; Davis et al. 1987), and these tumors are probably in the first line of metastatic tumor development. The role of regional lymph nodes in cancer metastasis at the early phase of tumor growth is indicated by the induction of T cells in regional lymph nodes. When a small number of tumor cells enters a regional lymph node, it operates as a temporary barrier against tumor growth. The suppressor activity induced by the tumor cells in the regional lymph node will facilitate tumor development and lead to lymphatic metastasis.

5.4.1.1 Neoplastic Diseases of the Thymus

Needle biopsy specimens taken from the anterior mediastinum is predominantly centred on the thymus encompassing a whole range of tumorous diseases including hyperplastic conditions, benign and malignant neoplasms. In addition, other neoplastic diseases typical for this location may be encountered such as extragonadal germ cell tumors, lymphomas, soft tissue tumors and conditions that simulate tumor development, cyst formation and inflammatory conditions (Den Bakker and Oosterhuis 2009). Lymphoma represents a common malignancy to selectively target this nodal chain, which usually results in the contiguous spread from the anterior mediastinal or paratracheal area to other mediastinal lymph node groups (Sachithanandan et al. 2002). The selectivity raises the question: Is it the altered thymus development that is responsible for these neoplastic changes, or could be parathymic lymph nodes involved? The question was answered by studies indicating that cytotoxic T lymphocyte antigen 4 (CTLA-4, also known as CD152), a protein that interacts with B7 ligands and transduces inhibitory signals to T7 lymphocytes, plays an important regulatory role in the primary immune response. Mice homozygous for null mutation in CTLA-4 suffered from lymphoproliferative disorder, under normal thymocyte development, leading to the conclusion that CTLA-4 is primarily involved in the regulation of peripheral T cell activation. The

apparent alteration in thymocytes and the abnormal T cell expansion in CTLA-4 deficient mice were not due to altered thymocyte development, rather to the enlargement of parathymic lymph nodes (PTNs) (Chambers et al. 1997).

5.4.1.2 Characterization of Parathymic Lymph Nodes

Parathymic lymph nodes lie in close proximity and are related to the thymus. The common arrangement is two to three nodes situated dorso-laterally to each lobe, but in some animals variations from one to four nodes occur. The lymph nodes of rats observed in the thoracic cavity are parathymic and posterior mediastinal lymph nodes. The common arrangement of murine parathymic lymph nodes was reported to be two to three nodes located dorso-laterally to each lobe of the thymus (Blau and Gaugas 1968). The number of parathymic lymph nodes varied between individual rats, but was regularly between three to four lymph nodes on each side (Takahashi and Patrick 1987). In mice, the parathymic lymph nodes weighing one-tenth to one-twentieth of the thymus are found inside the capsule, whereas in rats PTNs lie on the thymus capsule (Blau and Gaugas 1968). Tilney has mapped the lymphatic system of rats in detail and has described that the lymph to the parathymic lymph nodes comes from the peritoneal cavity, liver, pericardium and from the thymus and then pours into the mediastinal lymph trunk that enters the subclavian vein (Tilney 1971). Others reported three to four small murine nodes lying immediately behind the thymus that can be removed with the thymus (Dunn 1954). Due to the poor visibility of the parathymic lymph nodes, reports of phenotypic alterations in the murine thymus (Waterhouse et al. 1995; Tivol et al. 1995) could be explained by the inclusion or exclusion of parathymic lymph nodes and could have obscured the picture. To avoid such ambiguities it was advised to use rats to study parathymic lymph nodes that are positioned outside the thymus (Takashi et al. 2007). The presence of thymic lymphatics and thymus-specific lymph nodes has been reported in other animals, namely, in sheep (Yamashita et al. 1985) and in guinea pigs (Harris and Templeton 1966). Parathymic lymph nodes as potential metastatic sites of tumor progression have been neglected. Human parathymic lymph nodes have been reported only in two studies (Severeanu 1909; Tanegashima et al. 1999) probably because they are covered by the thymic capsule and indistinguishable from the thymus. Consequently, studies related to thymic tumors should be reinvestigated as some of them could be parathymic tumors.

Parathymic lymph nodes are known to be the major sites of antibody-producing cells containing as many antibody-forming cells as does the entire spleen and are considered the last defense line against antigen invasion. When the capacity of PTNs is exhausted and the lymph entered the major lymph vessel (*ductus thoracicus*) that pours into the blood circulation, the organism became defenseless. The vascular epithelial cells are normally resistant toward tumor invasion, consequently the tumor cells flow back to the primary tumor. Fatal spontaneous ruptures of hepatocellular carcinoma with massive hemorrhages in the primary tumor are known

to be followed by the development of metastases in the lung, pleura, spleen and peritoneum. Based on our data we hypothesize that parathymic lymph nodes are the primary sites of metastatic invasion of cancer cells that come from the abdominal cavity and spread to the thorax after crossing the diaphragm.

5.5 Importance of Size Distribution of Tumor Cells in Metastasis

5.5.1 Spread of Particles After i.v. Administration

As already noted, the vast majority (98 %) of *i.v.* administered colloidal particles in rats is extracted by the Kupffer cells of the reticuloendothelial system (RES), 1.5 % by the spleen and 0.2 % by the lung (Banfalvi et al. 1972). These early experiments indicated that the primary sites of extraction of colloidal particles from the circulation are not the primary lymphoid organs (bone marrow, thymus), but the secondary lymphoid organs.

There is a notable discrepancy between the size of nanoparticles (1–100 nm) and colloidal particles (1 nm–10 μm). Nano- and colloidal particles are recognized by the body as foreign.

Nanoparticles:

– viruses (HIV, ∼0.1 μm).

Colloidal particles:

– bacteria (few μm),
– yeast cells (baker's yeast 5–10 × 1–7 μm),
– tumor cells (small cancer cells 10–30 μm in diameter bearing higher metastatic potential), cell remnants,
– carbon particles (India ink, average 10 μm),
– fibrous crystals of asbestos (3–20 μm).

Nano- and colloidal particles are attacked by:

– granulocytes,
– monocytes (macrophages, dendritic cells),
– lymphocytes.

Microvessels, the smallest capillaries of the microcirculation that connect arterioles and venules are 5–10 μm in diameter. Particles larger than red blood cells (6–8 μm) injected intravenously may cause microembolization primarily in the lung. Microembolization may also occur when disease or injury to the sinus node is accompanied by thrombus formation in the human heart as a clinically important source for microembolization to lungs (James 1983). Coronary microembolization

was provoked with 25 μm microspheres in pigs (Arras et al. 1998). ^{113m}In–Fe(OH)$_3$ macroaggregates (^{113m}In–Fe-MAA) injected *i.v.* in rats generated microembolization in the lung of rats (Banfalvi and Csernay 1975). The heterogeneity of ^{113m}In–Fe-MAA containing larger than 50 μm particles made these preparations unsuitable for lung scintigraphy when injected *i.v.* to rats, causing temporary occlusions not only in the vessels of lung but also in other vital organs such as the heart. Although, the size of macroaggregated particles could be reduced to some extent by mechanical stirring, but it did not go under the safe 50 μm particle size for lung scintigraphy. To use microembolization for tumor metastasis in the lung, intravenously administered tumor cells should be larger than colloids (>10 μm), without exceeding the safe upper size limit of macroaggregates (<50 μm) (Banfalvi and Csernay 1975). In agreement with these findings hematogeneous tumor cells in clumps produced a significantly greater number of metastatic foci than did a similar number of single tumor cells; larger clumps produced significantly more metastatic foci than smaller clumps matched for the number of cells (Liotta et al. 1976). Based on these observations one can conclude that for metastatic studies, the administration of tumor cells locally or to the circulatory system should be avoided as it remains unpredictable where the tumor cells will adhere.

