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Cell and Molecular Biology of Endometrial Carcinoma

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Preface

These proceedings include communications presented at the 15th International Symposium of the Japan Human Cell Society (JHCS) on "Cell and Molecular Biology of Endometrial Carcinoma", held under the auspices of the 20th Annual Meeting of JHCS at Kitasato Institute, Kitasato University, Tokyo, on 30–31 August 2002, with truly international participation.

Hiroyuki Kuramoto was the first in the world to establish an in vitro cell line of human endometrial carcinoma, HEC-1, in 1968. Masato Nishida established a hormone-responsive endometrial carcinoma cell line, Ishikawa, in 1980. These two cell lines have been used to establish an in vitro experimental system to clarify the characteristic features of endometrial carcinoma and its carcinogenesis at both cell and molecular levels, and have contributed to create more than 500 published works. Twenty-one authors of these distinguished works, including nine from around the world, were invited to Tokyo. The Symposium was divided into ten categories including in vitro experimental systems; apoptosis, proliferation, and growth factors; cell cycle regulators; signaling pathways; angiogenesis; carcinogenesis; hormones and hormone receptors; genes and gene expression; endometrial receptivity; and chemo-resistance and -sensitivity. The Symposium was a privileged occasion to discuss the newest topics related to cell and molecular biology in the field of endometrial carcinoma.

An in vitro culture system is extremely useful, especially when human materials, including human endometrial carcinoma, are analyzed. Here, HEC-1 cells and Ishikawa cells, ideal materials for research into human endometrial carcinoma in an in vitro research system, are described, and the experiments carried out using these cells are reviewed.

Publication of the proceedings of this Symposium will allow the dissemination of information on the various aspects of research on endometrial carcinoma cells, presented by these leading speakers.

Finally, we extend our thanks to all those whose efforts made this Symposium possible: the members of the Organizing and Scientific Committees and Springer-Verlag, Tokyo who published this proceedings.

May 2003

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Organization of Symposium

The 15th International symposium of Japan Human Cell Society (JHCS) on "Cell and Molecular Biology of Endometrial Carcinoma" and the 20th Annual Meeting of JHCS

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Contributors

Part 1
In Vitro Experimental Systems

HEC-1 Cells: Establishment of an In Vitro Experimental System in Endometrial Carcinoma

Hiroyuki Kuramoto[1], Mieko Hamano[2], Manami Imai[3], Takesi Fujisawa[1], Yuko Kamata[1], Tsutomu Arai[1], and Miwa Kawaguchi[1]

Summary. The HEC-1 cell line was the first in vitro cell line of a human endometrial adenocarcinoma, which enabled us to perform research work on the endometrium and endometrial carcinoma at the level of a simplified cellular system; contributing to cell and molecular biological studies on endometrial carcinoma. Once a cell line is established, it provides a stable experimental system that facilitates and progresses the study of the tissues and/or neoplasias from which the cell line is derived. In this article, we report how HEC-1 cells have been established and cleared the proposed requirements to characterize an established cell line. In addition, in order to demonstrate the usefulness of the cell lines for research work once they have been established, we illustrate these concepts by recalling results obtained with HEC-1 and the HEC family of endometrial carcinoma cells and review the literature with regard to what has been achieved by using these cells.

Key words. HEC-1, in vitro, cell biology, human endometrial carcinoma, uterine neoplasm

Introduction

Cell and molecular biology has yielded a long record of achievements contributing to human welfare. Many of these advances have been achieved through in vivo studies, especially by using animal experimental models. However, in vitro experimental systems for such studies faced the difficulty of obtaining suitable cells, even though everybody recognized that it would be one of the most convenient experimental approaches, especially if human cells were used as research materials. Much effort has been devoted to obtaining stable cell systems, both in terms of quality and quantity, which would enable us to perform appropriate experiments. Cell lines derived from target tissues or organs, normal or neoplastic, are widely used once they have been established in vitro.

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The HEC-1 cell line [1,2], established in 1968, was the first to be derived from a human endometrial carcinoma, as well as human adenocarcinomas of any organ, except for HeLa cells. HEC-1 cells have been used for in vitro studies aiming to evaluate the characteristics of human endometrial carcinoma, thus resulting in a large number of articles that have been published to describe the characteristics of HEC-1 cells.

The present study article aims to describe how a useful cellular system, namely the HEC-1 cell line, which is suitable for in vitro cell and molecular biological studies, was established and to review the literature that has reported information on endometrial carcinomas obtained by using HEC-1 and its family of endometrial carcinoma cells.

Confirmation for Established Cell Lines

Requirement for Established Cell Lines

The Japan Human Cell Society has proposed the criteria [3] that a cell line should possess in order for it to qualify as an established cell line. These criteria are as follows:

(*1*) nuclear morphology with atypism;
(*2*) immortalized features (i.e. cells are cultured consecutively for at least 1 year, with more than 60 subcultures);
(*3*) loss of contact inhibition;
(*4*) heterotransplantability; and
(*5*) abnormal chromosome, both in number and karyotype.

Characterization of Cell Lines

Once a cell line is established according to the abovementioned criteria, features of the proposed cultured cells need to be described [3] and include the following:

(*1*) morphology, both microscopic and ultrastructural;
(*2*) biochemical and cytochemical features;
(*3*) growth, including the doubling time, cellular density, and colony forming rates;
(*4*) monolayer or floating culture;
(*5*) chromosome constitution;
(*6*) isozyme pattern;
(*7*) heterotransplantability;
(*8*) no microbial contamination;
(*9*) functional activity (hormone, tumor marker);
(*10*) oncogene, transformed or activated;
(*11*) surface antigen; and
(*12*) other specific features.

Characterizations of HEC-1 Cells

HEC-1 (human endometrial cancer-1) cells were established as the first cell line of human endometrial carcinoma [1,2,4]. Tissue material obtained from the uterus that had been removed from a 71-year-old female with endometrial carcinoma G2 (Fig. 1)

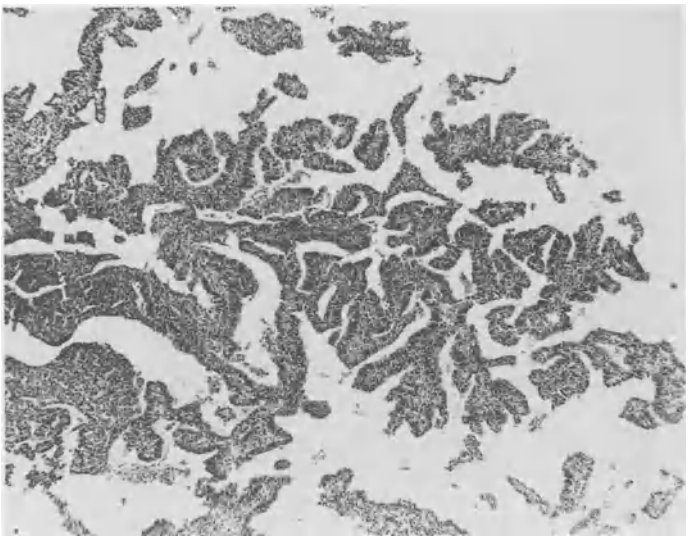

FIG. 1. Histologic findings of the original tumor showing endometrioid adenocarcinoma (hematoxylin and eosin stain)

was placed into culture on 16 May 1968. Initially, an explant plasma clot culture was made and the outgrown cells were subcultured through the first three generations, selecting for the epithelial cell type. Then, cells were transferred into the monolayer culture. Since then, the majority of monolayer-cultured cells (HEC-1-A) have shown a firm uninterrupted growth. In contrast, a flask of the cells (HEC-1-B) sustained a 2-month stationary period at the 8th–10th generations (135–190 culture days). The growth curve of HEC-1 cells revealed constant logarithmic growth, whereas two cases of normal endometrial cells ceased further growth after 100–150 days.

The population doubling time of HEC-1 cells was calculated to be 31 h with 13 h S phase, 12 h G1 phase, and 6 h G2 phase.

Cytological findings of HEC-1 cells reveal anaplastic features such as anisonucleosis and nucleolar pleomorphism. A jigsaw puzzle-like cellular arrangement (Fig. 2), with occasional dome formation and a piling-up tendency during the growing process are typical features of HEC-1 cells. A scanning electron microscopic study on the cell surface of HEC-1 cells with a synchronized culture was performed by Harada et al. [5], who showed that the cellular configuration changed depending on each phase of the cell cycle. Transmission electron microscopy has demonstrated that HEC-1 cells have desmosomes [6,7] and other characteristics of epithelia, whereas indirect immunofluorescence has been used to demonstrate the presence of cytokeratins and desmoplakins [6], revealing the epithelial phenotype. Negative expression of fibronectin has been confirmed by immunoblot [6].

The chromosomal distribution of HEC-1 cells has been stable at the diploid range [4]. However, the number of stem cells changed from 47 to 46 (HEC-1-A). One marker, which is the largest submetacentric chromosome, has been present since the beginning of culture. The stem cell of HEC-1-B was found to have 94 chromosomes and to

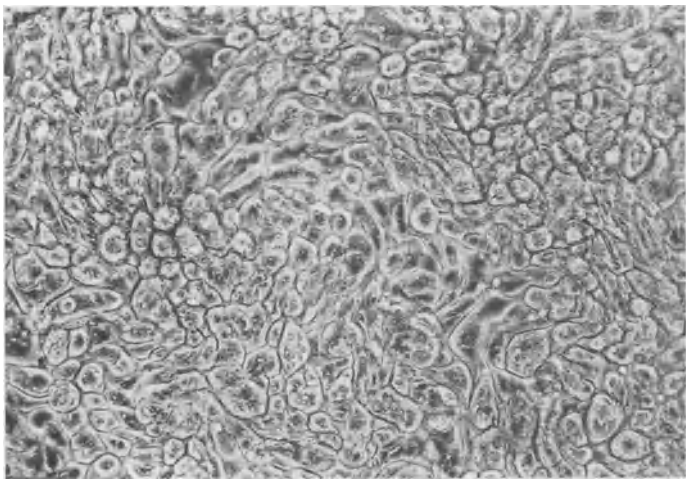

FIG. 2. Phase-contrast microscopic findings of monolayer-cultured cells revealing the sheet of polygonal cells with a jigsaw puzzle-like arrangement

be a duplicant of the original HEC-1 cell having two marker chromosomes. Noumoff et al. [8] analyzed the chromosomes of HEC-1 cells and reported, in 1988, that the marker chromosome was formed from an insertion of 2q21, probably representing an insertion of the missing chromosome 14. There was a translocation to the telomeric region of 1p and trisomies of 3, 7, and 17, which, hypothetically, could explain the increased expression of the epidermal growth factor (EGF) receptor and *erbB* gene present on chromosome 7 and the estrogen receptor and oncogene *erbA* present on chromosome 17.

HEC-1 cells repeatedly transplanted to hamster cheek pouches maintain the original endometrioid adenocarcinoma (Fig. 3). No differences have been observed in the histology of the tumor of HEC-1-B.

Human Endometrial Carcinoma Cells Established in our Laboratory

Including HEC-1 cells, we have established 12 cell lines of human endometrial carcinoma in our laboratory (Table 1). The cell lines consist of eight endometrioid adenocarcinomas, two adenoacanthomas (adenocarcinomas with squamous differentiation) and two serous adenocarcinomas in origin. The cell lines of the endometrioid adenocarcinomas include one well-differentiated (G1), five moderately differentiated (G2) and two poorly differentiated (G3) adenocarcinomas.

We have reported that the success rate of primary cultures was 57.7% [14]. The culture succeeded to the second generation in 19.5% of primary trials or 33.8% of successful primary cultures, whereas the third subculture was successful in 10.6% of

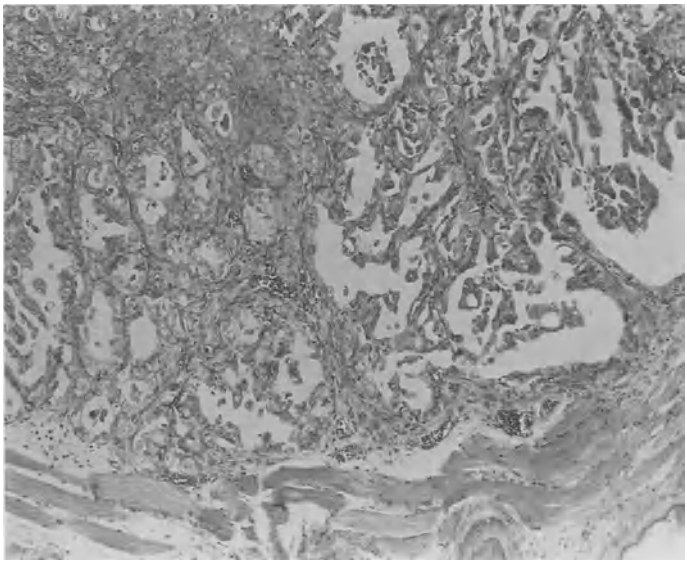

FIG. 3. Histologic findings of the grown tumor when transplanted into the hamster cheek pouch revealing adenocarcinoma that corresponds with the original tumor (31st passage generation; hematoxylin and eosin stain)

trials. Seven permanent cell lines were obtained in this series, revealing a 5.7% success rate in 123 trials. The results indicate that it should be possible for half the tissue specimens to be established into a cell line if the culture trial is able to subculture to the third passage.

HEC Cells for Cell and Molecular Biology

The established cell lines provide an eternal experimental system for research work once they have been established in vitro. Here, we show the role of HEC-1 cells and their families, as example, in establishing an in vitro experimental system for endometrial carcinoma and illustrate how cell lines are useful experimental tools.

Cell and molecular biological studies on human endometrial carcinoma using HEC-1 and its family of cells have been reviewed mainly through Index Medicus/ Medline and are summarized in the following section.

Hormone Studies

Estrogen

Gurpide et al. [14–22] started the in vitro estrogen study on endometrial carcinoma using HEC-1 cells.

TABLE 1. Human endometrial carcinoma cell lines established in our laboratory as of 27 November 2002

Name of cell line	Start of culture	Original histology	Origin of culture material	Culture method	Doubling time (h)	Passage generation	Chromosome mode	Transplant-ability	Remarks	References
HEC-1-A HEC-1-B	16 May 1968	G2 adenocarcinoma	Corpus	Plasma clot-monolayer	31	716 After thawing 233	47 94	+	EP sensitive EGFR ↑	1, 2
HEC-6	24 June 1970	Adenoacanthoma	Corpus	Monolayer	52	700	46, pseudodiploid	+	PAS	9
HEC-50	7 August 1975	G3 adenocarcinoma	Ascites	Monolayer	30.4	790	56	+	ALP, PAS, Alcian blue, EGFR ↑	10
HEC-59	14 February 1978	G2 adenocarcinoma	Corpus	Monolayer	24.8	312	47	+	ALP, PAS	11
HEC-88nu	2 December 1981	Adenoacanthoma	Tumor in nude mice	Monolayer	48	464	3n	+	ER, ALP. PAS, CEA	11
HEC-108	1 May 1984	G3 adenocarcinoma	Corpus	Monolayer	26.5	237	48	+	PAS	11
HEC-116	11 January 1985	G2 adenocarcinoma	Corpus	Monolayer	27.2	511	47	+	PAS	11
HEC-151	29 November 1988	G2 adenocarcinoma	Cervix	Monolayer	38	258	3n	+	CA125, E2DH EGFR ↑	12
HEC-155	24 January 1989	Serous adenocarcinoma	Corpus	Monolayer	34	246	Hyperdiploid	–	CA125	12
HEC-180	14 May 1991	Serous adenocarcinoma	Corpus	Monolayer	36	215	Hyperdiploid	–		
HEC-251	5 September 1997	G2 adenocarcinoma	Corpus	Monolayer	30	131	2–4n	+	Mutant p53	
HEC-265	5 June 1998	G1 adenocarcinoma	Corpus	Monolayer	36	114	2n	+	Wild-type p53, PR	13

EP, estrogen and progesterone; *EGFR*, epidermal growth factor receptor; *PAS*, periodic acid-Schiff; *ALP*, alkaline phosphatase; *ER*, estrogen receptor; *CEA*, carcinoembryonic antigen; *E2DH*, estradial dehydrogenase; *PR*, progesterone receptor

Recognition of Estrogen Receptor

Satyaswaroop et al. [15] recognized the estrogen receptor (ER) in HEC-1 cells by using sucrose density gradient ultracentrifugation. The presence of the ER was investigated in either whole cell, cytosol, or isolated nuclei, but mainly resided in the nucleus. A single peak of radioactivity was located in the 3S–4S region and this was blocked by the addition of unlabeled excess diethylstilbestrol. Scatchard analysis showed a concentration of binding sites of approximately 50 fmol/mg protein and a dissociation constant of approximately 5 nM.

Kassan et al. [16] examined cytosolic estrogen binders by applying Fourier-derived affinity spectrum analysis and found two binders in Ishikawa cells; however, in HEC-50 cells, the higher-affinity binder could not be demonstrated. Kassan et al. [16] suggested that the lack of responsiveness of HEC-50 cells to estrogens might be due to structural or functional alterations in the ER protein resulting in a loss of its capability to undergo estrogen-directed conformational changes required for biological activity.