5.5.2 Spread of Colloidal Particles from the Peritoneal Cavity

When the spread of intraperitoneal colloidal particles is chosen to follow metastasis it should be borne in mind that this transport is dependent on particle size and the particles can be taken up directly by the afferent lymph nodes or the particles can be carried by peritoneal macrophages (Marco et al. 1992). The lymphatic drainage of Evans blue dye complex from the peritoneal cavity has demonstrated the existence of anatomically distinct routes including: 1) transdiaphragmatic passage to sternal lymph nodes, 2) transport to cranial sternal lymph nodes, 3) transport to the caudal mediastinal lymph node, 4) uptake by the visceral lymphatics and 5) transport to the thoracic duct by efferent visceral lymphatics (Abernethy et al. 1991). Traumatic reticulo-peritonitis in buffaloes caused by the contiguous spread of bacteria resulting in mastitis (Morcos et al. 1977) indicated that colloidal particles from the peritoneal cavity could have moved to mammary lymph nodes. Moreover, the pathogenesis of malignant ascites formation may take additional nonlymphatic routes after the lymphatic pathway has been shut off (Nagy 1992).

To investigate the lymphatic drainage pattern from the peritoneal cavity, India ink colloidal particles (Rotring ART 5912) were injected intraperitoneally to rats. After 1 week the colloidal carbon deposited mainly in the parathymic lymph nodes and a smaller amount was collected in the left posterior mediastinal lymph nodes (Takahashi and Patrick 1987). Another study reported similarly to our observations that all the colloidal carbon injected intraperitoneally drained to the thorax and was extracted exclusively by the parathymic lymph nodes (Tilney 1971). That

parathymic lymph nodes represent the draining nodes for the peritoneal cavity (Finger et al. 1977) was confirmed by others who also injected *i.p.* into rats an empirically selected dose of 0.1 ml of a 1:2 dilution of Pelikan ink (Pitt and Anderson 1988). These authors found that peritoneal macrophages laden with carbon particles passed across the diaphragm deposited the colloidal carbon particles in the parathymic lymph nodes. The lung was not the predominant route of clearance of the peritoneal lymph (Pitt and Anderson 1988). As thymus excludes *versus* parathymic lymph nodes that take up the ink (Chambers et al. 1997; Mishell and Shiigii 1980), the best way to distinguish the thymus from PTNs in rodents, is to inject India ink intraperitoneally and then follow its accumulation in PTNs.

5.5.2.1 Metastatic Spread to Parathymic Lymph Nodes

The rat kidney capsule-PTN complex traces tumor progression directed towards potential thoracal metastatic sites. In conformity with the multistep nature and complexity of metastatogenesis none of the tumor models can imitate perfectly tumor progression or provide a common pathway. The kidney capsule-PTN model gives a reasonable explanation for at least the peritoneal, retroperitoneal and thoracal metastatic tumor development (Fig. 5.12). The major steps involved in this metastatic cascade upon syngenic heterotopic implantation of hepatocarcinoma and syngenic ectopic implantation of nephroblastoma cells under the capsule of rat kidney are summarized in six consecutive steps (Figs. 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 5.10, and 5.11) involving: (a) primary kidney or liver tumor formation upon chemical carcinogenesis, (b) peripheral angiogenesis in primary tumor, (c) necrosis inside the primary tumor, (d) appearance of blood and tumor cells in the interstitial space, (e) peripheral disruptions in the primary tumor, escape of tumor and blood cells, (f) lymphatic spread of tumor cells from the abdominal cavity to parathymic lymph nodes (metastasis). From the steps of this model the potential mechanism of metastatic tumor spread has been deduced (Fig. 5.12):

- After tumor cell (hepatocarcinoma or nephroblastoma) implantation primary tumor developed under the kidney capsule (Fig. 5.12a).
- Neovascularization takes place at the outer part of the tumor, the inner part becomes necrotized. Due to the delayed angiogenesis blood and tumor cells migrate to the interstitial space and leave the primary tumor through the tissue disruptions (Fig. 5.12b).
- Tumor cells appear in the abdominal cavity, cross the diaphragm through the lymphatic capillaries and enter the thoracal lymph nodes, primarily the parathymic lymph nodes, which are regarded as the sentinel lymph nodes, the fist sites of metastasis (Fig. 5.12c). Parathymic lymph nodes are among the smallest organs and accumulate tumor cells originating from the abdominal cavity and defend the organism from tumor cell invasion.
- When the capacity of the sentinel lymph nodes has been exhausted tumor cells move through the disruptions of the secondary PTN tumors to neighboring

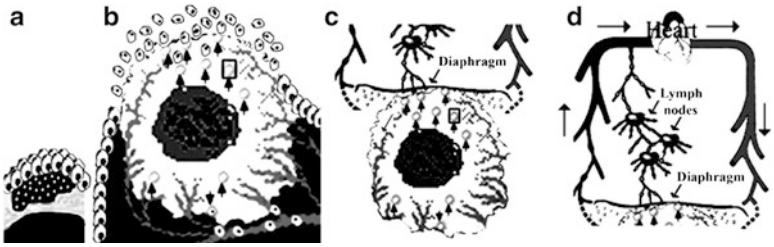

Fig. 5.12 Schematic view of metastatic spread of tumor cells from the primary tumor of kidney to thoracal lymph nodes. (**a**) Primary malignant neoplasm growing under the kidney capsule. (**b**) Lagging vascularization and necrosis inside the tumor release tumor cells through the disruptions indicated by the *black arrows*. Tumor cells enter abdominal cavity. (**c**) Transdiaphragmatic passage. Tumor cells enter thoracal lymph nodes, primarily parathymic lymph nodes. (**d**) Tumor cells reach blood circulation *via ductus thoracicus*, circulating tumor cells return and aggravate primary tumor formation. *DP* diaphragm, *PTN* parathymic lymph node

thoracal tissues and lymphatic vessels. Tertiary metastatic cells continue their migration through the thoracal lymph node chain, enter the *ductus thoracicus* that charges its content into the blood circulation *via vena cava inferior* (Fig. 5.12d).

5.5.2.2 Intravasation *Versus* Extravasation

As angiogenesis does not provide sufficient blood supply to the growing primary tumor, hypoxia develops inside the tumor causing necrosis. This observation suggests that the primary tumor grows significantly slower inside the host organ than outward favoring the idea of lymphatic extravasation rather than intravasation and vascular spread inside the primary tumor. The lymphatic interstitial → peritoneal → thoracal route of tumor spread is probably faster than the vascular spread that is delayed by the lagging angiogenesis, necrosis and the tissue disruptions in the primary tumors. The recognition of this type of lymphatic spread could open a new metastatic route and reduce the multitude of hypothetical alternatives of tumor spread.

Advantages of the renal-capsule parathymic metastatic model:

1. Tumor cell lines established from primary rat tumors allow the implantation of exact number of tumor cells under the kidney or liver capsules.
2. The temporal aspects of primary tumor growth and the secondary tumor spread (metastasis) can be followed more reliably than implanting tumor slices or injecting tumor cell suspensions.
3. Delayed and disrupted angiogenesis at the external part of the primary tumor, necrosis inside the tumor, combined with the lack or reduced venal back flow and elevated blood pressure are driving the blood and tumor cells to the interstitial fluid (Trencsenyi et al. 2010).

4. High glucose consumption in primary tumors can be evidenced by ^{18}FDG uptake generating excess acetyl-CoA. Under hypoxic conditions acetyl-CoA is unable to enter mitochondrial oxidative processes, including citric acid cycle, terminal oxidation and oxidative phosphorylation. Excess acetyl-CoA favors the lipogenic pathway manifested by lipid droplet formation at the frontline between tumor invasion and healthy liver tissue conforming to other observations (Banfalvi 2012a, b; Rozsa et al. 2009).
5. The inner part of the primary tumor is necrotized, the outer part is disrupted, blood and tumor cells filtrate from the primary tumor to the neighboring tissues and cavities (Trencsenyi et al. 2010).
6. Blood cells along with tumor cells emerging from the disruptions of the primary tumor are drained to the thoracal lymph nodes (Banfalvi 2012a, b), with parathymic lymph nodes being the metastatic sentinel lymph nodes (Trencsenyi et al. 2009, 2010; Rozsa et al. 2009).
7. Parathymic lymph nodes as sentinel lymph nodes of the metastatic process behave similarly to the primary tumors. Once the immunogenic capacity of PTNs is exhausted, and the metastatic tumor growth becomes faster at the peripheral parts than inside the lymph nodes, the PTNs are also subjected to hypoxic angiogenesis and tissue disruptions. The presence of lymph node metastases signifies further metastatic spread and poor patient survival (Moore et al. 1998). Unfortunately, the retention of tumor containing macrophages in the thoracal lymph nodes, particularly in parathymical lymph nodes has not been investigated. Cells contained in the efferent lymph are predominantly lymphocytes with a smaller population of macrophages (Morris and Courtice 1977). Tumor cells leaving the PTNs can invade the lower cavity of the thorax, spread to other tissues and lymph nodes and enter the blood transport elsewhere. An expected outcome of this process could be the redistribution of tumor cells to other thoracal lymph nodes and body parts (Kilburn 1984).
8. The metastatic tumor model supports the notion that similarly to bacteria, cancer cells cooperatively develop complex communities (primary tumors) with cell differentiation and distribution of tasks (Heppner 1993; Deisboeck and Couzin 2009). When injected systemically, bacteria specifically accumulate in tumours and migrate to distal regions far from the vasculature. These distal regions are typically hypoxic and hypoglycaemic and contain quiescent and necrotic cells (Forbes 2010).
9. The rational behind choosing rat rather than murine tumor model is the poor visibility of the 3–4 small murine parathymic lymph nodes lying inside the murine thymus capsule. Murine PTNs similarly to human parathymic lymph nodes, can be removed only with the thymus (Dunn 1954), unlike the separable rat PTNs positioned outside the thymus (Blau and Gaugas 1968) and their role in the metastatic process can be studied independently of the thymus.

5.5.3 *Major Steps from Carcinogenesis to Metastasis*

Hepatocarcinoma (HeDe) and nephroblastoma (NeDe) cancer growth and spread can be summarized as:

1. First tumor cells are generated by dimethylnitrosamine carcinogenesis
2. Local tumor formation at the site of tumor cell implantation (kidney, liver)
3. Local invasion damaging basement membrane
4. (a) Tumor angiogenesis
   (b) Lymphangiogenesis
   (c) Primary tumor expansion:
   - extravasation outward
   - necrosis inside
5. Disruption of primary tumor:
   - local invasion of surrounding tissues
   - travel to regional lymph nodes
   - local invasion inside tumor
6. Metastasis in sentinel lymph nodes (PTNs, exhausting their defense capacity)
7. Distant metastasis to other organs: micrometastasis → macrometastasis → organ failure.

These steps correspond to most of those shown in the map on "How cancer grows and spreads" presented by the Children's Hospital Boston in 2007. http://www.childrenshospital.org/research/_cancer/

5.6 Metastasis in Different Organs

The alphabetic (A to Z) list of cancers containing the incidence and mortality statistics of the USA is regularly reported (American Cancer Society 2013). These reports contain detailed information not only on the types of cancers, but also about treatment, causes, prevention, genetics, screening and testing, clinical trials and cancer literature, including National Cancer Institute-supported research and special reports. In Table 5.5 only those cancer types are mentioned based on their body location and incidence that metastasize and are regarded as major death causing cancers (Jemal et al. 2008) (Table 5.5).

Attention is called to the fact that with the notable exception of the lung all other primary tumors can give metastasis to the liver underlying the importance of the abdominal cavity in the progression of cancer and metastasis. Disruptions of liver and kidney tumors project tumor cells into the peritoneum that are taken up by thoracal lymph nodes. Next sections will deal only with major organs that either generate metastatic cancer cells or obtain cancer cells from other organs. The most important metastatogenic organs are lung, breast, liver, brain and kidney.

Table 5.5 Major metastatic sites in the human organism

Cancer type	Site of metastasis	References
Major types		
Lung	Adrenal glands, liver, bone, brain	Hirano et al. (2005)
Breast	Bone, lung, liver, brain	Lacroix (2006)
Colorectal	Liver, lung, peritoneum	Roth et al. (2009)
Kidney	Liver, lung, bone, brain	Campbell et al. (1968) and Petraki et al. (2003)
Pancreas	Liver, lung, peritoneum	Campen et al. (2011) and Yachida and Iacobuzio-Donahue (2009)
Melanoma	Lung, brain, liver, skin/muscle	Fidler (1995)
Ovary	Liver, lung, peritoneum	Woodward et al. (2004)
Prostate	Bone, lymph nodes, liver, lung	Bubendorf et al. (2000)
Minor types		
Bladder	Bone, liver, lung	Shinagare et al. (2011)
Stomach	Liver, peritoneum, lymph nodes	American Cancer Society (2013)
Thyroid	Bone, liver, lung	Hindie et al. (2007) and Schlumberger et al. (1988)
Uterus	Vagina, bone, liver, lung, peritoneum	Malek et al. (2012)

5.6.1 Lung Cancer

Based on GLOBOCAN estimates for 2008, lung cancer was and remained the leading cancer site in males, comprising 17 % of the total new cancer cases and 23 % of the total cancer deaths. GLOBOCAN managed by the Section of Cancer Information (CIN) of International Agency for Research for Cancer (IARC) contains information on the occurrence of cancer worldwide. The two main types of lung cancer are small-cell and non-small-cell lung carcinomas. Although, the frequency of non-small cell lung carcinoma is higher (∼80 %) than that of small cell lung carcinoma (∼17 %), it is less deadly and can be removed surgically, while small cell lung carcinoma responds only to chemotherapy and radiation (Vaporciyan et al. 2000). Lung cancer is the second most frequently occurring malignancy and the leading cause of cancer death worldwide. The mortality rate for lung cancers rose steadily through the 1980s and peaked in the early 1990s and then started to decrease gradually. As the prevalence of smoking peaked later in women than in men, the incidence and mortality rates has only recently begun to drop for women.

In terms of incidence and mortality, lung cancer has the highest rates in Europe (especially Eastern Europe) and North America. Highest lung cancer mortality rate was reported among Eastern European men, and significantly lower but consistently high rates among northern European and US women (Ferlay et al. 2010). The lifetime risk to develop lung cancer in the USA for men was estimated to be 8 % and for women 6 % (Horn et al. 2012).