Variability of Specific Estrogen Binding in Culture

Marked changes in the levels of specific estradiol (E2) binders occurred during the first 30 h after replating the culture, as noted when the cells were incubated with ^{3}H-E2. Marked increases and decreases were found to occur within periods as short as 2 h [17]. The rapidity of these changes suggests a possible relationship between the levels of estrogen-binding sites and the cell cycle, because elevated levels were found immediately before periods of accelerated increase in the amount of DNA per dish. After daily measurement of estradiol binding up to 8 days, it was realized that the concentrations of specific estradiol binders in HEC-1-B cells increased the first day after plating and declined thereafter [18]. The marked daily changes were also confirmed in primary cultures of epithelial and stromal cells from human endometrium [19].

Effects of Nucleotides on Estrogen Binding

The addition of molybdate (MoO_4^{2-}) caused substantial increases in cytoplasmic estradiol binding levels due to the activation of binding sites. This activation by MoO_4^{2-} requires cytosolic factors, as well as those associated with the cell membrane. In homogenates of HEC-1 cells, the addition of ATP, GTP, or cGMP was also found to increase estradiol binding. In contrast, cAMP lowered specific binding levels and counteracted the effects of MoO_4^{2-}, ATP, GTP, and cGMP [20]. A similar effect was observed in homogenates of normal human endometrium. Specifically, this effect was maximal with 1 μM cGMP. In contrast, cAMP decreased estradiol-binding levels under similar conditions [21,22]. The estrogen-binding sites sedimenting in the 4S and 8S regions of a low-salt glycerol gradient could be increased by cGMP. The amount of specifically bound radioactivity in each of these two regions was approximately doubled in cGMP-treated cytosol compared with untreated controls. The effects of cyclic nucleotides were completed within 15 min in the presence of Mg^{2+}. Both K^{+} and dithiothreitol affected these reactions [23]. The addition of sodium molybdate, ATP, and GTP to homogenates of HEC-1 cells produces an increase in estradiol binding

levels similar to those obtained by the addition of cGMP. However, these compounds were much less active when added to cytoplasm or cytosol. It is hypothesized that molybdate, ATP, and GTP affected estradiol binding levels primarily by increasing cGMP concentrations through a process involving a plasma membrane-bound guanylate cyclase. These findings suggest that cGMP and cAMP are the key compounds to control the level of estradiol binding to HEC-1 cells and changes in the levels of these two cyclic nucleotides may explain the fluctuations in concentrations of specific estrogen binders previously reported to occur in cultured endometrial cells [21,22]. The effects of the two nucleotides may be mediated by kinases and may involve phosphorylation.

Growth and Aggressiveness Control by Estrogen and Related Compounds

Ornithine decarboxylase (ODC) activity, high in proliferative endometrium, was present in HEC-1 and HEC-50 cells [10] and responded to medium renewal [24]. Difluoromethylornithine, an inhibitor of ODC activity, and sodium molybdate had marked antiproliferative effects in HEC-50 cultures.

Yoshizumi et al. investigated the effectiveness of Estracyt, a nitrogen mustard-conjugated 17β-estradiol, on the cell kinetics of the ER-positive human endometrial cancer cell line HEC-1 by using flow cytometry. Estracyt killed the cells in a dose- and time-dependent manner, accumulating in the cells in the G2+M phase [25].

Fujimoto et al. reported that invasiveness of Ishikawa, HEC-1-A, and HHUA cells to the interstitium was significantly enhanced by E2, whereas medroxyprogesterone acetate (MPA) significantly diminished the E2-enhanced invasive potential [26]. The migration potential through a basement membrane in these cell lines, in terms of strength, was also enhanced by E2, but not modified by progesterone and MPA, which inhibited estradiol-enhanced migration potential. The order of the level of ER in the cell lines is Ishikawa > HEC-1-A > HHUA cells [27]. Therefore, the results suggest that these cells may be activated by estradiol via ER.

Estrogen Metabolism

Fridman and Gurpide reported a superfusion method for the study of the metabolism of E2 and estrone (E1) in HEC-1 cells and showed that 20%–50% of superfused E2 and 40%–80% of E1 entered the cells before leaving the chamber and that most of the E2 and E1 entering the cell was not metabolized [28]. Castagnetta et al. [29] demonstrated that E2 and E1 comprised the majority of converted products in HEC-1A and Ishikawa cells; negligible amounts of other radiometabolites were observed and quite different conversion rates of E1 to E2 have been shown in HEC-1A cells (sixfold or more) with respect to Ishikawa cells. Castagnatta et al. [30] also reported that Ishikawa cells with E2 elevated formation of E2-sulfate, while HEC-1A cells with E2 elevated that of estrone, and that 17β-hydroxysteroid dehydrogenase (17β-HSD) activity diverged significantly in the two endometrial cancer cells. 17β-Hydroxysteroid oxidoreductase (17βHSOR) presides over E2 and E1 interconversion. Castagnetta et al. [31] clarified that the oxidative pathways (E2 $\rightarrow$ E_1) were dominant in nonresponsive, ER-poor HEC-1A cells, whereas reductive metabolism (E1 $\rightarrow$ E2) was dominant in ER-positive Ishikawa cells.

Responsiveness of Human Endometrial Carcinoma Cells to Estrogen

The physiological actions of estrogen on the endometrium include the induction of: (*1*) intracellular proteins, such as DNA polymerase α, alkaline phosphatase, and G-6-PD (glucose-6-phospho dehydrogenase); (*2*) secreting proteins, such as peroxidase and PA (plasminogen activator); (*3*) prostaglandins; (*4*) progesterone receptor; and (*5*) cellular growth. These inductive actions of estrogen are unfortunately not realized in HEC-1 cells, although the cells posses positive estrogen binding, which may not be followed by estrogen actions on gene expression.

However, ER- and progesterone receptor (PR)-positive Ishikawa cells [32] respond to estrogen stimulation and contribute to the progress of our knowledge on the estrogen responsiveness of endometrial carcinoma cells [33–40].

Tada et al. reported that, in HEC-59 [11], one of HEC family cell lines, 10^{-8}–10^{-11} M E2 had an effect on DNA synthesis of the cells [41].

The effects of E2 on DNA polymerase α activity were investigated by Gravanis and Gurpide [34]. Four- to fivefold increases in DNA polymerase α activity occurred when E2 was added to cultures of ER-positive Ishikawa cells and maximal stimulation was achieved at 18 h during incubation with 10^{-8} M E2, but ER-negative HEC-50 cells did not respond to incubation with E2.

Platelet-activating factor (PAF) has been shown to stimulate phospholipase D (PLD) activity in human endometrium. Armed et al. [42] found that E2 modulated PAF-evoked PLD activity in the endometrial cell line HEC-1B, but not inositide-lipid hydrolysis, and indicate that E2 can upregulate PAF-induced PLD activity in HEC-1B cells.

Progesterone

Nucleic Acid Synthesis and Mitotic Coefficient

Progesterone, at 2.5–20.0 μg/mL, suppressed DNA and RNA synthesis in HEC-1 cells by up to 52% and 43%, respectively, after 12 h of treatment [43]. The suppression of nucleic acid synthesis was as rapid as 15 min in onset. Progesterone, at 5 and 20 μg/mL, markedly suppressed the mitotic indices within the first 2 days, then gradually, and finally in 10 and 0 per 1000 cells at 2 weeks, respectively.

Morphology

The morphologic changes observed with both light and electron microscopy include an enlarged nucleus with nuclear undulation and nucleolar obliteration, and increases in Golgi complexes and lysosomal granules. Finally, the cells undergo an autolytic degenerative process, developing autophasic vacuoles, multinucleation, and nuclear picnosis, although the action does not seem to be homogeneous in the entire cell population [43].

Alkaline Phosphatase Activity

The alkaline phosphatase (ALP) activity of human endometrial carcinoma cells was investigated by using HEC-50-B cells, one of the HEC family cell lines [10]. The ALP activity of HEC-50-B cells increased sharply in the early stationary phase to reach an

activity almost 2.5-fold higher than that obtained in earlier stages culture. On administration of E2 to the culture medium, a sharp elevation of ALP activity was induced on average 2 days earlier (late logarithmic phase) than in the case of an ordinary culture (no hormone administration), without causing any notable changes in the cell growth pattern. In contrast, progesterone, at a low concentration that had very little effect on cell growth in culture, could clearly prevent the elevation in ALP activity [44]. This hormonal effect on ALP activity resembles that on enzyme activity in the endometrium of adult women [45].

The effects of various steroid hormones on ALP activity in HEC-1-B cells were evaluated bio- and histochemically. On administration of prednisolone, testosterone, and E2, each at 0.1 μg/mL for 24 h, ALP activity was induced, whereas ALP activity was suppressed by progesterone [46].

Suzuki et al. [47] characterized the biological properties of endometrial ALP, which was inhibited by sodium deoxycholate and imidazole and markedly inhibited by L-homoarginine. The enzyme also proved to be heat sensitive. The results indicated that endometrial ALP resembled hepatic ALP [47]. From the results of inhibition tests and immunocytochemical examinations, it was noted that the ALP of HEC-50-B cells had almost the same characteristics as that of ALP in normal endometrium, even after malignant transformation [47]. In contrast, the ALP of HEC-50-B cells showed slight heat stability, although it did not cross-react with placental ALP immunologically.

Cellular Growth

We evaluated the growth suppressive effects of progesterone on cells of the HEC family, including HEC-1-A, -1-B, -6, -50, -59, and -88nu [11], and on Ishikawa cells [48,49]. None of endometrial carcinoma cells in ordinary culture containing calf serum was inhibited in terms of growth by concentrations of 10^{-6} M or less of progesterone. At 10^{-5} M progesterone, the cells began to develop suppressive effects, depending on the cell line. HEC-1 was demonstrated to be the most resistant to progesterone among the various cell lines, whereas HEC-59 was the most sensitive. Satyaswaroop et al. also reported the same results in HEC-1 cells [50]. Sekiya and Takamizawa reported that HEC-1-B endometrial carcinoma cells were more sensitive to progesterone than other cell lines originating from carcinomas of the cervix and ovary [51]. Grenman et al. examined endometrial carcinoma cell lines, including HEC-1, and reported that none of the cell lines showed significant sensitivity to 10^{-7}–10^{-6} M MPA [52]. In contrast, 10 days exposure to 5 μM tamoxifen (TAM) induced an 83% inhibition of growth in HEC-1. The TAM-induced growth inhibition was reversible when TAM was removed from the culture medium. The addition of E2 to the medium did not accelerate recovery. Experiments using culture media containing serum may not be appropriate for the study of growth regulation by progesterone.

HEC-88nu, one of the cell lines of the HEC family that was first established in a nude mouse and is maintained in culture, is positive for ER and negative for PR. However, the PR is induced by giving a 72-mg pellet of E2. Tumor growth in vivo was suppressed by a 50-mg pellet of MPA and enhanced by E2. When both steroids were given simultaneously, MPA did not antagonize the growth stimulation of E2, whereas growth was most strongly suppressed when E2 + MPA administration was primed by the administration of E2 for 4 weeks [53]. This finding suggests that MPA acts through

mechanisms both dependent on and independent of the PR. Katsuki et al. [54] reported that dienogest, a novel synthetic progestational steroid, suppressed E2-dependent tumor growth of HEC-88nu cells transplanted into SCID mice, which were unresponsive to known progestins, such as MPA and norethisterone.

Apocrine Membrane Antigen

Forsman [55] reported that immunofluorescent staining of HEC-1-B cells, as well as MCF-7, revealed the presence of an apocrine epithelial differentiation antigen (AEA) using an antiserum against glycoproteins isolated from human milk fat globule membranes. The surface expression of the antigen was found to be regulated hormonally. The addition of prolactin to cultures of HEC-1-B and MCF-7 cells increased the amount of antigen. The prolactin-induced increased expression of AEA was further enhanced by progesterone (1 nM) in HEC-1-B cells and by E2 (1 nM) in MCF-7 cells.

Progesterone Metabolism

When progesterone was added to the medium of HEC-1-B cultures, it was rapidly metabolized ($t_{1/2} < 2$ h), whereas approximately 60% of the MPA remained unchanged after 24 h of incubation [15]. HEC-1 cells were incubated with ^{14}C-progesterone and four major metabolites were examined. 5α-Pregnane-3β,20α-diol was a characteristic metabolite of HEC-1, which was not detected in normal endometrium [50].

Antiestrogen

Hochner-Celnikier et al. [56] state that when examining ER-positive (Ishikawa) and ER-negative (HEC-1A) cells, tamoxifen produced estrogen agosistic effects on cell proliferation and plasminogen activator (PA) activity, and estrogen antagosistic effects on glycogen synthase and glycogen phosphorylase activities, but failed to regulate p53 and gelatinase expression. The tamoxifen-responsive systems were only observed in estrogen-responsive adenocarcinoma cells.

Gonadotropin-Releasing Hormone

Emons et al. [57] first showed that a specific high-affinity biding site for luteinizing hormone-releasing hormone (LHRH) was present in HEC-1A and Ishikawa cells, against which antiproliferative effects of LHRH antagonists and agonists were demonstrated. Irmer et al. [58] confirmed LHRH immunoactivity and bioactivity in these cell lines, as well as expression of LHRH mRNA, and assessed the existence of an autocrine regulatory system based on LHRH in endometrial cancer. Grüendker et al. [59] demonstrated the signaling pathway through which LHRH acted in endometrial carcinomas and provided evidence that the tumor LHRH receptor coupled to multiple G-proteins, but that antiproliferative signal transduction was mediated through the pertussis toxin (PTX)-sensitive G-protein α_i. The tumor LHRH receptor activated a PTP (phosphotyrosine phosphatase) counteracting EGF-induced tyrosine autophosphorylation of the EGF receptor, resulting in downregulation of mitogenic signal transduction and cell proliferation. Grüendker et al. [60] also demonstrated that activator protein (AP)-1 and c-jun N-terminal kinase (JNK) activity of HEC-1A and

Ishikawa cells was increased by the LHRH agonist triptorelin. The activation of AP-1 was blocked by PTX. c-jun mRNA expression and its phosphorylation were also obtained with triptorelin. These results and their early studies suggest that triptorelin stimulates the activity of the AP-1 through PTX-sensitive G-protein α_i and that triptorelin-induced activation of the JNK/AP-1 pathway in endometrial cancer cells is independent of the known AP-1 activators protein kinase C (PKC) or mitogen-activated protein kinase (MAPK).

In contrast, Chatzaki et al. [61] could not find high-affinity gonadotropin-releasing hormone (GnRH) binding sites or any effect of either GnRH or GnRH agonists on the growth of these cells. Kleinman et al. [62] demonstrated that the growth of HEC-1A cells was not affected by a GnRH antagonist (SB-75), but the growth of Ishikawa cells was, although the GnRH agonist did not suppress the growth of either cell line. Westphalen et al. [63] tried to develop targeted chemotherapy with a cytotoxic LHRH agonist (AN-152), in which doxorubicin is linked to the agonist carrier [D-Lys^6]LHRH, and reported that, in LHRH receptor-positive endometrial cell lines, including HEC-1A and Ishikawa cells, AN-152 was accumulated more frequently in nuclei and was more effective than doxorubicin in inhibiting cell proliferation. Davies et al. [64] showed that recombinant human luteinizing hormone (LH) and follicle-stimulating hormone (FSH) produced a concentration-dependent stimulation of HEC-1A in serum-free medium, whereas the growth of Ishikawa cells increased in the presence of LH, but not with FSH. Ishikawa cells expressed receptor mRNA of LH but not of FSH, whereas there was no expression of either mRNA by HEC-1A cells.

Testosterone

Yamamoto et al. [65] demonstrated that, among four endometrial cell lines, only HEC-59 cells had high aromatase activity and increased DNA synthesis in response to testosterone. They suggested that aromatase in cancer cells may contribute, in part, to cell proliferation if an androgen substance is provided.

Growth Factors and Their Receptors

EGF

The effect of growth factors on the mitotic activity of HEC-1-A cells in serum-free medium was demonstrated by Pearl et al. [66]. Mitotic activity was stimulated 3.5-fold by EGF, was less effectively stimulated by insulin-like growth factor (IGF)-I, IGF-II, and insulin, and was not stimulated by platelet-derived growth factor (PDGF). These findings suggest that EGF, IGF-I, IGF-II, and insulin may play a regulatory role in the proliferation of endometrial adenocarcinoma. Connor et al. [67] demonstrated that EGF and the tumor promoter phorbol myristate acetate (PMA) are mitogenic in HEC-1-A cells. The optimal incubation time for PKC activation in the cytosol by EGF was 5 min and enzyme saturation occurred at a concentration of 10 ng/mL EGF. The results suggest that EGF exerts its effects on the endometrial adenocarcinoma cell line by activating PKC through increased breakdown of phosphatidyl inositol (PI). The PI cascade appears to be an important signal transduction system mediating the growth-stimulatory effects of EGF on endometrial carcinoma. In contrast, there is a report that EGF was not detected in HEC-1A cells [68]. Burke et al. [69] investigated the

regulation of the expression of protein tyrosine phosphatase 1D (PTP1D) mRNA, which may play a role in uncontrolled cell proliferation and neoplastic transformation, by peptide growth factors, including forskolin (a stimulator of cAMP), EGF, and IGF-I.