5.6.1.1 Leading Causes of Lung Cancer

Most of the lung cancers are caused by long-term tobacco smoking (Merck Manual 2007; Trivedi et al. 2010) responsible for 80–90 % of lung cancers (Horn et al. 2012). Nonsmokers account for 10–15 % of lung cancer cases (Thun et al. 2008). Smoking (cigarettes, cigars, pipes, passive smoking) is the major risk factor counting for ∼90 % of lung cancer incidence (Biesalski et al. 1998). Lung cancer was uncommon before cigarette smoking, not even recognized as a distinct disease (Morgagni 1761). The incidence of lung cancer had increased from an estimated 0.3 % in 1852 to 8 % in 2012 in men. In developed countries 90 % of lung cancer deaths in men and 70 % of women were attributed to smoking (Peto et al. 1996). Other cases of lung cancer are attributed to genetic factors (Gorlova et al. 2007; Hackshaw et al. 1997; Alberg and Samet 2010), activation of proto-oncogenes primarily K-ras to oncogenes (Salgia and Skarin 1998; Herbst et al. 2008) and inactivation of tumor suppressor genes (Fong et al. 2003), particularly p53 tumor suppressor gene (Devereux et al. 1996), radon gas (Catelinois et al. 2006; Alberg and Samet 2010), asbestos (O'Reilly et al. 2007), air pollution (Kabir et al. 2007; Coyle et al. 2006; Chiu et al. 2006; Alberg and Samet 2010), and inhalation of secondhand smoke (Carmona 2006).

5.6.1.2 Diagnosis and Treatment of Lung Cancer

The risk of lung cancer can be reduced by eliminating or reducing exposure to any of the risk factors. Among the several relevant activities, the National Cancer Institute is running the Cancer Genome Atlas (TCGA) project that is systematically identifying the major genomic changes involved in more than 20 cancers. TCGA researchers use state-of-the-art genomic analysis technologies to identify genomic changes that divide lung cancer into two molecular subgroups and distinguish between lung squamous cell carcinoma and adenocarcinoma in smokers and nonsmokers. Standard treatments for lung cancer include surgery, radiation therapy, chemotherapy, targeted therapy, laser therapy, photodynamic therapy, cryosurgery, and electrocautery.

Since 1964, the Surgeon General's report in the USA has significantly changed the public attitude toward smoking. The most dramatic warning was given in 1982 when "The Surgeon General Has Determined That Cigarette Smoking Is Dangerous to Your Health."

5.6.2 *Breast Cancer*

Worldwide, breast cancer is the leading cause of cancer death among women. In spite of all efforts breast cancer it is still not curable for the vast majority of patients. In developed countries one-third of early stage breast cancer patients develop metastasis (O'Shaughnessy 2005). In the third world, where breast cancer is diagnosed in a later stage, the metastatic rate and the death toll are much higher (Anyanwu 2008).

Breast Cancer – GLOBOCAN 2008 Data
Breast cancer is by far the most frequent cancer among women. In 2008 an estimated 1.38 million new cases were registered representing 23 % of all cancers and ranked second overall with ~11 % of all cancers. Incidence rates varied from 19 per 100,000 women in Eastern Africa, 40 per 100,000 in Japan, 90 per 100,000 women in Western Europe and more than 80 per 100,000 in other developing regions. The average rate of mortality is between 6–19 deaths per 100,000 due to the higher survival in developed regions. Breast cancer ranked as the fifth cause of death from all cancer, but still the most frequent cause of death in women almost equal to the estimated number of death from lung cancer.
http://globocan.iarc.fr/factsheet.asp

5.6.2.1 Breast Cancer Theories

Recently a more detailed review on breast cancer has been compiled (Banfalvi 2012a). Here only a short description of breast cancer theories is given.

5.6.2.1.1 Humoral Theory of Breast Cancer

In the Sect. 5.1 devoted to the "Historical view of metastasis" it was mentioned that ancient Egyptian physicians Hippocrates and Galen thought that the "black bile" of breast cancer poisoned that whole body. Only palliative therapy of breast cancer was recommended as it could not be healed and in spite of surgical removal new tumors would be created.

5.6.2.1.2 Local Tumor Theory of Breast Cancer

After the discovery of lymphatic vessels (Loris 1970) and the recognition that *ductus thoracicus*, the major thoracic vessel charges its content into the blood circulation the humoral theory of breast cancer was challenged (James 2002). The

demonstration that the breast cancer did not contain bile and that the localized tumor could be surgically removed (Doe 1960) lead to "radical mastectomy" (Halsted 1894). Super-radical mastectomy with *en bloc* removal of the entire internal mammary lymph node chain (Urban and Baker 1952) served then as a model for the removal of other tumors.

5.6.2.1.3 Systemic Disease Theory of Breast Cancer

The regrowth of breast tumors even after radical mastectomy was accounted for by the secretory function of hormones. The treatment resulted in starving the tumors by the removal of ovaries, but brought only temporary relief to the patients (Stockwell 1983). Controversial results indicated that reproductive events and early age pregnancy and lactation reduced the risk of breast cancer (Chodosh et al. 1999; MacMahon et al. 1970; Layde et al. 1989) and that beside estrogen-like substances there are chemicals that evoke mammary carcinoma in rats (Huggins et al. 1961, 1970, 1981). The disturbing observation that breast cancers continued to kill even after extreme surgeries, disproved the local tumor theory (Crile 1967) and yielded in the spectral view of breast cancer (Travis 2005), also known as systemic disease that is not localized but can spread as metastasis.

5.6.2.1.4 Olygometastasis Theory of Breast Cancer

The breast cancer that was regarded first as a lymphoid disorder, then changed in the last century to a hematogen and recently to a lymphoid-hematogen disease brought further progression without offering a final solution. Among the causes of breast cancer are intrinsic (hereditary, dietary, hormonal) and extrinsic (chemical, radiation, viral, bacterial) factors. The oligometastasis theory postulates a limited number and sites of tumors with restricted tumor causing potential (Weichselbaum and Hellman 2011) implying that the survival and spread of tumor is not a random event, but a pre-existing metastatic subpopulation is responsible for the metastatic spread (Hart and Fidler 1980).

5.6.2.2 Metastatic View of Breast Cancer

The metastatic view of breast cancer theory assumes a continuous spread and recurrence of tumor cells several years later no matter how many tumor cells have been left after any radical therapy. It differs from the olygometastasis view by postulating a hidden, pre-existent tumor subpopulation that bursts into metastases. The metastatic theory brings us back to the original lymphatic view of Egyptians. The metastatic theory regards breast cancer as a metastasis, rather than a primary tumor. Breast cancer can metastasize to further tissues and organs (bone, lungs, regional lymph nodes, liver, brain, etc.). This does not mean that primary breast

tumors could not develop. There are at least a dozen of arguments that support the notion that tumor bearing regional lymph nodes among them mammary lymph nodes are not the primary sites of cancer, but can be the consequence of metastasis (Banfalvi 2012a):

1. The metastatic view is supported by the idea of Hippocrates and Bernard Fisher (Travis 2005) that cancer cells travel throughout the circulatory and lymphatic systems, consequently surgery alone would not cure breast cancer. Metastases are spread to distant locations (Chambers et al. 2002; Fidler 2003) occurring several years after drastic resection of the primary breast tumor.
2. The metastatic view is in conformity with the heterogeneity of the tumor cells of the breast cancer. Properties of metastatic breast cancer cells often differ from the primary breast cancer. The incidence and the type of tumors elicited are strongly influenced by host factors (Akewanlop et al. 2001).
3. All malignant tumors metastasize to a lesser or greater extent through the lymphatic system (Hsieh and Trichopoulos 1991) and explain the primary role of thoracal lymph nodes in preneoplastic processes.
4. Tumor cells of tissues heavily exposed to stress (environmental, chemical, genetic) grow under hypoxic conditions with limited hemangio- and lymphangiogenesis. Similarly to chemical carcinogenesis, exemplified by the nephroblastoma (NeDe) cell line, the arterial capillary vessels of the primary tumors are not connected to venal capillaries. The central part of the tumor becomes necrotic and as a result of the capillary pressure the peripheral tissue of the primary tumor is disrupted and induces metastasis (Wood et al. 2008).
5. Blind-ended angiogenesis in the primary tumor drives the blood and tumor cells to the interstitial fluid. Blood and tumor cells enter the lymphatic capillaries and are retained by the thoracal lymph nodes, known as the primary site of metastasis. Sentinel lymph nodes may have different locations (PTNs, mammary lymph nodes).
6. Liver plays a key role in metabolic processes, detoxification of foreign substances and metastatogenesis. In liver, the principal site of metabolism, cancer is caused by gastrointestinal and liver toxicants. Due to limited angiogenesis the outer part of the primary tumor is disrupted and blood cells along with tumor cells emerge in the peritoneal fluid of the abdominal cavity.
7. The drainage of tumor cells observed in isolated model systems has shown that tumor cells in the abdominal cavity pass the lymphatic vessels of the diaphragm, primarily through the parasternal lymphatic vessels.
8. After exhausting the defense capacity of the primary sites of metastasis (parathymic lymph nodes and/or internal mammary lymph nodes), cancer cells in the lymphatic trunks, flow to the vascular system through the main thoracal lymph vessel (*ductus thoracicus*) that discharges lymph into the subclavian vein at *angulus venosus*.
9. The tight junctions of the endothelial cells tile the walls of the closed vascular system and prevent the spread of circulating tumor cells. Under ischemic conditions local vascular development is hampered by delayed angio-, and

vasculogenesis. Through the openings of the fenestrated capillaries of sinusoid vessels (30–40 μm) blood and tumor cells pass the membrane. The lining wall of the lymphatic system is developmentally an offshoot of the venous system and is composed of elongated endothelial cells protecting from leakage. Exceptions are sinusoid blood vessels located in the bone marrow and lymph nodes that remain permeable. Professional phagocytes (neutrophils and monocytes) in the blood attack tumor cells. Antibody-induced phagocytosis of breast cancer cells is mediating specific T-cell activation (Suwatanapongched and Gierada 2006). After returning to the primary tumor, cancer cells reenter the lymphatic and vascular circulation and generate a vicious cycle (Fig. 5.12).

10. Cancer cells channeled to interconnected thoracal nodes could cause metastatic spread of cancer to mammary lymph nodes. Local injuries and damages in the vascular and lymphatic systems of the breast are potential sites of tumor cell infiltration to neighboring tissues. As an example bra cup size is mentioned, which is related to mammary gland size, but a risk factor for breast cancer when too tight (Vendrell-Torné et al. 1972). Nearly 75–85 % of women wear incorrect bra size (Johnson and Shah 2005) causing thoracic pain that can lead to vascular damages and to the establishment of primary breast tumors and metastases.
11. Colloidal particles (viruses, bacteria, cancer cells, India ink) released into the peritoneal cavity move to thoracal (parathymic and internal mammary) lymph nodes. Lymph nodes filled with lymphocytes act as filters and destroy bacteria, viruses and tumor cells. It is accepted but not completely understood how particle size, shape or tumor type can influence the deposition of cancer cells in thoracal lymph nodes. After the capacity of the inferior and superior mediastinal lymph node chains is exhausted tumor cells can spread to other lymph nodes. The lymphatic connection between the axillary and sternal lymph nodes of the Bitch (Patsikas and Dessiris 1996) indicates that similar connections may exist among other lymph nodes including mammary and thoracal glands.
12. Tumor staging determines how far the disease has progressed. Breast cancer stages (from 0 to IV) are based on several criteria including the size of the cancer, its invasive or non-invasive nature, whether the cancer is in local lymph nodes or spread to other parts of the body beyond the breast. Another system known as TNM takes into consideration tumor size (T), the involvement of nodes (N) and metastasis (M), with many subcategories of breast cancer indicating that the tumor cells are invading first a limited area, then gradually invading the healthy tissues inside the breast and gradually spreading to neighboring tissues (cancer) and to distant parts of the body (metastasis). The idea of progressive tumor spread implies that breast cancer can be a metastasis already at its initial stage of tumor growth and similar stages of metastatic development could have occurred in other tissues before and after the manisfestations of breast cancer.

Human breast metastases originate primarily from lymphomas, leukemias, gynecologic cancers, lung cancer, soft tissues and are more common in younger women

(Jardines et al. 2012). The connection between parathymic and mammary lymph nodes is not known, only suspected, but could explain the metastatic spread both to PTNs and mammary lymph nodes.

5.6.3 Liver Cancer

Types of gastrointestinal cancers include the upper and lower tracts: esophageal cancer, stomach cancer (gastric cancer), liver cancer (mainly hepatocellular carcinoma and hepatoma), gallbladder cancer, pancreatic cancer and colorectal cancer. Liver cancer being the most prevalent one, will be discussed separately.

Human liver is the largest parenchymal organ sitting in the upper right portion of the abdomen, above the stomach, beneath the diaphragm. Liver cancer is the third most common form of cancer, less common in developed than in undeveloped countries. Primary liver cancers that start to grow in the liver account for only a few percents in developed countries, but represent more than 50 % in some third world countries due to the high incidence of contagious viruses, primarily hepatitis B and C.

Benign liver tumors including hemangioma, hepatic adenoma, focal nodular hyperplasia, cysts, lipoma, fibroma, leiomyoma are similar to other benign tumors (discussed in Chap. 4). These tumors are not treated as liver cancer and if they are causing bleeding or pain can be removed surgically.

Liver cancers have two major types: hepatocellular carcinoma with its cholangiocarcinoma variant (cancer of the bile duct) and hemangioendothelioma arising from the blood vessels of the liver. The frequency of liver cancer does not mean that all liver cancers originate from the liver itself. Several other areas of the body, most often colon, lung and breast may project tumor cells to the liver causing metastatic liver cancer.

Liver Cancer
Quotation from GLOBOCAN 2008
Liver cancer is the fifth most common cancer in men (523 000 cases, 7.9 % of the total) and the seventh in women (226 000 cases, 6.5 % of the total), and most of the burden is in developing countries, where almost 85 % of the cases occur, and particularly in men: the overall sex ratio male: female is 2.4. The regions of high incidence are Eastern and South-Eastern Asia, Middle and Western Africa, but also Melanesia and Micronesia/Polynesia (particularly in men). Low rates are estimated in developed regions, with the exception of Southern Europe where the incidence in men (ASR 10.5 per 100,000) is significantly higher than in other developed regions. There were an estimated 694 000 deaths from liver cancer in 2008 (477 000 in men, 217

(continued)

(continued)
000 in women), and because of its high fatality (overall ratio of mortality to incidence of 0.93), liver cancer is the third most common cause of death from cancer worldwide. The geographical distribution of the mortality rates is similar to that observed for incidence.
http://globocan.iarc.fr/factsheet.asp

Liver metastasis should not be confused with liver cancer. Liver metastases originate from other organs and are named after the organ where primary tumor development began in the body and migrated to the liver. Metastases of the liver come most frequently from the gastrointestinal tract (colon cancer, carcinoid tumor of the appendix), but also from breast, lung, kidney, prostate, etc.