The EGF receptor was found to be present in HEC-1-A cells by Lelle et al. [70]. Scatchard analysis revealed dissociation constants of 0.23 nM. Other peptides, such as IGF-I, IGF-II, and insulin, did not compete for the receptor. Watson et al. [71] demonstrated an EGF receptor in HEC-1-B cells with a K_d of 3.09 nM and binding of 845 fmol/mg protein. The PKC activator PMA increased the K_d of the EGF receptor in a dose-dependent manner up to 50 nM, an effect that was overcome by the PKC inhibitor staurosporine. Both E2 (1 nM) and progesterone (1 μM), added to the medium for 6 days, increased EGF receptor numbers with maximum five- and seven-fold increases. The result suggested the presence of a high-affinity binding site for EGF in HEC-1-B cells that is regulated by both ovarian hormones.

IGF

Reynolds et al. [68] evaluated several endometrial cell lines, including HEC-1A and Ishikawa cells, for IGF-I, transforming growth factor (TGF)-α, and EGF, and demonstrated that autocrine production and stimulation by TGF-α and IGF-I, but not by EGF, existed in endometrial carcinoma cell lines.

IGF-Binding Proteins

Lamson et al. [72] examined the expression of IGF-binding protein (IGFBP) in HEC-1 cells and identified two binding proteins, one of which was immunoprecipitated with an antiserum made to BP-53. BP-53 is known to exist in serum. The second protein was a 32K protein consistent with one predominant in cerebrospinal fluid [73]. Pekonen et al. reported that HEC-1-B cells secreted IGFBP-1 and contained IGF-I receptors. Progesterone and clomiphene inhibited the production of IGFBP-1, whereas estrogen, cortisol, and insulin had no effect on IGFBP-1 secretion [73]. The secretion of IGFBP-3 was also recognized in both HEC-1-B and HEC-1-A cells. IGFBP-1 secreted in HEC-1-B cells was shown to be mostly in the nonphosphorylated form [74]. Camacho-Hubner et al. [75] identified the expression of three IGFBPs (IGFBP-1, -2, and -3) and suggested that at least one form secreted by HEC-1-B cells may act as a positive autocrine modulator of the growth-stimulation actions of IGF-1. Gao and Tseng [76] transfected a PR (B form) expression vector into HEC-1B cells and found that progestin reduced the levels of secreted IGFBP-1 and IGFBP-1 mRNA, and IGFBP-1 promoter activity.

Gong et al. [77] demonstrated that the addition of PMA to HEC-1-B and HEC-50 cells resulted in changes in morphology, growth inhibition, activation of PKC, and an increase in expression of IGFBP-1, whereas PMA had no effect on Ishikawa cells [12,32], which did not express IGFBP-1. PMA also increased IGFBP-3 in HEC-50 cells and IGFBP-6 in HEC-1-B cells, whereas it reduced IGFBP-2 and -3 in HEC-1-B cells [79]. These data establish a role for the PKC pathway in the regulation of expression of IGFBPs in endometrial adenocarcinoma cells and illustrate the complexity of cell type-specific expression of the IGFBPs [76]. Talavera et al. [78] reported that forskolin, a stimulator of cAMP production, and, to a lesser extent, PMA, a stimulator of PKC, exert their effects at the G1 phase of the cycle to enhance IGF-I effects in HEC-1-A cell proliferation.

Gao et al. demonstrated that most of the regulation of the *IGFBP-1* gene in HEC-1-B cells was derived from the distal promoter region confined to the *Rsa* I-*Cla* I fragment and that the same region mediated an inhibitory effect of the PR [76,79].

TGFs

Boyd and Kaufman examined the effects of TGF-β1 on eight cell lines of endometrial carcinoma [80]. The growth of five cell lines, including HEC-1-A and HEC-1-B cells, was inhibited by TGF-β1 (10 ng/mL) with morphological alterations, whereas the growth of three cell lines was not inhibited. Correspondingly, the former cell lines, which responded to TGF-β1, expressed much lower levels of TGF-β1 mRNA. Estradiol and 4-hydroxy tamoxifen (OH-Tam) significantly increased TGF-β1 mRNA levels in HEC-1-A xenografts in nude mice, as well as significantly reducing tumor size [81]. MPA had no effects on either tumor size or TGF-β1 expression in HEC-1-A xenografts. Bergman et al. [82] demonstrated that basal and EGF-stimulated proliferation was inhibited by TGF-β. TGF-β also reversibly decreased EGF-induced *c-fos* mRNA expression in a dose- and time-dependent manner, showing that TGF-β negatively modulates EGF-induced *c-fos* expression, which may be related to the inhibition of carcinoma cell proliferation.

TGF-α expression in Ishikawa cell xenografts was significantly reduced by MPA [81]. Gong et al. [83] and Murphy et al. [84] compared *TGF* gene expression in Ishikawa and HEC-50 cells. Gong et al. [83] examined the effects of MPA on cell proliferation and the expression of *TGF*-α and *TGF*-β genes and found that a significant decrease in *TGF*-α mRNA was apparent 6 h after exposure to MPA, whereas MPA had no effect on the expression of *TGF* genes by HEC-50 cells. An antiEGF receptor monoclonal antibody inhibited Ishikawa cell growth, but had no effect on HEC-50 cell proliferation. Exogenous TGF-α stimulated the proliferation of both cell lines, but Ishikawa cells were significantly more sensitive to exogenous TGF-α. The antiproliferative effects of progestin on Ishikawa cells were mediated by decreased expression and autocrine action of TGF-α, whereas in HEC-50 cells, in which growth inhibition also seen, progestin had no effect on TGF-α expression.

Murphy et al. [84] examined the effects of OH-Tam in addition to MPA on cell proliferation and the expression of TGF-α and TGF-β genes in Ishikawa and HEC-50 cells. In both cell lines, MPA produced a time- and dose-dependent inhibition of cell proliferation, whereas OH-Tam had no effect on HEC-50 cell proliferation. TGF-α mRNA abundance was reduced in Ishikawa cells with OH-Tam. Exogenous TGF-α stimulated proliferation of both cell lines, whereas exogenous TGF-β inhibited the proliferation of Ishikawa cells but stimulated the proliferation of HEC-50 cells. Gong et al. [85] showed that, in HEC-50 cells, neither E2 nor three antiestrogens had any effect on cell proliferation or TGF mRNA abundance. In contrast, the antiestrogens had different effects on Ishikawa cells, depending on the culture conditions. In medium containing 5% fetal bovine serum (FBS), the antiestrogens inhibited cell proliferation and significantly decreased TGF-α mRNA abundance and TGF-α secretion. In medium supplemented with 1% ctFBS (twice charcoal-treated FBS), E2 increased cell proliferation and TGF-α mRNA and TGF-α secretion, whereas OH-Tam, which also stimulated cell proliferation, reduced TGF-α mRNA abundance and had no significant effect on TGF-α secretion.

Hata et al. [86] demonstrated that both TGF-α and EGF stimulated the proliferation of Ishikawa cells at doses of 0.1 and 1 nM. Anti TGF-α antibody and anti EGF receptor antibody eliminated the stimulatory effects on these cells. The production of TGF-α into culture media was 5–40 pg/10 cells per 24 h in nine endometrial cancer cell lines, including HEC-1 and Ishikawa cells. At 10 nM, E2 increased the TGF-α production by Ishikawa cells, but not that by HEC-50 cells. Both E2 and TGF-α increased C-erbB-2 expression in Ishikawa cells, whereas, in HEC-50 cells, TGF-α increased C-erbB-2 expression, but E2 had no effect on the expression.

PAF and its Receptors

Maggi et al. [87] reported that HEC-1-A cells not only synthesized and released PAF (platelet activating factor), but also responded to PAF. PAF induced a time-dependent increase in the expression of the nuclear protooncogene *c-fos* and stimulated DNA synthesis of HEC-1-A. The presence of two populations of PAF receptors was also identified. The results provide evidence for the existence of an autocrine proliferative loop involving PAF in the endometrial cancer cell line HEC-1-A. PAF is thought to be an important mediator of embryo–endometrial interactions in early pregnancy. Ahmed et al. [88] demonstrated the presence of mRNA encoding PAF receptors in the endometrial epithelial cell line HEC-1-B that were functional and linked to inositol-lipid hydrolysis, calcium mobilization, and tyrosine kinase activity. Dearn et al. [89] reported that activation of PAF receptor stimulated nitric oxide (NO) release via PKC-α in HEC-1B cells and postulated that PAF-stimulated NO release via PKCα may regulate endometrial functions, such as implantation and menstruation. Munir et al. [90] demonstrated that MAPK was activated with PAF and hCG in HEC-1B cells, and that cyclooxygenase (COX)-2 was stimulated through the MAPK activation pathway with hCG and the wortmannin (phosphatidylinositol 3-kinase (PI3-K) inhibitor)-sensitive pathway with PAF. Bonaccorsi et al. [91] indicated that PAF-induced mitogenesis in HEC-1A cells was mediated by the activation of multiple signaling pathways, involving protein tyrosine kinase (PTK), MAPK, and PKC activation.

Giannni et al. [92] reported that the presence of two IGFBPs, namely IGFBP-2 and -3, in HEC-1A-conditioned medium and incubation with PAF (1 μM) significantly increased the release of the two IGFBPs from the cells, demonstrating the existence of an autocrine growth loop driven by PAF.

Uteroglobin is a protein synthesized and secreted by most epithelia, including the endometrium, that exerts antiproliferative and antimetastatic effects in cancer cells via a membrane receptor. HEC-1A cells were transfected with human uteroglobin cDNA and the transfectant showed markedly reduced proliferative potential and significantly impaired synthesis of PAF, suggesting that the antineoplastic properties of uteroglobin may be due to the inhibition of the synthesis of PAF [93].

Basic Fibroblast Growth Factor

Presta [94] demonstrated the presence of basic fibroblast growth factor (bFGF) in extracts of HEC-1-A and HEC-1-B cells that was assayed on the basis of the ability of the extracts to stimulate plasminogen activator (PA) production in bovine capillary endothelial cells. E2 stimulated the synthesis of bFGF, whereas progesterone antago-

nized it [94]. The data demonstrate the capacity of sex hormones to regulate bFGF synthesis in tumor endometrial cells and suggest that bFGF may play a role in the vascularization of the endometrial adenocarcinoma, as well as of the normal endometrium. HEC-1-A and HEC-1-B cells that posses high-affinity binding sites for bFGF responded to the addition of human recombinant bFGF with an increase of the synthesis and secretion of urokinase- and tissue-type PAs (uPA and tPA, respectively) and with an increase in cell proliferation. An expression vector harboring the human bFGF cDNA was transfected into HEC-1-B cells by Coltrini et al. [95], who found that a significant amount of bFGF was present in conditioned medium and extracellular matrix of the bFGF-B9 clone. The cloned cells formed highly vascularized tumors that grew faster than parental cells when injected s.c. into nude mice. In addition, the cloned cells were more potent than nontrasfected cells in inducing an angiogenic response in the rabbit cornea assay. bFGF export by human endometrial adenocarcinoma cells results in autocrine and paracrine effects that confer a growth advantage in vivo associated with increased neovascularization.

TGF-β also induced an increase in PA synthesis in both HEC-1-A and HEC-1-B cells and stimulated the production of the endothelial cell-type PA inhibitor in HEC-1-A cells. However, TGF-β inhibited basal proliferation of both cell lines and suppressed the mitogenic activity of bFGF [96].

Vascular Endothelial Growth Factor

The repair of the human endometrium after menstruation and preparation of the endometrium for implantation involve profound angiogenic changes that are controlled by vascular endothelial growth factor (VEGF). The endometrial carcinoma cell lines HEC-1-A, HEC-1-B, and Ishikawa express all four species of VEGF. E2 increased mRNA encoding VEGF in a dose- and time-dependent manner in HEC-1-A cells. The data show that VEGFs are the principal angiogenic growth factors secreted by HEC-1-A and HEC-1-B cells, because mRNA for acidic FGF or bFGF was not demonstrated in these cells [97].

Yanase et al. reported the antitumor activity of angiogenesis inhibitor TNP-470 on HEC-1-A, HEC-6 [2] and HEC-50 cells in vitro [98].

Endothelin (ET)-1 is a vasoconstrictor peptide. The expression of ET-1 was detected in two of five cell lines out of human endometrial adenocarcinoma, including HEC-1-A cells. The HEC-1-A cells were also shown to express ET-1 mRNA. HEC-1-B cells, which did not produce measurable amounts of immunoreactive ET-1, also contained ET-1-specific mRNA [99].

Leukemia Inhibitory Factor

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine that has been found to be expressed in the human endometrium and to play a role in reproduction. Bamberger et al. [100] investigated the expression and regulation of the human LIF promoter in HEC-1B cells, which are able to produce endogenous LIF mRNA. The activity of the LIF promoter was stimulated by a combination of phorbol ester (TPA) and ionomycin, but not by MPA treatment in the presence of transfected PR-B expression vector in HEC-1B cells. Therefore, the results indicate that the LIF promoter in uterine tumor cells is not always regulated by progestin.

Hepatocyte Growth Factor

Tumor progression is often regulated through interactions between carcinoma cells and host stromal cells. Yoshida et al. [101] demonstrated that conditioned medium from normal epithelial cells and the cancer cell lines HEC-1 and Ishikawa induced hepatocyte growth factor (HGF) production in normal stromal cells and identified bFGF as an HGF inducer derived from endometrial cancer cell lines. Yoshida et al. [101] suggest that there is a mechanism potentially involving HGF in cancer–stromal interactions.

Calcyclin

Calcyclin is a member of the S-100 family of calcium-binding proteins, the expression of which is enhanced when quiescent cells are exposed to mitogenic signals. The addition of PMA to HEC-1-B and HEC-50 cell cultures resulted in a change in cell morphology, an inhibition of proliferation, and an increase in calcyclin transcription rate, as well as an increase in calcyclin mRNA and calcyclin protein levels [102]. The results demonstrate dissociation between calcyclin expression and cellular proliferation and suggest that the enhanced calcyclin expression may result from activation of the PKC system.

COX-2

Munir et al. [103] demonstrated that expression of COX-2 (cyclooxygenase 2) was regulated by PGE_2 in HEC-1B cells in addition to regulation by PAF (see the section on PAF [90], PGE_2 also induced activation of MAPK and protein kinase B (PKB), and these PGE_2-induced kinase activations were sensitive to MAPK kinase (MEK) and PI3-K inhibitors, respectively. Munir et al. [103] suggest that both these pathways are involved in the expression of COX-2.

Purinergic Receptors

ATP acts as an extracellular messenger for purinergic receptors, including P2Y receptors. Katzur et al. [104] demonstrated that ATP in HEC-1A and Ishikawa cells induced a rapid increase in extracellular Ca^{2+} in a dose-dependent manner and that the activation of the $P2Y_2$ receptor by the ATP analog ATP-γ-S was associated with a significant suppression of cell proliferation, without any effect on cellular apoptosis. The results indicate that $P2Y_2$ receptors may participate in the control of the cell cycle of endometrial carcinoma cells.

Neuropeptide Y Receptor

The activity of neuropeptide Y (NPY), which is widely distributed in both the central and peripheral nervous systems, is mediated by several NPY receptor subtypes. The NPY receptors act to downregulate adenylyl cyclase activity. Moser et al. [105] devel-

oped a functional assay method for the human NPY Y_5 receptor based on adenylyl cyclase activity. HEC-1B cells, in which cAMP synthesis is efficiently stimulated with forskolin, were selected for transfection with cDNA of the receptor and it was found that functional assays were only feasible with HEC-1B cells stably expressing the Y_5 receptor. At 10 nM, pNPY (porcine NPY) inhibited forskolin-stimulated cAMP synthesis by 75%. Bischoff et al. [106] concluded that, at least upon expression in HEC-1B cells, the signal transduction of Y_5 receptors is limited to inhibition of cAMP accumulation.

Genes

Evi-1

The expression of the *Evi-1* gene is one of the most common events associated with transformation in murine myeloid leukemia. Morishita et al. [107] found that the *Evi-1* gene was uniquely expressed at low levels in HEC-1-A cells and at high levels in HEC-1-B cells. cDNA clones were isolated and sequenced from the HEC-1-B cell line. The human gene is highly homologous to the murine gene.

ras

Eleven endometrial carcinoma cell lines were examined for possible point mutations of *ras* protooncogenes, which were found in seven of 11 (64%) tumors that consist of three in Ha-*ras* and four, including HEC-1-A (Gly → Asp), in Ki-*ras* [108]. No evidence was found for amplification or overexpression of the c-erbB2 or EGF receptor genes in any endometrial carcinoma.

p53

Yaginuma and Westphal presented a *p53* gene analysis of six endometrial carcinoma cell lines and compared them with seven cervical carcinoma cell lines [109]. These studies revealed mutations changing the p53 amino acid composition in all endometrial carcinomas and in two human papilloma virus-negative cervical carcinomas, whereas five papilloma virus-positive cervical carcinomas contained wild-type *p53* gene sequences.