5.6.3.1 Major Causes of Liver Cancer

The major causes and risk factors of liver cancer (hepatocellular carcinoma) can be summarized as:

- Hepatitis C virus became the major cause of liver cancer not only in the United States (El-Serag and Mason 1999; Rosen 2011), but similar trends in the incidence of hepatocellular carcinoma have been reported worldwide (Saracci and Repetto 1980). The risk of most tumors is age-dependent and increasing with age. Hepatocellular carcinoma is an extremely age-dependent cancer, but inversely related to it (Kaczynski and Oden 1999). The age-specific rising of hepatocellular carcinoma has progressively shifted toward the younger generation (El-Serag and Mason 1999).
- Chronic hepatitis B infection. The most likely reason for the rising incidence of liver carcinoma is the spread of hepatitis virus infection in the population. Two viruses cause almost all hepatocellular carcinoma tumors: hepatitis B and hepatitis C viruses (Ince and Wands 1999). Those patients who have both hepatitis B and C infection and consume alcohol, have an increased risk of developing liver cancer compared to patients abstaining from alcohol consumption (Tagger et al. 1999). The spread of hepatocarcinoma is fueled by the risk of family members who have first-degree relatives suffering in hepatitis B infection (Yu et al. 2000).
- *Alcohol abuse.* Chronic alcohol consumption is associated with liver cancer causing cirrhosis. Autopsy is providing early evidence in at least half of the alcoholics to have unsuspected cancer in their liver. Often alcoholic cirrhosis is associated with chronic hepatitis C virus infection. Unexpectedly actively drinking alcoholics normally do not develop liver cancer, nevertheless die earlier than from non-cancer related liver diseases such as liver failure. It is more likely that in individuals who stopped drinking the liver starts to regenerate, but due to the high number of mutations liver cancer ensues.

- Aflatoxin has also emerged as a factor in the etiology of primary liver cancer in those areas of the world where this mycotoxin is present in ingested food (Blumberg et al. 1975; Alpert et al. 1971).
- Vinyl chloride. Workers who were exposed to vinyl chloride dust developed sarcomas in the liver, most commonly angiosarcomas. Other sarcomas (smooth muscular and vascular) were also observed. Other chemicals causing liver tumor are: herbicides, arsenic, thorotrast dye (thorium dioxide, ThO_2, used as a radiocontrast agent) smoking, hormones (androgens, estrogens), iron overload in liver (causing hemochromatosis).
- Obesity, fatty acid disease and birth defects are also risk factors implicated in the increased rate of nonalcoholic hepatocellular carcinoma (El-Serag 2011).

5.6.3.2 Diagnosis of Liver Cancer

Regular control of elevated blood level of alpha-fetoprotein and the combination of imaging techniques (ultrasound, CT, MRI scanning) contribute to early, liver biopsy to definitive diagnosis of liver cancer. As long as the liver tumor in a non-cirrhotic patient is smaller than 1 cm in diameter, the patient has more than 50 % chance of surviving at least 3 years without additional therapy. Many patients do not develop symptoms until advanced stages of liver cancer. By the time patients develop diagnosis the prognosis is usually poor and the majority of patients die within 1 year upon diagnosis of liver cancer. Patients with multicentric tumors and signs of liver failure (decompensated cirrhosis) are unlikely to survive more than a few months, even under treatment.

5.6.3.3 Medical Treatment of Liver Cancer

The best way would be to prevent this disease by social and lifestyle changes mentioned in the causes of liver tumor. The treatment depends largely on the stage and severity of the liver cancer. Ablative and local techniques (chemoembolization, radioembolization, radiofrequency cryoablation, stereotactic radiosurgery) help to control individual cancers for a prolonged period of time. Surgical removal may be sufficient for small and localized tumors that did not infiltrate into neighboring tissues. For patients with small tumors liver transplantation offers the best chance for cure. Medical treatment of liver tumor, primarily chemotherapy can be effective, but often disappointing carrying the risk of drug resistance. Combination of chemotherapy and radiotherapy are preferentially used.

5.6.3.4 Liver Metastasis

Almost any cancerous tumor can spread to the liver from somewhere else in the body. Major types of cancer that may spread to the liver include, but are not limited to: breast cancer, colorectal cancer, esophageal cancer, lung cancer,

melanoma, pancreatic cancer, stomach cancer. Colon carcinoma cells injected into the circulation of rats passed microvessels of all organs without size restriction, showed high adhesion potential within the liver and lung but rarely in other organs (Schlüter et al. 2006). The distribution of colon cells was explained by their size and shape. The invasive capacity of colon cells was strongly correlated with cells having a spindle cell shape (de Both et al. 1999). That the size matters in organ distribution has been shown by injecting *i.v.* into rats colloidal particles and macroaggregates accumulating in the liver and lung, respectively (Banfalvi and Csernay 1975).

Other organs (central nervous system, adrenal glands, spleen, skeleton, skin) together accounted for less than 10 % of the colorectal metastases (Hermanek et al. 1994; Haier 1997). Colorectal carcinomas are the most important malignancies of the gut and the third leading cause of cancer-related deaths among women and men (American Cancer Society 1997). Liver metastasis may also develop from an original liver or from other primary cancer that was diagnosed, occurred earlier and has been removed. The risk and growth of primary cancer spreading to the liver depends on the site of the original cancer. In several cases there are either no symptoms of metastasis or can be mistakenly general such as anorexia, confusion, fever, jaundice, nausea, upper right abdominal pain, sweats, weight loss, etc.

5.6.3.5 Diagnosis and Treatment of Liver Metastasis

Similarly to liver cancer, by the time proper diagnosis has been obtained from the liver function tests, liver biopsy, CT, PET, MRI and ultrasound scans the cancer could have invaded the liver at multifocal locations and to such extent, that treatment will be too late. When metastatic spread is limited to the liver, individual tumors can be removed surgically. Other treatments may imply whole-body (systemic) chemotherapy and/or radiation therapy, embolization of blood vessels to prevent blood supply and to starve the tumor to death. Although, occasionally the surgical removal of liver tumor may lead to cure, but the outlook of liver metastasis is generally bleak. Although, treatment may help to shrink, limit or at least curb metastatic growth, to relieve symptoms of complications, improve life expectancy, metastatic liver patients die of their disease.

5.6.4 Brain Cancer

The definitions of intracranial cancerous or non-cancerous neoplasms differ from those used in other types of malignant or benign neoplasms in the body. Unlike other tumors it is not only the type of the brain tumor, but there are other factors that pose potential threat, such as the location and the size of the tumor. Although, the brain is well protected by the skull, but this may be also a disadvantage from the point of view of diagnosis and the limited space of tumor development resulting in diagnosis of advanced stages causing unusual symptoms. Although, primary tumors

can develop anywhere in the brain, there is an age-specific distribution: in children tumors are formed primarily in the brainstem and cerebellum, while in adults it is the anterior part of the cerebral hemispheres that is more likely to be affected. Genetic analysis of high-grade tumors revealed a high frequency of aberrations affecting the RTK/RAS/PI3K signaling pathways, with RTK and EGFR being most commonly mutated, but mutations also frequently found in platelet derived growth factor alpha polypeptide (PDGFRA), v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2) and met proto-oncogene hepatocyte growth factor receptor (MET) (Cancer Genome Atlas Research Network 2008).

5.6.4.1 Diagnosis of Brain Cancer

1. Physical examination includes the assessment of symptoms that may suggest brain cancer personal and family health history and neurological examination. Neurological examinations consist of checking the patient's vision (following a moving finger), hearing, sensation, coordination (walk heel-to-toe), balance, strength, reflexes and ability to think and remember. Symptoms, family history combined with neurological examination may help not only to verify brain tumor, but also its likely localization in the brain.
2. Diagnostic test of brain cancer. The two most commonly used examinations to diagnose brain cancer are magnetic resonance imaging (MRI) and computed tomography (CT).
   (a) MRI scan. The machine of MRI consist of a strong magnet linked to a computer to create detailed pictures of areas of the brain occasionally after injecting a special contrast material into the blood vessel to highlight abnormal areas such as tumors in the brain. Functional MRI (fMRI) has been devised to determine the specific location of brain, where certain function (speech, motor) takes place.
   (b) CT scan. X-ray imaging is combined with a computer to generate pictures of the head. Special dye may also be used as contrast material to get a better resolution of abnormal areas of the brain.
3. Other tests. As there are several possibilities for brain tumor diagnosis, only the most important ones will be mentioned briefly:
   (a) Angiogram. Dye injected into the bloodstream makes blood vessels in the brain visible.
   (b) Spinal tap. Removal and laboratory check of a sample of cerebrospinal fluid.
   (c) Biopsy. Removal of brain tissue sample to look for tumor cells during brain surgery (open biopsy), performed as a separate procedure (needle biopsy), stereotactic biopsy (computer directed needle biopsy).
   (d) Diffusion tension imaging. Measuring the flow of physiological solution through the white matter tract of the brain to visualize its circularity.
   (e) Blood and urine tests to diagnose pituitary tumors.
   (f) Bone scan to diagnose skull base brain tumors.