Telomere Reduction

Genomic instability in carcinomas may be due to the loss of telomeric sequences. A reduction of telomeric repeat sequences in tumor versus normal tissue was found in 10 of 11 cases of endometrial carcinoma. It was also seen in four of five endometrial carcinoma cell lines, including HEC-1-A and HEC-1-B cells [110].

Wnt

Wnt genes are transforming mouse breast epithelium and are hormonally regulated in vivo. Bui et al. [111] examined seven *Wnt* genes in normal and malignant endometrial cells, including HEC-1-A and Ishikawa cells, and found that *Wnt7a* and *Wnt7b*

mRNA was expressed in both cell lines. In contrast, *Wnt2*, *Wnt3*, *Wnt4*, and *Wnt5a* mRNA was expressed in normal epithelial and stromal cells and was independent hormonally. Bui et al. [111] suggest that *Wnt4* gene down-regulation, as well as the down-regulation of *Wnt2*, *Wnt3*, and *Wnt5a*, may be associated with endometrial carcinogenesis.

PMS2

HEC-1-A cells are defective in the mismatch repair gene *hPMS2*. Kato et al. [112] examined the mutations at the hypoxanthine–guanine phosphoribosyltransferase (*hprt*) locus in the cells and detected 58 mutations, which included frameshifts (66%) and the reduced incidence of C → T transitions at the CpG site. Kato et al. [112] suggest that the hPMS2 gene product is involved in the avoidance of ±1 bp events at repeating sequence sites and in the repair of the mismatch at CpG sites. Risinger et al. [113] transfected wild-type *hpMS2* cDNA into HEC-1-A cells and found that the cells had a reduced mutation rate at the *hprt* locus and that the extract from the cells was able to perform strand-specific mismatch repair. The *gPMS2* gene is integral to the maintenance of genome stability.

PTEN

PTEN gene mutation is most frequently involved in endometrial carcinoma, occurring in approximately 45% of cases. Among the HEC family of endometrial carcinoma cells, the *PTEN* tumor suppressor gene was mutated in HEC-6, HEC-59, HEC-88, HEC-108, HEC-116, and HEC-151 cells, whereas it was not mutated in HEC-1 and HEC-50 cells [114]. The *PTEN* gene encodes a multifunctional phosphatase that plays an important role in inhibiting the PI3-K pathway and downstream functions that include activation of AKT/PKB, cell survival and cell proliferation. AKT, of which PTEN is the negative regulator, is elevated in Ishikawa cells with a mutant *PTEN* gene, whereas in HEC-1-A cells with a wild-type *PTEN* gene AKT levels were not elevated [114,115]. Lilja et al. [115] reported that expression of PTEN significantly suppressed the growth of Ishikawa cells when the cells were transfected with wild-type *PTEN* cDNA, but not when cells had been transfected with mutant *PTEN* (C124S). PTEN also inhibited malignant transformation mediated by *ras* and *c-myc* oncogenes. Matsushima-Nishiu et al. [114], using microarrays and subsequent semiquantitative RT-PCR analysis, revealed transcriptional stimulation of 99 genes, including cyclin-dependent inhibitor 1B (p27Kip1) and 2B (p151NK4B), and repression of 72 genes. Unoki et al. [116] reported that eight genes tested, including the novel gene *PINK1* (PTEN-induced putative kinase), were activated in HEC-151 and Ishikawa 3-H-12 cells, when an exogenous *PTEN* gene was transfected and that two genes, namely *EGR2* and *BPOZ*, were able to significantly suppress the growth of cancer cells.

p27

p27 is regarded as a cyclin-dependent kinase inhibitor of G1–S cell cycle progression that is activated during the secretory phase of the endometrium [117]. Kanai et al.

[13] reported that *p27* expression in PR-positive HEC-265 cells was enhanced by MPA, but not in PR-negative HEC-50B cells.

Using hisotologic specimens of endometrial carcinoma, Watanabe et al. [117] investigated paradoxical expression of p27 in a higher grade of endometrial adenocarcinoma (endometrioid type), which overexpressed cell cycle promoters, including Ki-67. p27 expressed in the higher-grade carcinoma may have lost physiological activity.

c-jun

Alkhalaf et al. [118] reported that, under conditions of MPA-induced growth inhibition, *c-jun* mRNA and protein levels were decreased in Ishikawa cells and this decrease was accompanied by an overall decrease in endogenous AP-1 activity. In contrast, in HEC-50 cells, only a transient decrease in *c-jun* mRNA levels was seen, without any effect on endogenous AP-1 activity.

Müllerian Inhibiting Substance

Müllerian inhibiting substance (MIS), which is produced by the fetal testis, causes regression of the Müllerian duct in the male fetus. Bovine MIS has been reported to inhibit the growth of some gynecological tumors. Chin et al. [119] demonstrated that recombinant human MIS caused significant inhibition of gynecological carcinoma cell lines, including HEC-1-A cells. Rosenwaks et al. [120] reported also that rat testicular homogenates inhibited HEC-1 cell growth. However, the bioactive anti-Müllerian hormone purified from the testicular agent by monoclonal antibody chromatography had no effect on the growth of malignant endometrial cells [121].

Extracellular Matrix

Laminin

In vitro studies of endometrial carcinogenesis have been hampered by limited differentiation of the cells in culture. Hopfer et al. [122] established culture conditions that preserve a more differentiated state of HEC-1B (L) cells (subclone of HEC-1B) using the reconstituted basement membrane Matrigel (SERVA, Heidelberg). Features of differentiation included a web-like structure and two additional secretory proteins, 31 and 77 kDa in size, suggesting a regulatory role for the extracellular matrix (ECM) in the differentiation of the HEC-1B cell line. Behrens et al. [123] reported that basement membrane (Matrigel)-induced differentiation of HEC-1B cells was mediated by laminin, but not collagen type IV. Both antilaminin antibodies and laminin-specific peptides suppressed Matrigel-induced formation of the 77 kDa secretory protein by HEC-1B cells. Tan et al. [124] demonstrated that culturing cells on Matrigel downregulated the steady state mRNA levels of the proliferation-associated protein Ki-67 in HEC-1B and Ishikawa cells after 48–96 h of culture on the matrix substrate; proliferation could be stimulated again by functional domains of tenascin-C.

Matrix Metalloproteinase

Park et al. [125] established a three-dimensional culture system in which normal endometrial stromal cells and endometrial cancer cells were cocultured under defined hormonal conditions in order to investigate the potential paracrine effect on synthesis and secretion of matrix metalloproteinases (MMPs). Using their experimental system, Park et al. [125] concluded that stromal cell-derived MMP-2 was translocated to the surface of HEC-1A cells. The integrin β3-subunit may contribute to providing a binding site for MMP-2. Thus, HEC-1A cells invaded by recruiting MMP-2 secreted by stromal cells, which was greatly enhanced in the presence of E2. Sillem et al. [126] reported that secretion and regulation of the MMPs and tissue-specific inhibitors of metalloproteinases (TIMP), as well as uPA and its inhibitors in endometrial cancer cell lines, including HEC-1A and Ishikawa cells, seemed to be unrelated to the invasiveness of the cells.

Integrin

The integrin family of adhesion molecules consists of heterodimeric receptors involved in cell–cell and cell–ECM (extracellular matrix) interactions. Prifti et al. [127] reported that endometrial cancer cells, including HEC-1A and Ishikawa cells, express the $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ integrins, which modulate adhesion as well as migration into the artificial matrix Matrigel of endometrial tumor cell lines. Struck et al. [128] also found that Matrigel regulated the expression of various forms of the integrin β4-subunit in HEC1B(L) cells and suggested a regulatory role for the integrin β4 isoform in the process of in vitro differentiation of HEC1B(L).

Tenascin-C

Tenascin-C (TN-C) is a large hexameric glycoprotein of the ECM that is localized in the mesenchymal tissue of organs and in the stroma of malignant tumors, including those of the endometrium. Vollmer et al. [129] reported that five of six endometrial cancer cells, including HEC-1A, HEC-1B, HEC-1B(L), and Ishikawa cells, produced TN-C mRNA as well as endometrial stromal cells analyzed by RT-PCR. The expression of TN-C was increased by adding either serum or 20 ng/mL TGF-β. Evidence is provided that TN-C can originate from both tissue compartments of the human endometrial stroma and tumor epithelium.

icb-1 and Other Genes

Culturing HEC1B(L) cells on an ECM, Struck et al. [130] isolated two DNA fragments, MESR4/13 and MES13/15, by using the method of differential display RT-PCR. The former was downregulated by ECM and contains a TG-rich sequence, whereas the latter demonstrates high sequence similarity to MESR4/13, except for the TG-rich region, and was induced by ECM.

Treeck et al. [131] cultured HEC1B(L) cells in Matrigel and isolated a 2.4 kb Matrigel-induced transcript *icb-1* containing an open reading frame predicting a 31.7-kDa protein. The time-dependent induction of *icb-1* gene expression by basement membrane was confirmed by Northern blot experiments. *icb-1* is a new

gene induced by basement membrane that enables the study of cell–matrix interactions.

Chemosensitivity

By using HEC-1A, HEC-50B, HEC-59, HEC-108, and Ishikawa cells, Hiramatsu et al. [132] examined the chemosensitivity of endometrial carcinomas and reported that paclitaxel was effective followed by aclarubicin and actinomycin D, whereas cisplatin and ifosfamide were not effective. Being used in combination, paclitaxel plus SN-38 (the active metabolite of CTP-11) and paclitaxel plus cisplatin as well as actinomycin D and SN-38 resulted in synergistic effects followed by paclitaxel plus SN-38. Examining actin polymerization dynamics, Stournaras et al. [133] demonstrated that malignant cells were characterized by higher G/total actin ratio, decreased F- and total-actin content, and lower resistance to cytochalasin B. Microfilaments in poorly differentiated endometrial adenocarcinoma HEC-50B were more destabilized than those in the well-differentiated Ishikawa cells. It seems worthwhile to determine whether the differential sensitivity of malignant cells to anticytoskeletal drugs may provide a valuable approach to the treatment of malignant cells.

Photodynamic Therapy

The response of the human gynecological cell line HEC-1-A to photodynamic therapy in vitro was examined by using the porphyrin compound Photosan III (PhIII) for photosensitization of cells and laser light at 630 nm for irradiation. HEC-1-A cells did not survive photodynamic therapy with 10 J/cm^2 after incubation with 5 μg/ml for 48 h. After a shorter incubation time of 24 h, 10 μg/ml PhIII was necessary to achieve the same effect [134].

Microorganisms

Interferon

Kuwata et al. first mentioned that HEC-1 cells were resistant to the antiviral and anticellular actions of interferon (IFN), although the cells are susceptible to the cytotoxicity of natural killer (NK) cells [135]. Two double-stranded RNA-dependent enzymatic activities (an oligoisoadenylate synthetase and a protein phosphokinase), which were induced by IFN, were originally high in untreated HEC-1 cells [136]. 2′,5′-Oligoadenylate synthetase was not induced in the cloned cell lines HEC-1C and HEC-1D by IFN treatment [137] and the results suggest that the cells may have the structural gene for the IFN receptor but that functional receptor sites may be absent. The synthesis of PGE and $PGF_{2\alpha}$ in IFN-sensitive cell lines, which was stimulated by human IFN-β, was not stimulated in IFN-resistant HEC-1 cells [138]. 2′-Phosphodiesterase activity, which is usually stimulated by IFN, was not affected in HEC-1 cells, whereas HEC-50 cells [10] were sensitive to IFN [139]. HEC-1 cells were shown to have only low-affinity sites for IFN-α [140]. HEC-1 cells, which are totally refractory to the

actions of human IFN, appeared most resistant to ultraviolet (UV) light. In contrast, UV-sensitive cells appeared highly susceptible to human IFN-β [141]. The antiproliferative activity of human IFN was enhanced by dipyridamole. However, HEC-1 cells were not susceptible to these two combinations [142]. The human endometrial carcinoma cells HEC-50 and Ishikawa are sensitive to the cytotoxic activity of peripheral blood lymphocytes (PBLs). Rossiello et al. [143] reported that the addition of IFN-β to the culture increased tumor target cell sensitivity to the lytic activity of untreated PBL.

Neisseria gonorrhoeae

There is no animal model for identification of virulence factors for *Neisseria gonorrhoeae*. An alternative model for the assessment of gonococcal virulence was reported by Chen et al. [144], who developed an invasion assay using the adenocarcinoma endometrial cell line HEC-1-B. Members of the opacity associated (Opa) outer membrane protein family of *N. gonorrhoeae* were proposed to mediate adherence to and invasion of cultured human endometrial epithelial cells HEC-1-B, indicating that at least one gonococcal Opa protein is an invasion protein [145]. Griffiss et al. [146] examined concomitant roles of pili as well as Opa in promoting *N. gonorrhoeae* adherence to and invasion of human endometrial HEC-1-B cells and reported that *N. gonorrhoeae* coordinately uses pili and Opa to activate HEC-1-B cell microvilli, which causes engulfment of the gonococci. The genes encoding the glycosyltrasferases responsible for the addition of the five sugars in the α-oligosaccaride (α-OS) moiety of lipooligosaccharide (LOS) have been identified. Minor et al. [147] suggested that the proximal glucose residue of gonococcal α-OS chain of LOS was required for efficient invasion of gonococci into host mucosa.

Chlamydia trachomatis

In order to identify *Chlamydia trachomatis* genes involved in its attachment to host cells, a *Chlamydia* genomic library was screened on the basis of binding characteristics. Individual recombinant *Escherichia coli* clones were assayed for adherence to two eukaryotic cells, including the human endometrial epithelial cell line HEC-1-B, and revealed the presence of chlamydial sequences inserted in the plasmids and the expression of novel 18-, 28-, and <82-kDa proteins [148]. *Chlamydia trachomatis* serovar L2 is an obligate intracellular bacterium that is internalized in target epithelial cells (HEC-1B) by endocytosis and resides within a membrane-bound vesicle. Schramm et al. [149] reported that the inactivation of Na^+/K^+-ATPases, the ion pumps responsible for maintaining a pH above 6 within early endocytic vesicles, inhibited the growth of *C. trachomatis* within epithelial cells. Thus, chlamydiae do not reside within highly acidic vesicles and avoid the pathway leading to lysosomes.

Haemophilus ducreyi

Electron microscopy used to examine *Haemophilus ducreyi* adherence to, and entry into, eukaryotic cells of genital origin revealed that the strain 90–244 entered HEC-1-B cells but did not enter HeLa cells of cervical carcinoma origin [150]. The authors

suggest that this model of in vitro *H. ducreyi* infection of eukaryotic cells will allow for more specific studies to determine the virulence of *H. ducreyi*.

Mycobacterium haemophilum

Fischer et al. [151] developed an in vitro model to study the temperature-regulated cytotoxicity and intracellular growth of *Mycobacterium haemophilum* in cultured human epithelial cells (HEC-1-B). Intracellular replication of *M. haemophilum* was 1000-fold greater at 33 than at 37°C.

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Ishikawa Cells: Opening of In Vitro Hormone Research on Endometrial Carcinoma

MASATO NISHIDA

Summary. More than 20 years have passed since the Ishikawa cell line, a well-differentiated human endometrial adenocarcinoma cell line, was established. Because this cell line bears estrogen and progesterone receptors, the cells have been used in numerous basic research areas, such as reproductive biology and molecular science, and have been distributed to more than 100 institutes. However, even the Ishikawa cells, after long-term culture, tend to transform into undifferentiated cells. In addition, it has been reported that estrogen and progesterone receptors disappeared from the cells that I have distributed. I therefore attempted to establish well-differentiated cells from the parent Ishikawa cells and to produce a new and good-quality supply of this cell line. I believe that it is very important for the investigator who established a cell line to be responsible for maintaining the quality of the cells. That is why I have not deposited this cell line in any cell bank.

I would like to take this opportunity to report the history of Ishikawa cells from establishment to the present day.

Key words. Ishikawa cells, endometrial carcinoma, cell line, estrogen receptor, progesterone receptor

Establishment and Characterization of Ishikawa Cells

With improvements in tissue culture techniques, numerous human cancer cell lines have been established. These cell lines contribute to research into carcinoma by providing an excellent experimental system. With regard to endometrial carcinoma, since Kuramoto first established the human endometrial cancer cell line HEC-1 cells in 1968 [1], several cell lines have been established. However, a single human cancer cell line may not possess the universal characteristics of all human cancers. Therefore, it is important to compare investigation results among several cell lines. Thus, the establishment of new cell lines is important. I succeeded in establishing a well-differentiated endometrial adenocarcinoma cell line, Ishikawa cells [2], from a 39-year-old Japanese patient. This cell line has the special characteristic of bearing estrogen and progesterone receptors (ER and PR, respectively). I would like to describe the establishment and characteristics of Ishikawa cells.