5.6.4.2 Risk Factors of Brain Cancer

Studies have found relatively few risk factors for brain cancer:

- Family history. Brain cancers have a poor family history. It is rare that families have several members with brain tumors.
- Environmental factors. There are several potential environmental factors, among them vinyl chloride and other chemicals at work, ionizing radiation and exposure to magnetic fields are known as risk factors. Mobile phone radiation has been suspected to be a potential risk of brain tumors (Frei et al. 2011). This notion has been confirmed by the World Health Organization by classifying cell phone radiation on the IARC scale into Group 2B as a possibly carcinogenic risk factor. As consistent links between mobile phone radiation and brain tumors have not been proved, further studies are needed.

5.6.4.3 Brain Metastasis

Malignant brain tumors (brain cancers) could have started to grow as primary tumors or have spread to the brain as metastases from other parts of the body. Specific adhesion molecules, primarily neutrophins attach to microvessels of endothelial cells of the brain and help to invade the central nervous system (Nicolson et al. 1994). Additionally autocrine growth factors, paracrine growth factors and other factors contribute to the successful invasion, colonization and growth in the central nervous system (Menter et al. 1995). Metastatic brain tumors may originate from lung cancer, breast cancer, kidney cancer, melanoma and any other types of cancer spreading to the brain.

5.6.5 Kidney Cancer

In 2004 the incidence of renal cancer was estimated to be ~3 % in the United States (American Cancer Society 2004). In 2004 kidney cancers accounted for somewhat less than 2 % of all cancers worldwide (Lindblad 2004). The incidence globally of kidney cancer varies considerably among different populations and regions. The rates are highest in Western and Eastern Europe, North America, Australia and Scandinavia. Within Europe there is a considerably variation in incidence rates. The incidence of kidney cancer is intermediate in Southern Europe and Japan, and low elsewhere in Asia, Africa and the Pacific. The incidence of kidney cancer increased during the last decades in Norway and Finland with about 1–3 % per year, respectively. The greatest increase in incidence rates of kidney cancer was observed in some areas in Japan, Italy and Eastern part of Germany, for men with 7, 5 and 5 % per year, respectively. The corresponding increases for women were 4, 5 and 4 %, respectively (Lindblad 2004). The reason why more kidney cancers are detected is

not clear. One possible explanation could be that much more imaging techniques are being used than earlier, leading to the discovery of more kidney cancers.

Renal carcinoma is characterized by developing into organ specific metastasis. The chemokine stromal derived factor-1 and its receptor seem to be responsible for the regulation of organ specific regulation not only in kidney but also in other cancers. Chemokines are small (8–10 kD) proteins, that attract blood leukocytes to sites of infection and inflammation. Chemokines function as regulatory molecules in leukocyte maturation, homing of lymphocytes (Baggiolini 1998) which play a pivotal role in the regulation of leukocyte trafficking and extravasation into sites of tissue inflammation (Premack and Schall 1996; Zlotnik and Yoshie 2000). It is hypothesized that the interaction of the stromal derived factor with its receptor is the major mechanism of renal cell carcinoma (Pan et al. 2006). To examine intratumor heterogeneity, exome sequencing, chromosome-aberration analysis, and ploidy profiling on multiple spatially separated samples were performed in primary renal carcinomas and associated metastatic sites. The high degree of mutational diversity suggested that these mutations serve as substrate for Darwinian selection and evolutionary adaptation of tumor progression.

The two common types of kidney cancer are renal cell carcinoma (hypernephroma) localized in the renal tubules and urothelial carcinoma in the renal pelvis. The most common type of kidney cancer is renal cell carcinoma comprising >80 % of kidney cancers (Mulders et al. 2008), regularly confined to the renal parenchyma and commonly treated by partial or radical nephrectomy (Rini et al. 2008). Renal cell carcinoma develops within the microscopic filtering system of the kidney, in the lining of tiny tubes that lead to the bladder. Nephrectomy of renal cell carcinoma provides a 60–70 % survival in 5 years, but this rate is less than 10 % for distant metastatic renal cell carcinoma (Chow et al. 1999). The 5-year survival for distant metastatic renal cell carcinoma is less than 10 % (Motzer et al. 1996; Javidan et al. 1999; Chow et al. 1999; Kinouchi et al. 1999; Pantuck et al. 2001; Cheville et al. 2003). The resistance of renal cell carcinoma to radiation therapy and chemotherapy contribute to the reduced odds of survival.

The other major kidney cancer type is urothelial carcinoma also called transitional cell carcinoma. This cancer type develops in the area of the kidney where urine is collected before moving to the bladder. It is similar to and treated like bladder cancer and is accounting for 10–15 % of kidney cancers in adults.

Less common types of kidney cancer are: squamous cell carcinoma, reninoma, angiomyolipoma, renal oncocytoma, Bellini duct carcinoma, Wilms' tumor, clear-cell sarcoma, mesoblastic nephroma and mixed epithelial stromal tumor (Thyavihally et al. 2005). Wilms' tumor is common in children under age 5 and is treated differently than the kidney cancer of adults. Other rare kidney cancers include: clear cell adenocarcinoma, transitional cell carcinoma, renal lymphoma, teratoma, inverted papilloma, carcinosarcoma, carcinoid renal pelvis tumor (Nzegwu et al. 2007; Chiu et al. 2008; Kuroda et al. 2008).

5.6.5.1 Risk Factors of Kidney Cancer

Although, the causes of kidney cancer are not known, but certain factors increase the risk of getting kidney cancer:

- age – kidney cancer occurs primarily in people older than age 40,
- smoking – the risk for kidney cancer is twice that of nonsmokers,
- being male – twice as many men get kidney cancer as women,
- being black – for unknown reason the risk is higher than those of whites,
- obesity – hormonal changes increase the risk of kidney cancer,
- prolonged pain medication – long-term use of medicine and dialysis increases the risk,
- genetic conditions – family history of kidney disease, kidney cancer or Hippel-Lindau disease predisposes to kidney cancer,
- exposure to certain chemicals – e.g. asbestos, carcinogens,
- high blood pressure – extra burden of kidney may lead to cancer,
- lymphoma patients have a higher risk to get kidney cancer. The reason is not known.

5.6.5.2 Diagnosis and Treatment of Kidney Cancer and Metastasis

Tests and procedures to diagnose kidney cancer include: blood and urine tests, imaging tests (CT, MRI), removing samples of kidney tissues (biopsy). Unfortunately blood and urine tests do not detect directly kidney tumors. When kidney tumor is suspected imaging techniques, usually ultrasound and CT scans are performed. When cancer spread (metastasis) is suspected evaluation will include abdominal CT scan or MRI, chest X-ray and blood tests. Bone scan could indicate metastatic spread from kidney to the bone. Kidney cancer has also the tendency to grow into veins generating a "tumor thrombus" that can be diagnosed by MRI and CT scans.