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Methods

On 14 October 1980, we operated on a 39-year-old parous female diagnosed with endometrial adenocarcinoma stage 2. She visited Tsukuba University Hospital with a chief complaint of vaginal bleeding. She had taken birth control pills for several years after giving birth to her second child at age 29. She underwent chemotherapy after surgery and left the hospital on 27 December. She is currently healthy and has not suffered a recurrence.

From the uterus, we took a tiny specimen for primary culture, which ultimately resulted in the Ishikawa cells. The tumor was so fragile that the epithelial part was easily divided from the stroma. Epithelial parts of the tumor were rinsed twice with a solution of antibiotics, minced, and stirred in phosphate-buffered saline (PBS) (−) containing 0.25% trypsin at room temperature. The trypsin solution was changed every 10 min and mixing was maintained. The solution was then centrifuged at 1000 r.p.m. (190 g) for 5 min. The sediment was resuspended in growth medium, placed in 6-cm plastic dishes, and incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide in air. The growth medium used was Eagle's minimum essential medium (MEM; Nissui, Tokyo, Japan) supplemented with 15% fetal bovine serum (FBS; Flow Laboratories, Irvine, Scotland). The medium was changed twice a week. A solution of 0.02% EDTA and 0.1% trypsin was used for subculturing in order to exfoliate the cells from the dish. From 4 November, cells were cultured in a closed manner.

Morphological Study

The original tumor and mouse grafts were fixed with 10% formalin solution, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin (HE). The cultured cells were observed with a phase-contrast microscope, fixed with 95% ethanol solution, and stained with Papanicolaou's solution.

Growth Characteristics

To study the growth curve (GC), approximately 3×10^5 cells/5 mL were placed into 6-cm plastic dishes and cultured for 14 days. Cells were counted every 2 days and the GCs were drawn. The population doubling time (DT) and saturation density (SD) were determined from the GC.

Chromosome Analysis

For chromosomal analysis, approximately 4×10^6 cells/10 mL were placed into 10-cm plastic dishes and cultured for 3 days. On the 3rd day, 0.1 μg/mL Colcemid (GIBCO Laboratories, Gaithersburg, MD, USA) was added and incubated for 2 h. Cells were exfoliated by 0.25% trypsin, flame fixed, and stained with 15% Giemsa for 15 min.

Heterotransplantation

Approximately 1×10^7 suspended cells were implanted subcutaneously into a BALB/c-nu mouse (5-week-old female). The tumors were spherical and the average length of the tumors was measured every week with calipers. The size of the grafts (maximum

length of tumor × minimum diameter^2/2) was measured every week after transplantation. Mice were killed after 8 weeks and the tumors were examined histologically.

ER and PR contents of the original and heterotransplanted tumors in nude mice were determined by the dextran-coated charcoal (DCC) assay, as reported by McGuire et al. [3]. The ER and PR contents of in vitro-cultivated cells were also determined in the same manner.

Results

Histopathology of Tumor

The uterus was larger than a human fist and the tumor covered the entire surface of the uterine cavity, protruding in a papillary fashion. Myometrial invasion exceeded one-half of the uterine wall, but there was no metastasis to lymph nodes (Fig. 1). Histologically, most of the tumor was well-differentiated adenocarcinoma, with a small poorly differentiated part, and was accompanied by squamous metaplasia. The tumor was positively stained by periodic acid-Schiff (PAS) and Alcian blue stain (Fig. 2).

Establishment of the Cell Line

Initially, both epithelial colonies and a few fibroblasts were present in the primary culture. However, the epithelial cells grew rapidly and pure epithelial cells were soon obtained from the primary culture without the colony isolation technique. On the 6th

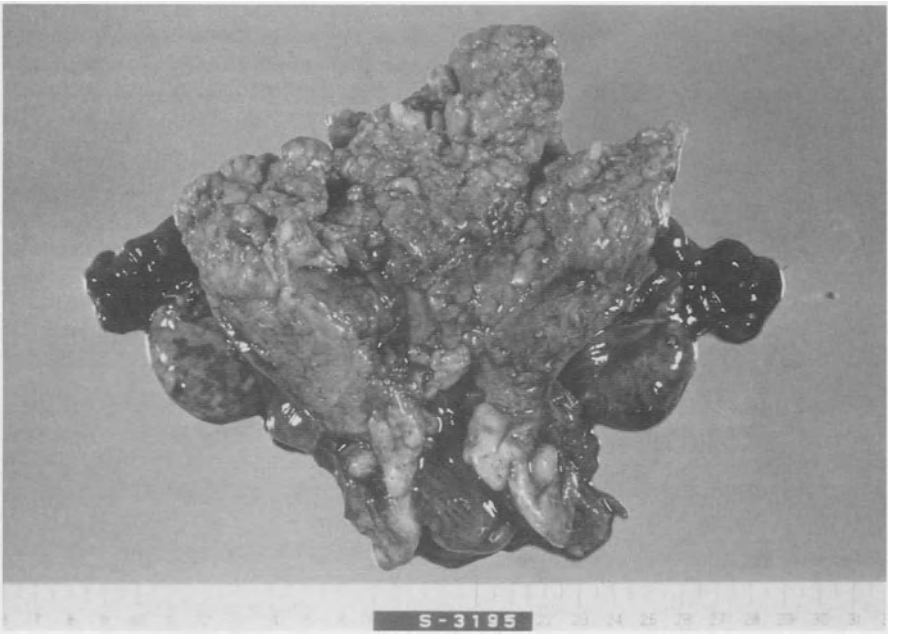

FIG. 1. Primary tumor of Ishikawa cells

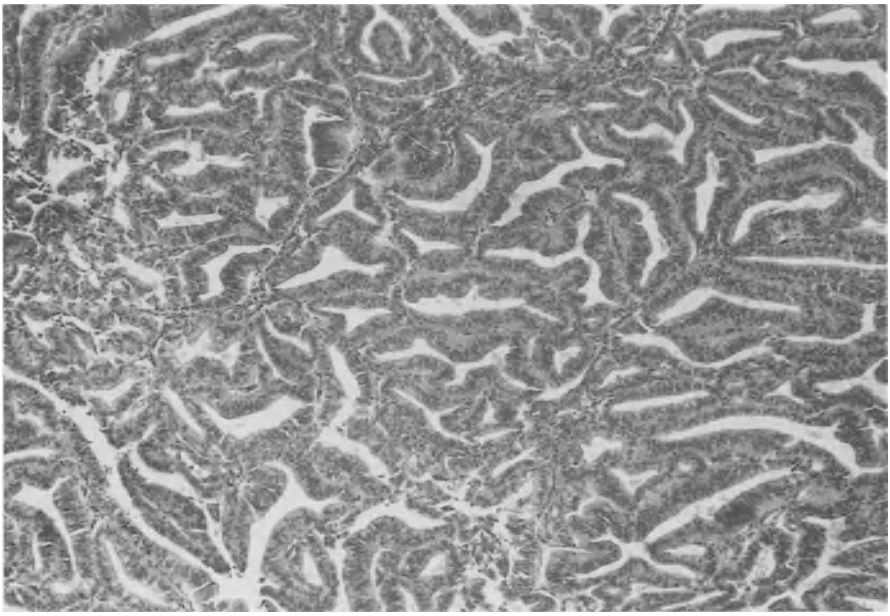

FIG. 2. Histologic findings of the primary tumor, well-differentiated adenocarcinoma

day of culture, the first subculture was performed. The Ishikawa cells then grew without interruption for more than 20 years.

Morphology of Cultured Cells

Cells are arranged in a monolayer fashion. At confluence, cells take on a botryoidal appearance and dome structures are prominent. The borderlines of the cells are well demarcated. The cells were polygonal and showed pleomorphic features, such as fine chromatin granules, irregularly shaped nuclei, and small prominent nucleoli. These cells often have small vacuoles, giving the impression that mucus was produced within the cells (Fig. 3). With Papanicolaou staining, the cytoplasm appeared foamy and pale, and the circumscribed border was indistinct. Nuclei were pleomorphic but nuclear chromatin glanules were fine and evenly distributed. The cytoplasm stained weakly with PAS and Alcian blue (Fig. 4). Although these morphological findings were constant until approximately the 45th generation, characteristic findings of undifferentiated cells, such as cells becoming the same size and showing indistinct borders, as well as having fewer vacuoles and granules, have been recognized since.

Growth Characteristics

The population DT of Ishikawa cells was studied at passages 9, 40, and 50. The growth rate accelerated with increasing passage number. The population DT was 36, 29, and 27 h at passages 9, 40, and 50, respectively.

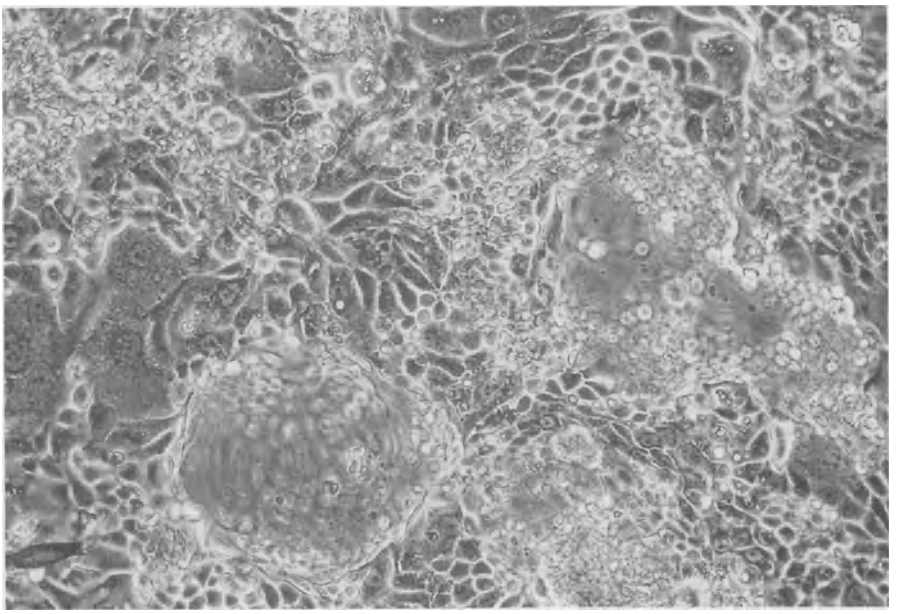

FIG. 3. Phase-contrast microscopic findings of the Ishikawa cells in vitro

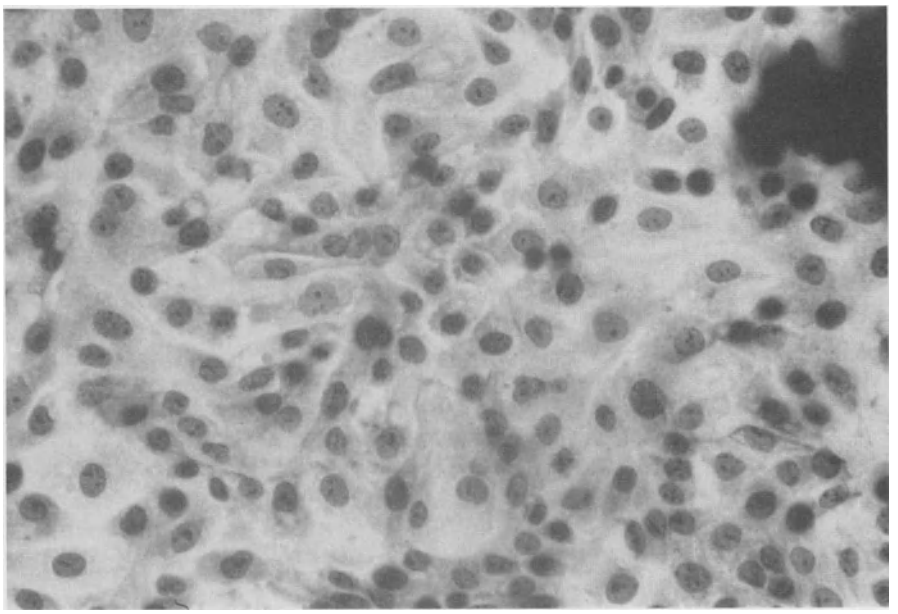

FIG. 4. Microscopic findings of the Ishikawa cells in vitro (Papanicolaou stain)

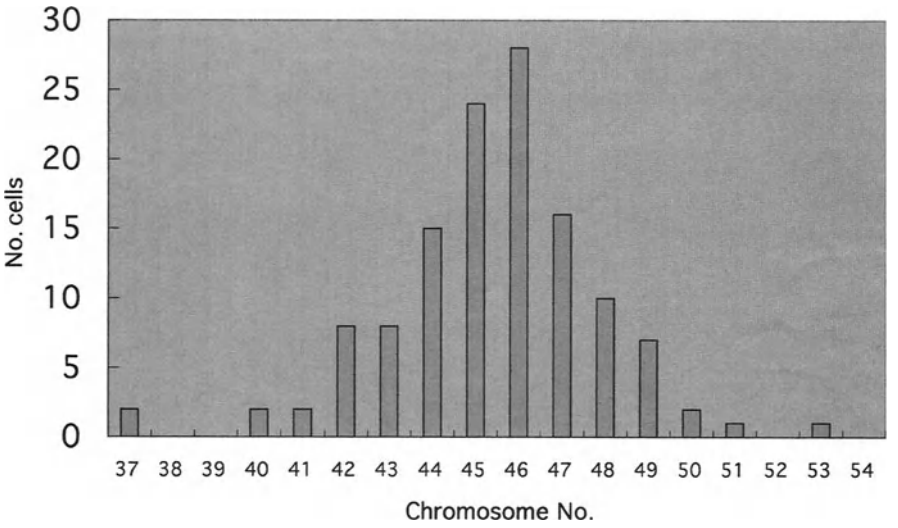

FIG. 5. Distribution of chromosome number in the diploid range, showing 46 as the peak

Chromosome Analysis

Chromosome analysis was performed at passage 22. Histograms of chromosome number distribution were determined from 166 metaphases. The chromosome number varied widely, from 19 to 163. However, the modal chromosomal number was (125/166) of the diploid range. Chromosomal distribution in modal number is shown in Fig. 5 (46 is the peak). One of the karyotypes in the diploid range is shown in Fig. 6.

Heterotransplantation

Ishikawa cells (1×10^7) injected into the subcutis of nude mice produced tumors in all transplanted nude mice. The transplanted tumor did not increase in size after the tumor measured 1000 mm^3. The grafts were solid tumor and interpreted histopathologically as well-differentiated endometrial adenocarcinoma with PAS- and Alcian blue-positive substances in the glands (Fig. 7). Tumor GCs for the tumor tissue transplanted into five nude mice are shown in Fig. 8. When the tumor volume reached 1000–2000 mm^3, growth plateaued. The tumor DT was calculated from the GCs as 2.7 days. The tumor tends to ulcerate on the surface and necrose in the center after tumor growth stops.

Estrogen and Progesterone Receptors

ER and PR were positively detected in tumors transplanted at the 25th passage into nude mice. The ER and PR values were 238 and 50 fmol/mg protein, respectively. In contrast, concerning in vitro cultured cells, we attempted to detect receptors under various culture conditions, as shown in Table 1. ER and PR were detected in the early

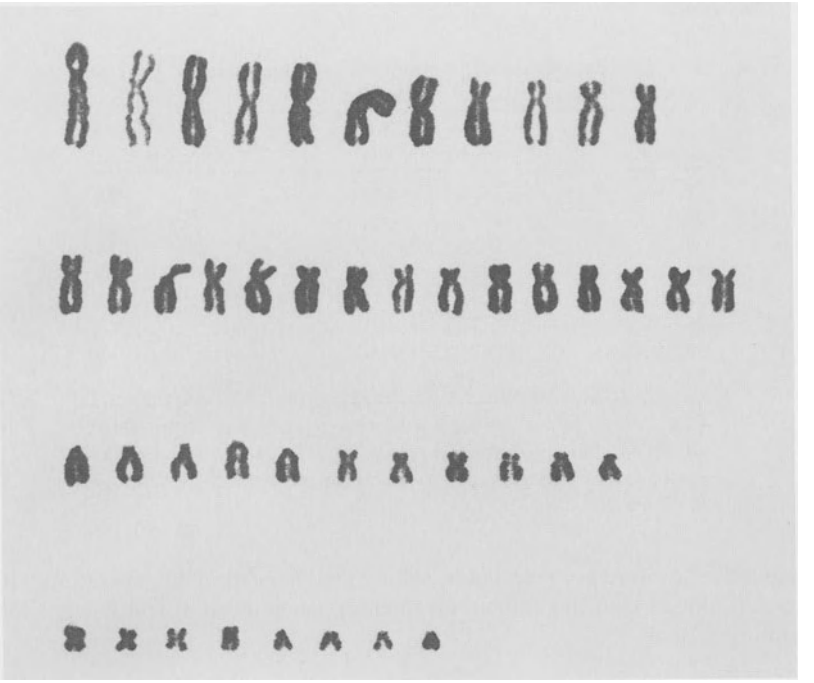

FIG. 6. Karyotype of Ishikawa cells

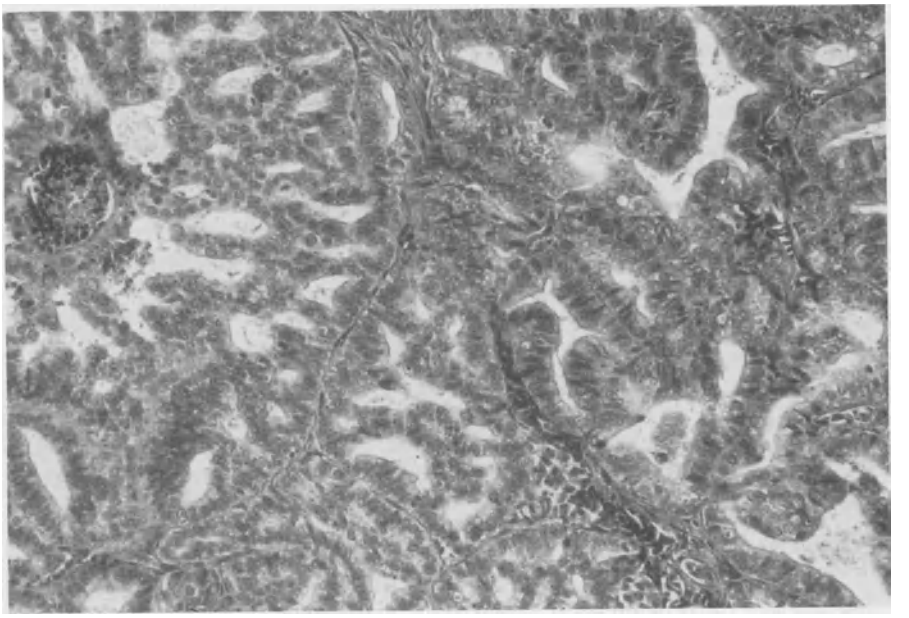

FIG. 7. Histologic findings of heterotransplanted tumor into nude mice

TABLE 1. Estrogen and progesterone receptor values of Ishikawa cells in vitro under various conditions

Generation	Culture conditions	ER (fmol/mg protein)	PR (fmol/mg protein)
28	DCCFCS + HMG	—	18
28	DCCFCS	—	—
30	DCCFCS	103	14
30	DCCFCS + HMG	47	30
33	FCS	26	9
36	DCCFCS + HMG	91	—
50	DCCFCS + HMG	—	—
50	DCCFCS	—	—

FCS, culture media prepared with 15% fetal bovine serum (FBS); *DCCFCS*, culture media prepared with 15% FBS treated with dextran-coated charcoal; *HMG*, prepared with human menopausal gonadotropin at 100 mIU/mL

passage cells, but were not detected in culture cells over the 50th generation. ER and PR tended to increase when human menopausal gonadotropin (HMG) was added to the culture media.

Single Cell Cloning of Ishikawa Cells

The cultured cell system is an excellent experimental system that presents innumerable advantages. In addition, if the cells are of human origin, the results obtained are of much more value in human medicine than those obtained using cells of animal origin. In contrast, the minimum requirement for a cell system is that it should yield constant, reproducible results. However, cultured cells undergo gradual but steady changes after repeated subcultures.

The Ishikawa cell line, which was established from a well-differentiated human endometrial carcinoma and bears both ER and PR, tends to transform into undifferentiated cells after long-term culture. Because the stock of well-differentiated cells preserved in liquid nitrogen is destined to be exhausted, we cloned Ishikawa cells using the limiting dilution method to preserve well-differentiated cells [4].

Methods

Cells

Ishikawa cells from the 25th passage, which had been frozen on 12 May 1982, were thawed and cultured as monolayers. After several subcultures, cells were cloned using the limiting dilution method.

Cloning

Cells were suspended in MEM supplemented with 15% FBS at a cell density of 3 cells/mL. The cell suspension was dispensed into three 96-well flat-bottomed

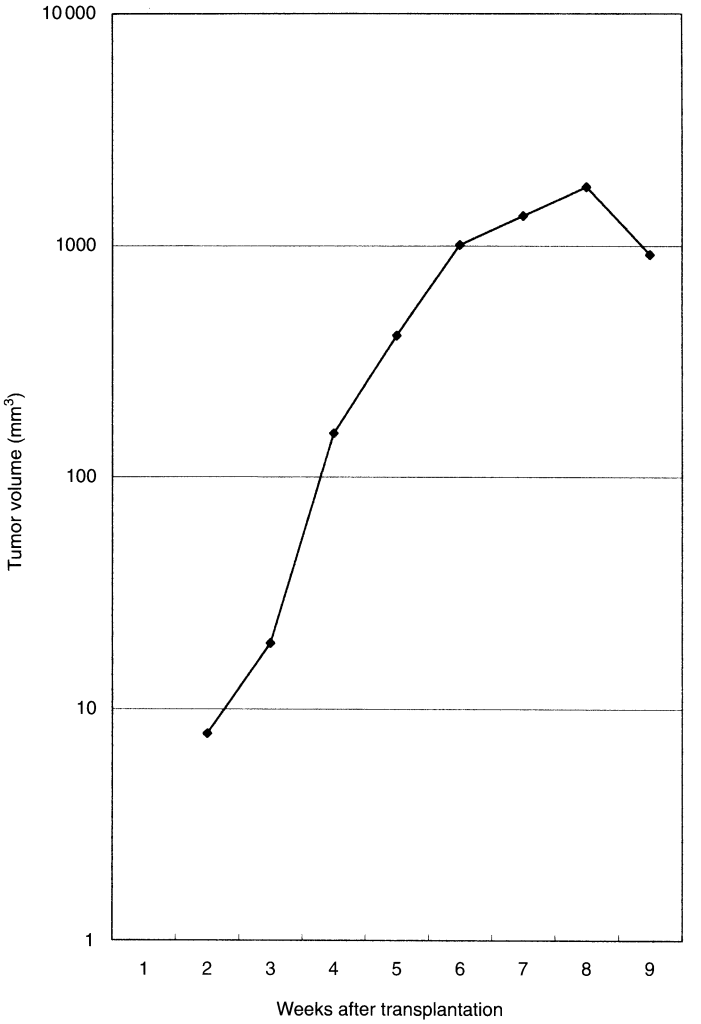

FIG. 8. Growth curve of the tumor transplanted into nude mice

microplates (Falcon, Becton-Dickinson, Oxnard, CA, USA) at a rate of 200 µL/well; the plates were then placed in a CO_2-incubator filled with 5% CO_2 and 95% air. The plates were examined under a phase-contrast microscope every day to detect the colonies formed. The wells in which aggregates of two or more cells were detected immediately after seeding and those in which two or more colonies were found thereafter were excluded from the experiment. When the cells started to proliferate, the medium was renewed as required. When the colonies covered more than two-thirds of the bottom of the wells, the cells were dispersed with 0.02% EDTA plus 0.1% trypsin solution and were seeded in petri dishes (ø 3.5 cm, Lux; NUNC, Naperville, IL, USA). When

they formed a confluent monolayer, cells were transferred to plastic petri dishes (ø 6 cm, Lux) and finally to a plastic flask (Corning Costar, Cambridge, MA, USA) to continue culture in a closed system.

A code number was used to distinguish each cell line.

Microscopic Characteristics

Cloned cells were compared with those of the tumors, obtained by transplanting these cells subcutaneously onto the back of nude mice, with regard to their morphological characteristics under a phase-contrast microscope.

Heterotransplantation

Cloned cells (approximately 1×10^7) were injected subcutaneously on both sides of the dorsum of 5-week-old female BALB/c nude mice to check their transplantability and examine the histological characteristics of the transplanted tumors. When tumors did not form after the first trial, transplantation was attempted several times.

Determination of ER and PR

The tumors thus formed in the nude mice were allowed to grow up to a certain size and were then fixed in formalin for morphological examination; half of each tumor was preserved at –80°C and then assayed for the presence of ER and PR according to the method reported previously [1].

Growth Kinetics

After trypsinization, cells were washed in PBS and suspended in culture medium at a cell density of 1×10^5 cells/mL; 3×10^5 cells was seeded in plastic petri dishes (ø 60 mm, Lux) and grown in duplicate in a humidified atmosphere containing 5% CO_2 and 95% air. Every 2 days, cells from two dishes were counted and the mean value was plotted to obtain the GC. The population DT and SD were calculated from the GC.

Plating Efficiency

Cells were seeded into plastic dishes at a density of 300 cells/dish (ø 6 cm, Lux). Colonies visible after 10 days of culture were counted and the plating efficiency was expressed as a percentage.

Results

Characteristics of Cloned Cells

Eighteen clones were obtained (Table 2). Regarding their degree of differentiation in vitro, there were four well-differentiated clones, eight moderately differentiated clones, and six poorly differentiated clones. Specifically, the well-differentiated clones consisted of cells with distinct pleomorphism, varying in size, but showing clear-cut

TABLE 2. Clone characteristics

Clone code	Differentiation		ER[a]	PR[b]	PIS[c]	PDT (days)	SD (h)	CFRF ($\times 10^4$ cm^2) (%)
	In vivo[d]	In vitro[e]						
1-A-9	WD	WD	89	116	52	34.3	91.4	35.9
2-F-4	WD	WD	183	697	55	31.8	64.0	37.7
3-D-7	WD	MD	0	319	20	25.2	35.5	24.4
3-D-9	WD	WD	0	NI	20	40.8	25.0	7.2
3-H-4	WD	MD	365	325	20	31.6	31.9	39.6
3-H-7	WD	MD	36	251	20	26.4	44.5	18.9
1-C-10	MD	MD	145	881	20	31.2	27.6	24.4
1-F-6	MD	MD	70	675	52	37.9	64.0	38.2
1-H-6	MD	MD	117	873	52	40.1	38.1	28.8
2-C-3	MD	MD	61	1000<	69	26.4	64.6	28.4
2-E-7	MD	PD	NI	156	20	38.4	49.3	27.8
3-H-3	MD	MD	31	352	69	44.4	26.9	17.7
3-H-12	MD	PD	146	574	69	24.0	37.9	34.7
1-E-8	PD	PD	70	308	20	38.9	58.5	31.7
2-B-12	PD	PD	155	74	20	36.5	77.7	11.8
2-C-7	PD	PD	16	NI	69	38.4	40.3	11.3
1-A-12	ASC	WD	23	59	52	40.8	34.9	13.7
1-D-9	ASC	PD	172	457	52	35.0	64.0	30.6

[a] Estradiol-binding capacity (fmol/mg cytosol protein)
[b] Progesterone-binding capacity (fmol/mg cytosol protein)
[c] Period from inoculation to subculture (days)
[d] Heterotransplanted tumor
[e] Cloned cells in vitro
PDT, population doubling time; *SD*, saturation density; *CFR*, colony formation rate; *WD*, well-differentiated type; *MD*, moderately differentiated type; *PD*, poorly differentiated type; *ASC*, adenosquamous carcinoma; *NI*, not investigated

borderlines, giving the impression that the cells were thick. Furthermore, these cells often had small vacuoles; giving the impression that mucin was produced within the cells (Fig. 9). When monolayers become confluent, cells pile up as a bunch of grapes and the borderlines of the cells are well demarcated (Fig. 10). In contrast, poorly differentiated clones were characterized by cells of almost the same size and indistinct borders. Moreover, the cell structures were hard to distinguish, giving the impression that the cells were thin. Furthermore, these cells had fewer vacuoles and granules than the well-differentiated clones (Fig. 11). When they become confluent, the piled-up cells run parallel to each other, like piled-up fibroblasts; individual cells are not clearly distinguishable and the borders of the cells between the areas of high cell density and the monolayer are indistinguishable (Fig. 12). The moderately differentiated clones had features in between the highly differentiated and poorly differentiated clones (Fig. 13).

Morphology of Heterotransplanted Tumors

All 18 clones are transplantable into nude mice. However, transplantability varies from clone to clone. Three clones were transplanted successfully at the first trial, whereas

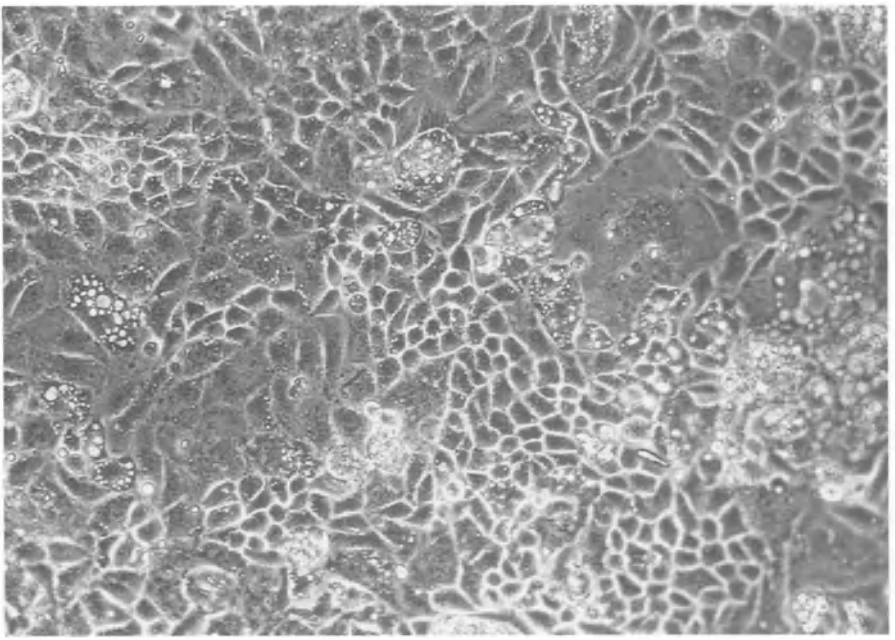

FIG. 9. Phase-contrast microscopic findings presumed to be of the well-differentiated type (3-D-9). Pleomorphism, clear-cut borderlines, distinct contrast of various parts of each cell, and cells of different sizes can be observed. These cells often have small vacuoles of mucin (original magnification ×100)

five clones could be transplanted after more than five trials. Most formed solid tumors, whereas one clone (2-E-7) formed a small tumor that tended to form cysts.

Four different histological types were obtained (Table 2). These were six tumors of well-differentiated adenocarcinoma cells (Fig. 14), seven of moderately differentiated cells (Fig. 15), three of poorly differentiated cells (Fig. 16), and two of adenosquamous carcinoma cells (Fig. 17).

Morphological Comparison Between Cells Propagated In Vitro and Those Propagated In Vivo

Histological findings in the transplanted tumor were compared with the morphological characteristics of cells examined under a phase-contrast microscope in vitro (Table 2). Of the four clones found to be well differentiated in vitro, three formed well-differentiated tumors after transplantation into nude mice and one formed an adenosquamous carcinoma. Of the eight clones found to be moderately differentiated in vitro, three formed well-differentiated tumors and five formed moderately differentiated tumors. Of the six clones found to be poorly differentiated in vitro, two formed moderately differentiated tumors after transplantation, three formed poorly differentiated tumors, and one formed an adenosquamous carcinoma in vivo.

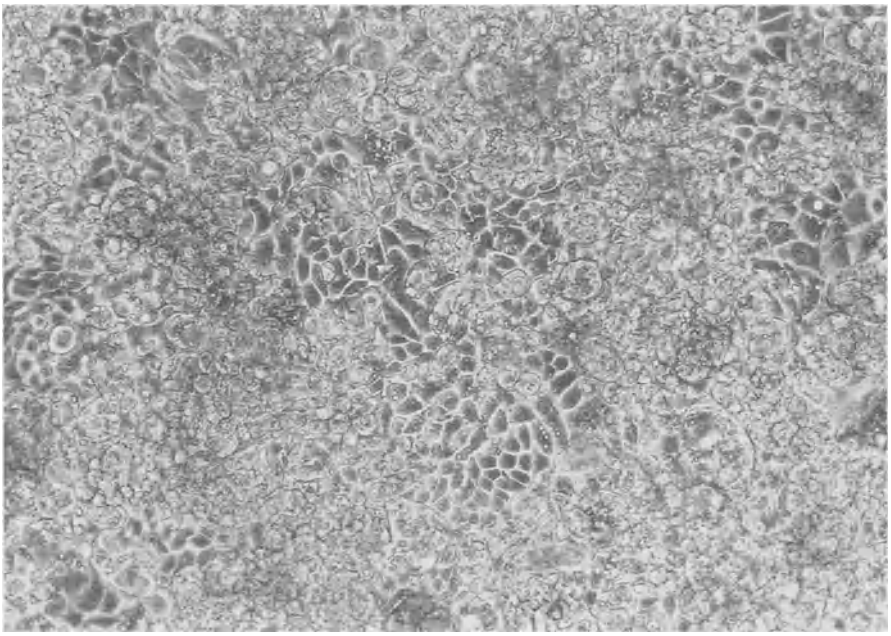

FIG. 10. Phase-contrast microscopic findings presumed to be of the well-differentiated type (3-D-9). When the cells become confluent, they pile up as a bunch of grapes and the borderlines of the cells are well demarcated (original magnification ×100)

Hormone Receptors

Not all heterotransplanted tumors were analyzed for the presence of ER and PR because some were not large enough. ER were investigated in 17 of 18 tumors (not in 2-E-7) and only two clones (3-D-7 and 3-D-9) were ER negative (Table 2). The mean value of ER was 102.3 fmol/mg protein and the maximum was 365 fmol/mg protein. As for the relationship between histological differentiation and the amount of ER, the mean value of ER in the well-differentiated, moderately differentiated, and poorly differentiated types of tumor, and in the adenosquamous carcinoma was 112.2 ± 141.7, 105.0 ± 58.5, 80.3 ± 70.1, and 97.5 fmol/mg protein, respectively, showing no significant differences among the four tumor types.