Most of the renal cancers are resistant to chemotherapy and radiotherapy, thus these treatments are not being used. Exception is the Wilms' tumor affecting children where chemotherapy and radiotherapy are accepted treatments beside surgery. Individual cancers are removed by laparoscopic and robot-assisted surgical approaches, partial nephrectomy, occasionally nephrectomy is applied to remove the whole kidney. Even if one kidney is removed by nephrectomy or its tissue destroyed by ablation or embolization, the remaining functional kidney tissue usually works sufficiently to avoid problems. When the tumor is in clinical stage T3 or T4, or the patient has bilateral kidney tumors or a solitary adrenal gland, sparing the adrenal gland is recommended. Surgery can be supported by ablation: freezing or burning the tumor away by cryotherapy or radiofrequency ablation, respectively. Embolization is used to stop the bleeding and permits to stabilize the patient before surgery. Adjuvant therapy helps to improve the survival rate. Additional options of treatment could include immunotherapy and biological therapies using natural

substances. The most common immunotherapeutic agents are interleukin-2 (IL-2) and interferon. Combinations of therapies may include nephrectomy followed by immunotherapy or antiangiogenic treatment.

5.6.6 Prostate Cancer

^{111}Indium-capromab pendetide turned out to be useful for the evaluation of patients with residual or recurrent prostate cancer after radical prostatectomy (Kahn et al. 1998). Prostate specific antigen (PSA) test is favored by patients to detect early prostate cancer rather than physical checkup or invasive biopsy tests. Prostate cancer and metastasis to other organs are modeled in mice by surgical orthotopic implantation (An et al. 1998). Knock-in prostate cancer model demonstrated biology similar to that of human prostate cancer and suitable for preclinical studies (Gabril et al. 2005). The latest murine tumor model was published this year (Irshad and Abate-shen 2013) and the new book on prostate cancer provides a comprehensive view on the biochemistry, molecular biology and genetics of prostate cancer characteristic to men older than 50 years (Tindall 2013).

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Abbreviations

AcetylCoA	Acetyl coenzyme A
ACTH	Adenocorticotropic hormone
AIDS	Acquired immune deficiency syndrome
ADH	Antidiuretic hormone
ADP	Adenosine-5′-diphosphate
AFP	Alpha-fetoprotein
ALARP	As Low As Reasonably Practicable (3D nuclear imaging)
ALAT	Alanine aminotransferase (glutamate pyruvate transaminase = GPT)
AMP	Adenosine-5′-monophosphate
Ara C	Cytosine arabinoside, cytarabine
ASAT	Aspartate aminotransferase (glutamate oxaloacetate transaminase = GOT)
ATCC	American Type Culture Collection
ATP	Adenosine-5′-triphosphate
AVP	Arginine vasopressin hormone
BME	Basal Medium Eagle
BRCA1	Breast cancer-associated gene 1
BSL	Biosafety level
Cal	Calorie
cAMP	Cyclic AMP
cGMP	Cyclic GMP
CBC	Complete blood count
CCC	Complete cell count
CCD	Charge-coupled device
CCL	Chemokine C-C motif ligand
CD4	Cluster of differentiation 4 (Surface glycoprotein of T cells)
CD8	Cluster of differentiation 8 (Two chain glycoprotein on circulating T cells)
CDC	Centers of Disease Control and Prevention
CEA	Carcinoembryonic antigen
CCLE	Cancer Cell Line Encyclopedia

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CHNOPS	C, H, N, O, P and S bioelements
CNS	Central nervous system
CT	Computer tomography
CTH	Corticotropin releasing hormone
DAPI	4′,6-Diamidino-2-phenylindole (fluorescent stain)
DC	Dendritic cell
DME	Dulbecco's Modified Eagle's Medium
DOPA	3,4-Dihydroxyphenylalanine
DSMZ	Deutsche Sammlung von Microorganismen und Zellkulturen Gmbh,German Collection of Microorganisms and Cell Cultures
EACC	European Collection of Cell Cultures
EPO	Erythropoietin
ESCs	Pluripotent embryonic stem cells
DAR	Differential absorption ratio
ΔG	Gibbs's free energy change
ΔH	Change in heat content
dNTP	Deoxyribonucleoside triphosphate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ΔS	Entropy change
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
F	Faraday constant (96,500 C)
FACS	Fluorescent-activated cell sorting
FAD	Flavin adenine dinucleotide (oxidized form)
$FADH_2$	Flavin adenine dinucleotide (reduced form)
FDA	Food and Drug Administration
^{18}FDG	^{18}F labeled fluorodeoxyglucose
GABA	Gamma-amino butyric acid
GLUT	Glucose transporter protein
GPCR	G-protein-coupled receptor Guanine nucleotide-binding protein
G-protein	Guanine nucleotide-binding protein
GTP	Guanosine-5′-triphosphate
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HeDe	Hepatocarcinoma Debreceniensis
HEPA	High-efficiency particulate air (filter)
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HMGB	High mobility group box
HMG-Coa	3-hydroxy-3-methylglutaryl CoA
HPV	Human papillomavirus
HSP	Heatshock protein
HTLV	Human T-lymphotropic virus

i.a.	Intra-arterial injection
IARC	International Agency for Research on Cancer
i.c.	Intracardial injection
i.d.	Intradermal injection
IL	Interleukin
i.m.	Intramuscular injection
i.p.	Intraperitoneal injection
IP3	Inositol trisphosphate
iPSCs	Induced pluripotent stem cells
i.v.	Intravenous injection
Kd	Dissociation constant
KSHV	Kaposi's sarcoma-associated herpesvirus
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LUCA	Last universal common ancestor
MB	Medulloblastoma
MCV	Merkel cell polyomavirus
MEM	Minimal Essential Medium
MET	met proto-oncogene (hepatocyte growth factor receptor)
MRI	Magnetic resonance imaging
My1/De	Myeloblastoma Debreceniensis type 1
NAD	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NeDe	Nephroblastoma Debreceniensis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMRI	Nuclear magnetic resonance imaging
NK cells	Natural killing cells
NTP	Ribonucleoside triphosphate or National Toxicological Program
Osm	Osmotic concentration – 6×10^{23} soluble particles dissolved in 1 kg distilled water
p53	p53 tumor suppressor protein
PAP	Prostatic acid phosphate
Pap test	Papillomavirus test
PBS	Phosphate-buffered saline
PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide
PET	Positron emission tomography
PFK-1	Phosphofructokinase 1
Pi	Inorganic phosphate
pH	Negative logarithm of hydrogen ion concentration
pK	Negative logarithm of dissociation constant
PNETs	Primitive neuroectodermal tumors
PPi	Pyrophosphate
Ppm	Part per million – One part per million (1 mg/l) or (1 mg/kg)
PSA	Prostate specific antigen
PTN	Parathymical lymph node

PTX	Pentaxin related protein
PZD	Post-synaptic density (domain)
Q	Mass action ratio
QT	interval Time elapsing in heart from the QRS complex to the end of the T wave
R	Universal gas constant
s.c.	Subcutaneous injection
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species
r.p.	Retroperitoneal injection
RPMI	Roswell Park Memorial Institute
SCID	Severe combined immune deficiency
SHC	Sterilizing grade high capacity (filter)
SPECT	Single photon emission computed tomography
SUV	Standardized uptake value
SV40	Simian virus 40
TCA	Tricarboxylic acid (Krebs) cycle
TLR	Toll-like receptor
TGF	Transforming growth factor
UCP	Uncoupling protein (thermogenin)
UNEP	United Nations Environmental Program
USPHS	US Public Health Service (overseen by the Surgeon General)
UVB	Ultraviolet B irradiation
VEGF	Vascular endothelial growth factor
Vmax	Maximum reaction rate
WHO	World Health Organization
XMRV	Xenotropic murine leukemia virus-related virus

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