PR were examined in 16 of 18 clones and all clones were positive for the PR (Table 2). PR values were generally higher than those for the ER, with a mean value of 444.8 fmol/mg protein; the PR value in the 2-C-3 clone was more than 1000 fmol/mg protein. The PR value in the well-differentiated, moderately differentiated, and poorly differentiated types of tumor, and in the adenosquamous carcinoma was 341.6 ± 215.8, 644.4 ± 306.8, 191.0 ± 165.5, and 518.0 fmol/mg protein, respectively, showing no significant differences among the four tumor types.

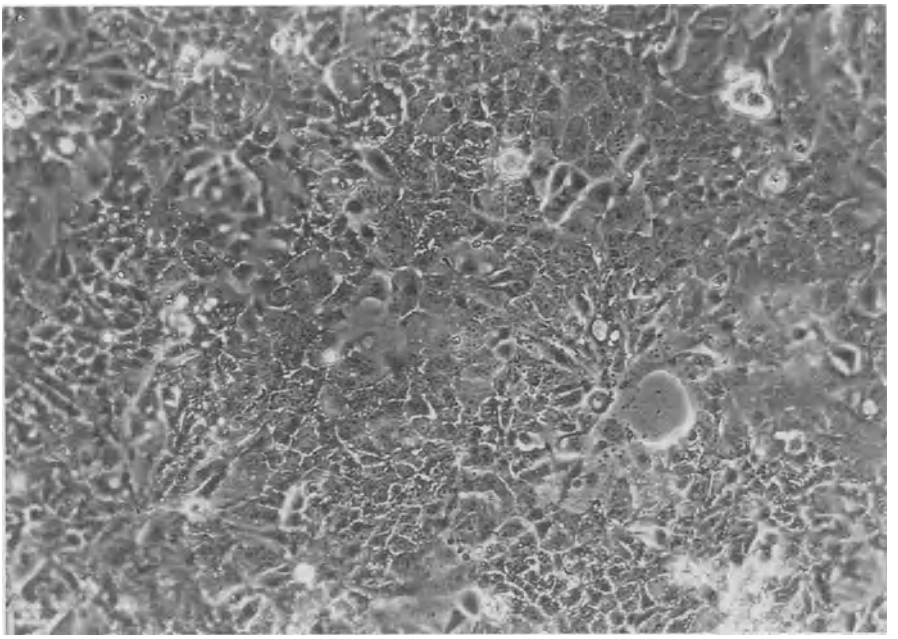

FIG. 11. Phase-contrast microscopic findings presumed to be of the poorly differentiated type (2-B-12). The cells are almost the same size and have indistinct borders. The cell structures are hard to distinguish. These cells have fewer vacuoles and granules than well-differentiated cells (original magnification ×100)

Growth Kinetics

Transfer from microplates to petri dishes was possible 20 days after cloning in eight cases, but in other cases it took between 52 and 69 days (Table 2).

The shortest population DT was 24.0 h and the longest was 44.4 h (Table 2). The mean DT of six well-differentiated clones and seven moderately differentiated clones was 31.7 ± 5.7 and 34.6 ± 7.6 h, respectively, whereas that of three poorly differentiated clones and two adenosquamous carcinoma clones was 37.9 ± 1.3 and 37.9 h, respectively. The population DT tended to be shorter for more differentiated clones, although there was no significant difference among the four types of tumor. The average population DT of the clone subcultured 20 days after cloning was 33.6 h; in case of those subcultured at 52 and 69 days, the population DT was 37.6 and 33.3 h, respectively, showing no firm relationship between the population DT and time elapsed before the first subculture.

The highest SD was 91.4×10^4 cells/cm^2 (clone 1-A-9) and the lowest was 25×10^4 cells/cm^2 (clone 3-D-9). The mean (± SD) density for well-differentiated, moderately differentiated, and poorly differentiated clones was $48.7 \pm 24.9 \times 10^4$, $44.1 \pm 15.7 \times 10^4$, and $58.8 \pm 18.7 \times 10^4$ cells/cm^2, respectively, showing no significant differences among them. The overall mean value was 48.6×10^4 cells/cm^2.

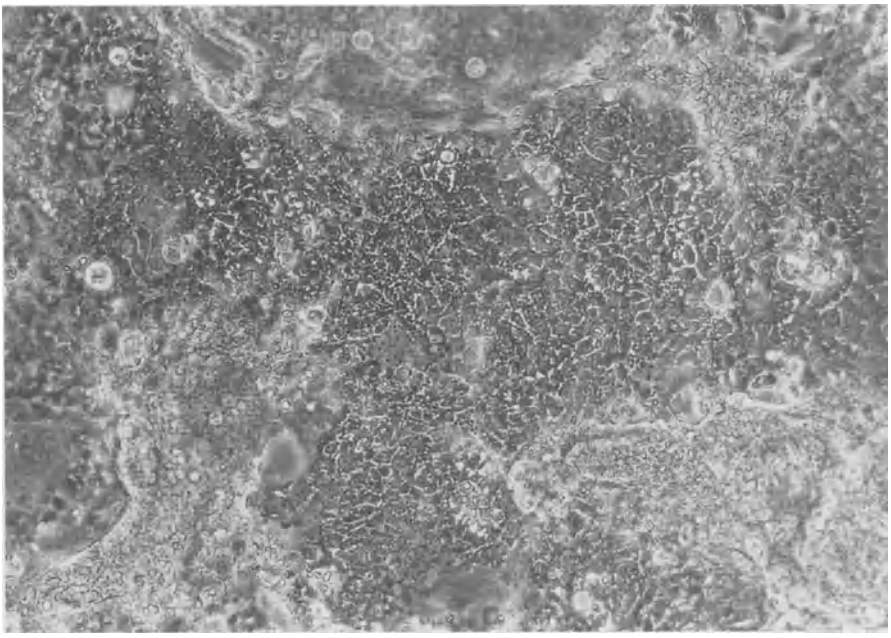

FIG. 12. Phase-contrast microscopic findings presumed to be of the poorly differentiated type (2-B-12). When the cells become confluent, the piled-up cells run parallel to each other, like piled-up fibroblasts; individual cells are not clearly distinguishable and the borders of the cells between the areas of high cell density and the monolayer are indistinguishable (original magnification ×100)

Plating Efficiency

There were significant differences among the clones regarding colony formation rate. The colony formation rates were distributed from 7.2% to 39.6% and the mean colony formation rate was 25.7% (Table 2). The colony formation rate for well-differentiated, moderately differentiated, and poorly differentiated clones was 27.3 ± 12.8%, 28.6 ± 6.7%, and 18.3 ± 11.6%, respectively. There were no significant differences among the three types.

Distribution of Ishikawa Cells

Ishikawa cells have been distributed to all investigators who wished to use the cells for their research. At first, parent Ishikawa cells were distributed but, after the cloned cells were established, subclone 3-H-4 was distributed from 1993 and subclone 3-H-12 has been distributed from 1996 until the present day. A serial number was put on each of the subclones (Table 3). A Medline search over these past 10 years using the search term 'Ishikawa cells' revealed approximately 220 publications.

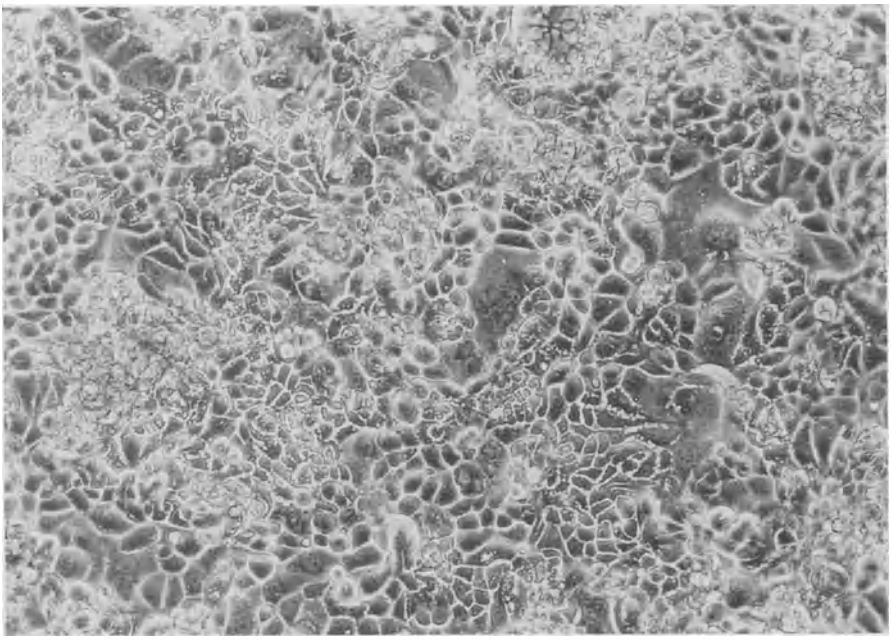

FIG. 13. Phase-contrast microscopic findings presumed to be of the moderately differentiated type (3-H-3). The moderately differentiated clones had features in between the highly differentiated and poorly differentiated clones (original magnification ×100)

Discussion

Morphological Heterogeneity of Established Cell Lines

Eighteen clones with different degrees of cell differentiation were obtained. Therefore, we assume that cells, even those of an established cancer cell line that are supposed to have a high degree of homogeneity, show morphological heterogeneity.

Morphological Characteristics In Vitro

When adenocarcinoma tissues are of the well-differentiated type, the cancer cells would aggregate to form a glandular or a papillary structure that secretes mucin. These morphological characteristics were reflected in vitro in the form of secretory granules within the cytoplasm and the clear borderlines of individual cells. On the basis of these findings, we distinguished well-differentiated clones from poorly differentiated clones.

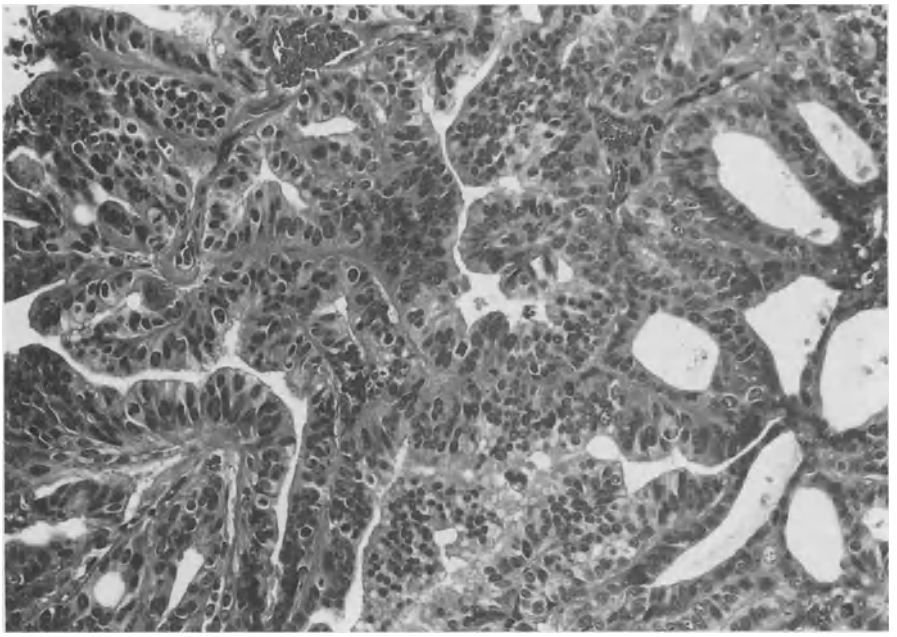

FIG. 14. Histological findings of the tumor obtained after transplantation of 3-H-4 cells, a well-differentiated clone, into nude mice (original magnification ×100)

Differences Between Endometrial Carcinoma of the Poorly Differentiated Type and Clones of Poorly Differentiated Cells

Clinically, it has been reported that poorly differentiated endometrial adenocarcinomas have small levels of both ER and PR [5–7]. Of the clones established in the present study, three were adenocarcinomas of the poorly differentiated type and two were positive for both ER and PR. On clone (2-C-7) was positive for ER only, but no further analysis was possible. On the basis of this finding, we consider that the poorly differentiated adenocarcinoma cells derived from well-differentiated cells after long-term culture are different from cells derived from primary poorly differentiated adenocarcinomas.

Histological Grading and Steroid Hormone Receptors

It has been reported that well-differentiated endometrial cancer cells contain high levels of ER and PR and that these levels are decreased in poorly differentiated cells [5–7]. Of the 18 clones we obtained, 17 were examined for ER and 15 tested as positive, whereas all 16 clones examined for PR were positive. Regarding their degree of differentiation, there were no significant differences among the mean values of ER or PR for well-differentiated clones, moderately differentiated clones, and poorly

TABLE 3. Scientists who have received Ishikawa cells

Scientists	Institutes	Date of distribution
H. Kuramoto	Dept. OB&GYN, School of Medicine, Kitasato Univ.	1981.9.29
S. Uehara	Dept. OB&GYN, School of Medicine, Tohoku Univ.	1981.11.27
I. Nishiya	Dept. OB&GYN, Iwate Medical Univ.	1982.4.22
N. Terakawa	Dept. OB&GYN, Faculty of Medicine, Osaka Univ.	1983.5.24
M. Nanba	Dept. Pathology, Kawasaki Medical School	1983.12.14
N. Terakawa	Dept. OB&GYN, Faculty of Medicine, Osaka Univ.	1984.3.3
Y. Tsuji	Dept. OB&GYN, Hyogo College of Medicine	1984.3.3
A. Takeda	Laboratory of Ultrastructure Research, Aichi Cancer Center Research Institute	1984.4.12
M. Inoue	Dept. OB&GYN, Faculty of Medicine, Osaka Univ.	1984.6.5
R. Kudo	Dept. OB&GYN, Sapporo Medical Univ.	1984.7.2
E. Gurpide	The Mount Sinai Medical Center, USA	1984.9.10
H. Kanma	Dept. Pathology, Institute of Basic Medical Science, Univ. of Tsukuba	1984.11.29
H. Kanma	Dept. Pathology, Institute of Basic Medical Science, Univ. of Tsukuba	1984.12.22
R. Kudo	Dept. OB&GYN, Sapporo Medical Univ.	1985.1.23
K. Hayashi	National Defense Medical College	1985.9.3
N. Terakawa	Dept. OB&GYN, Faculty of Medicine, Osaka Univ.	1985.10.23
N. Terakawa	Dept. OB&GYN, Faculty of Medicine, Osaka Univ.	1985.11.6
N. Terakawa	Dept. OB&GYN, Faculty of Medicine, Osaka Univ.	1985.11.13
H. Tejima	Dept. OB&GYN, Faculty of Medicine, Osaka Univ.	1985.11.20
H. Kuramoto	Dept. OB&GYN, School of Medicine, Kitasato Univ.	1985.12.18
N. Terakawa	Dept. OB&GYN, Faculty of Medicine, Osaka Univ.	1985.12.20
A. Takeda	Laboratory of Ultrastructure Research, Aichi Cancer Center Research Institute	1986.4.23
J. Kitawaki	Medical Foundation of Baffalo Inc., USA	1986.6.11
H. Noguchi	Dept. OB&GYN, Shinshu Univ., School of Medicine	1986.6.14
N. Terakawa	Dept. OB&GYN, Faculty of Medicine, Osaka Univ.	1986.6.25
R. Lyttle	Univ. of Pensylvania School of Med., USA	1986.6.25
S. Tanaka	Dept. OB&GYN, Sapporo Medical Univ.	1986.10.14
R. Kudo	Dept. OB&GYN, Sapporo Medical Univ.	1986.12.10
K. Sato	Dept. OB&GYN, Iwate Medical Univ.	1987.2.4
N. Terakawa	Dept. OB&GYN, Faculty of Medicine, Osaka Univ.	1987.4.30
M. Noguchi	Dept. OB&GYN, Aichi Medical Univ.	1987.5.28
A. Oliff	Merck Sharp & Dohme Research Laboratories	1987.6.4
H. Noguchi	Dept. OB&GYN, Shinshu Univ., School of Medicine	1987.6.24
S. R. Glasser	Texas Medical Center, Cell Biology, USA	1987.11.26
B. Green	Bioscience and Biotechnology, Univ. Strathclyde, UK	1988.3.7
N. Terakawa	Dept. OB&GYN, Faculty of Medicine, Osaka Univ.	1988.5.18
M. Noguchi	Dept. OB&GYN, Aichi Medical Univ.	1988.5.18
T. Yamamoto	Dept. OB&GYN, Kyoto Prefectural Univ. of Medicine	1988.10.11
K. Katase	Dept. Gynecology, Cancer Institute Hospital	1988.10.26
G. Davis	Ludwig Inst. for Cancer Res. Middlesex/Univ. xzC College Branch, England	1988.11.8
R. W. Kelly	Med. Res. Council Reproductive Biology Unit, Edinborough, UK	1988.11.28
T. Idutsu	Dept. OB&GYN, Iwate Medical Univ.	1988.11.30
R. Knuppen	Inst. fur Biochemische Endokrinologie Medizinisch Univ.,West Germany	1989.1.25
Y. Yaginuma	Health & Human Services, NIH, USA	1989.8.28

TABLE 3. *Continued*

Scientists	Institutes	Date of distribution
K. Kasahawa	Dept. OB&GYN, Tokyo Kosei-Nenkin Hospital	1989.9.26
H. Kuramoto	Dept. OB&GYN, School of Medicine, Kitasato Univ.	1989.10.11
J. Aplin	Manchester Univ., UK	1989.11.8
H. Nagasaka	Yamanashi Medical Univ.	1989.11.8
J. Nakamura	Snow Brand Milk Products Co. Ltd	1989.11.8
M. Murarnatsu	Dept. Biochemistry, Faculty of Medicine, Univ. of Tokyo	1989.12.7
I. Sekiguchi	Dept. OB&GYN, Jichi Medical School	1989.12.7
M. Yasumizu	Dept. OB&GYN, Yamanashi Medical Univ.	1990.2.19
K. Takagi	Tokyo Women's Medical Univ.	1990.9.4
M. Nozue	Dept. Surgery, Institute of Clinical Medicine, Univ. of Tsukuba	1990.9.28
K. Kasahawa	Dept. OB&GYN, Tokyo Kosei-Nenkin Hospital	1991.1.16
M. Seppala	OB/GYN Helsinki University	1991.2.6
H. Fushiki	Dept. OB&GYN, Toyama Medical and Pharmaceutical Univ., Faculty of Medicine	1991.2.6
J. F. Randolph Jr.	Univ. of Michigan Medical School	1991.2.6
S. K. Smith	Univ. of Cambridge Clinical School, UK	1991.2.6
M. Seppala	Helsinki Univ. Central Hosp., Finland	1991.2.6
K. Otani	Dept. OB&GYN, Nihon Univ., School of Medicine	1991.4.9
K. Otani	Dept. OB&GYN, Nihon Univ., School of Medicine	1991.5.7
M. Ueda	Dept. OB&GYN, Osaka Medical College	1991.5.7
P. G. Baer	Glaxo Inc. Research Inst.	1991.8.21
	The Jikei University School of Medicine	1991.8.21
K. Nakayama	Biosciences Research Laboratory, Mochida Pharmaceutical Co. Ltd	1991.11.26
M. Suzuki	Dept. OB&GYN, Jichi Medical School	1992.2.14
E. Kita	Dept. Bacteriology, Nara Medical Univ.	1992.2.18
I. Ishiwata	Ishiwata Obstet. & Gynecologic. Hospital	1992.3.31
H. Ikegami	Dept. OB&GYN, Faculty of Medicine, Osaka Univ.	1992.3.31
M. Mochizuki	Dept. OB&GYN, Kobe University School of Medicine	1992.4.28
T. Hamada	Dept. OB&GYN, Ehime University School of Medicine	1992.4.28
T. Fujimoto	Okazaki National Research Institutes	1992.7.28
H. Takahashi	Iwate Prefectual Livestock Experiment Station	1992.12.24
H. Sakamoto	Dept. OB&GYN, Nihon Univ., School of Medicine	1993.4.14
M. Matsuyama	Taisho Pharmaceutical Co. Ltd	1993.4.14
I. Konishi	Dept. OB&GYN, Kyoto Univ., Faculty of Medicine	1993.4.14
K. E. Monagham	Central Research Division, Pfizer Inc.	1993.5.12
G. Vollmer	Lubeck Univ., Germany	1993.5.12
B. Fournier	Ciba-Geigy Ltd Switzerland	1993.5.12
Y. Akao	Dept. Anatomy, Osaka Medical College	1993.5.12
T. Yano	Dept. OB&GYN, Faculty of Medicine, Univ. of Tokyo	1993.5.18
T. Shiozawa	Dept. OB&GYN, Shinshu Univ., School of Medicine	1993.7.27
Subclone 3-H-4		
K. Tsukazaki	Dept. OB&GYN, School of Medicine, Keio Univ.	1993.11.16
M. Matsuyama	Dept. Applied Biology, Research Center, Taisho Pharmaceutical Co. Ltd	1993.11.16
I. Konishi	Dept. OB&GYN, Kyoto Univ., Faculty of Medicine	1993.11.16
T. Tanaka	Dept. Laboratory Medicine, Miyazaki Medical College	1993.11.16
B. Fournier	Ciba-Geigy Ltd Switzerland	1993.12.7
M. Ogino	Dept. OB&GYN, Teikyo University School of Medicine	1993.12.7

TABLE 3. *Continued*

Scientists	Institutes	Date of distribution
S. Sugaya	Dept. OB&GYN, Niigata Univ., Faculty of Medicine	1993.12.7
E. J. Thomas	Univ. of Southampton, UK	1993.12.24
M. Jouvenot	INSERM U., France	1994.2.1
A. F. Stewart	European Molecular Biology Laboratory, Federal Republic of Germany	1994.3.18
H. Sakamoto	Dept. OB&GYN, Nihon Univ., School of Medicine	1994.5.9
T. Yano	Dept. OB&GYN, Faculty of Medicine, Univ. of Tokyo	1994.10.4
R. Dijkema	N. V. Organon, The Netherlands	1994.10.4
R. W. Kelly	MRC Reproductive Biology Unit, UK	1994.10.18
T. Shiozawa	Dept. OB&GYN, Shinshu Univ., School of Medicine	1995.2.24
S. Nishiwaki	Central Research Laboratories, SS Pharmaceutical Co. Ltd	1995.2.24
D. Bertolini	Miles Inc., Bayer, USA	1995.2.24
M. Carson	Clin. Chemist., Meilahti Hosp., Finland	1995.3.7
B. Fournier	Ciba–Geigy Ltd Switzerland	1995.3.7
T. Shiozawa	Dept. OB&GYN, Shinshu Univ., School of Medicine	1995.3.14
Subclone 3-H-12		
T. Shiozawa	Dept. OB&GYN, Shinshu Univ., School of Medicine	1996.3.5
M. Yajima	Dept. OB&GYN, Tokyo Women's Medical Univ.	1996.3.5
F. Yoshikawa	Dept. OB&GYN, Nagoya Univ., School of Medicine	1996.3.5
T. Fujii	Pharmacological Research, Dept., Teikoku Hormone Manufacturing Co. Ltd	1996.3.5
M. Carson	Clin. Chemist., Meilahti Hosp., Finland	1996.3.5
M. Sillem	Gyn. End., Ruprecht-Karls-Univ., Heidelberg	1996.3.5
B. van den Burg	Hubrecht Lab., The Netherlands	1996.3.5
H. Mizunuma	Dept. OB&GYN, Gunma Univ. School of Medicine	1996.3.21
K. Takeda	Dept. Pharmacy, Univ. of Tsukuba	1996.4.5
J. White	OB&GYN, Hammersmith Hospital, UK	1996.5.28
C. F. Singer	ICP, Belgium	1996.6.5
C. Coutifaris	Univ. of Pennsylvania Medical Center	1996.9.10
N. B. Moisson	Centre Alexis Vautrin, France	1996.9.10
Y. Sato	Dept. OB&GYN, Institute of Clinical Medicine, Univ. of Tsukuba	1996.9.10
M. Hori	Dept. Pathology, Ibaraki Prefectual Central Hospital	1996.9.25
B. Cypriani	Univ. de Besancon, France	1996.10.22
T. Kyo	Dept. OB&GYN, School of Medicine, Kanazawa Univ.	1996.10.22
Y. Katsuki	Mochida Pharmaceutical Co. Ltd	1996.12.25
T.-M. Lin	Univ. of Wisconsin, Madison	1997.1.22
A. Darnel	The Lawson Research Institute, Canada	1997.1.28
H. Hiramatsu	Dept. OB&GYN, National Defense Medical College	1997.2.12
E. Koopman	Erasmus University, Rotterdam, The Netherlands	1997.5.20
T. Suzuki	POLA R&D Laboratories, POLA Corporation	1997.5.20
A. M. Cummings	U.S. Environmental Protection Agency, USA	1997.6.17
A. Horii	Dept. Molecular Pathology, Tohoku University School of Medicine	1997.8.26
T. Takeda	Dept. OB&GYN, Faculty of Medicine, Osaka Univ.	1997.9.30
M. Hori	Dept. Pathology, Ibaraki Prefectural Central Hospital	1998.4.13
Y. Ikeda	Dept. Pharmacology, Kanebo Ltd	1998.7.1
E. Koopman	Erasmus University, Rotterdam, The Netherlands	1998.7.1
H. Okada	Dept. OB&GYN, Kansai Medical Univ.	1998.8.5

TABLE 3. *Continued*

Scientists	Institutes	Date of distribution
Y. Tabata	Pharmaceutical Research Center, Meiji Seika Kaisha Ltd	1998.8.5
C. Amezcua	Univ. of Southern California	1998.9.25
H. Sasaki	Dept. OB&GYN, The Jikei University School of Medicine	1998.12.15
S. Kawai	Graduate School of Human Sciences, Kobe College	1999.1.12
M. H. A. Kester	Erasmus University, Rotterdam, The Netherlands	1999.1.14
R. Sasaki	Div. Integrated Life Science, Graduate School of Biostudies, Kyoto Univ.	1999.2.12
I. Oizumi	Pharmaceutical Technology Laboratory II, Chugai Pharmaceutical Co. Ltd	1999.2.12
M. Dowsett	Royal Marsden NHS Trust, Academic Biochem., UK	1999.2.23
P. Amato	Univ. of CA–San Diego	1999.7.13
S. Araki	Tsukuba Research Laboratories, Eisai Co. Ltd	1999.9.2
Y. Nakamura	The Institute of Medical Science, Univ. of Tokyo	1999.9.16
A. Schneyer	Harvard Medical School Reproductive Endocrine Unit	1999.9.22
E. Koopman	Erasmus University, Rotterdam, The Netherlands	1999.10.8
J. Kuwajima	Dept. of Pharmacol., Discovery Res. Lab., Dainippon Pharmaceutical Co. Ltd	1999.11.16
S. Yoshida	Dept. OB&GYN, Tottori Univ. Faculty of Medicine	1999.12.1
D. Singleton	Univ. of Cincinnati	2000.1.26
R. Minami	Dept. Pathology, Institute of Basic Medical Science, Univ. of Tsukuba	2000.4.14
T. Sakamoto	Dept. OB&GYN, Teikyo Univ. School of Medicine	2000.5.19
N. Sakuragi	Dept. OB&GYN, Hokkaido Univ., School of Medicine	2000.9.8
H. Sasaki	Dept. OB&GYN, The Jikei Univ., School of Medicine	2000.9.8
F. Narita	Dept. OB&GYN, Kobe Univ., School of Medicine	2000.9.13
T. Yamamoto	Dept. OB&GYN, Nihon Univ., School of Medicine	2001.2.6
T. Nishihara	Graduate School of Pharmaceutical Sciences, Osaka University	2001.4.9
H. Shigeta	Dept. OB&GYN, Yokohama Municipal Citizen's Hospital	2001.12.25
S. Hayashi	Division of Endocrinology, Saitama Cancer Center Research Institute	2001.12.25
K. Ueki	Dept. OB&GYN, Osaka Medical College	2001.12.25
N. Harada	Dept. Biochemistry, School of Medicine, Fujita Health Univ.	2001.12.25
D. Singleton	Univ. of Cincinnati	2001.12.25
A. Sakurada	Dept. Thoracic Surgery, Institute of Development, Aging and Cancer, Tohoku Univ.	2001.12.25
R. M. Bigsby	Research Labs, USA	2002.5.13
T. Shiozawa	Dept. OB&GYN, Shinshu Univ., School of Medicine	2002.5.13
K. Ikeda	Saitama Medical School Research Center for Genornic Medicine	2002.5.13
J. Hongo	Dept. OB&GYN, Okayama Univ. Medical School	2002.5.23
G. Vollmer	Molekulare Zellphyslologie, Technische Univ., Dresden	2002.10.11
N. Sakurai	Frontier Collaborative Research Center, Tokyo Institute of Technology	2002.10.11
C. Morimoto	Division of Clin. Immunology, Inst. Med. Science, Univ. of Tokyo	2002.10.11
B. A. Lessey	Univ. of North Carolina	2002.10.11
S. W. Jiang	Dept. Int. Medicine, Mayo Clinic	2002.10.11

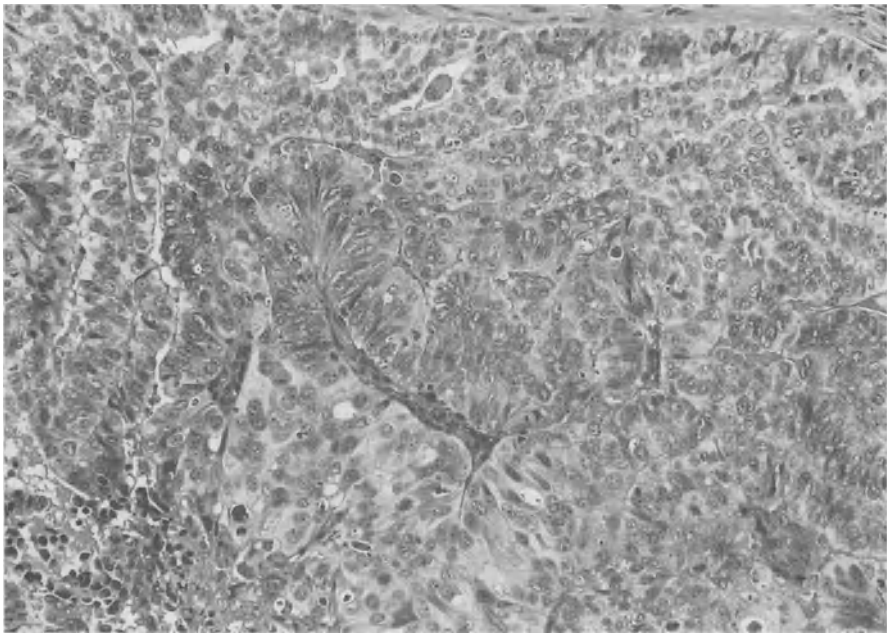

FIG. 15. Histological findings of the tumor obtained after transplantation of clone 1-F-6 cells, a moderately differentiated clone, into nude mice (original magnification ×100)

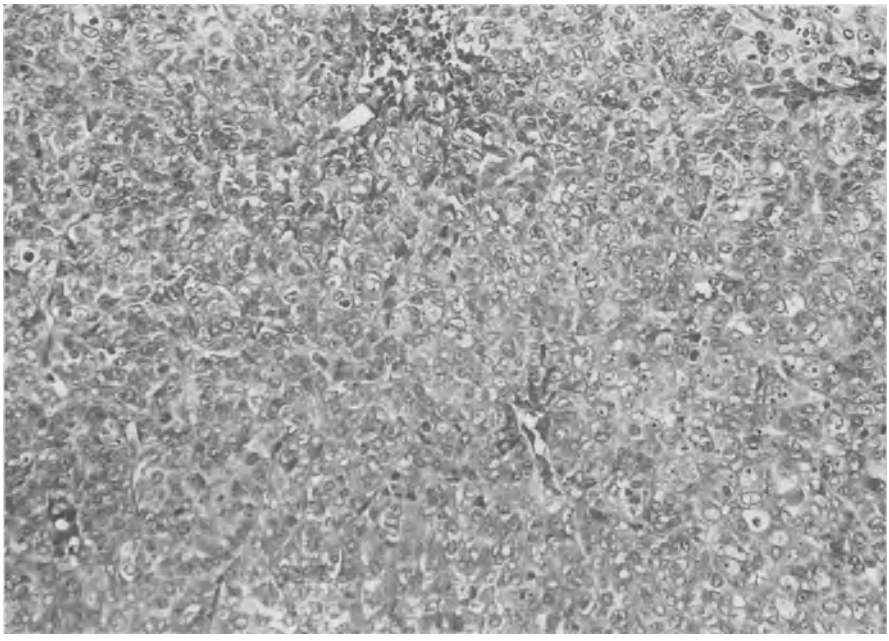

FIG. 16. Histological findings of the tumor obtained after transplantation of clone 2-B-12 cells, a poorly differentiated clone, into nude mice (original magnification ×100)

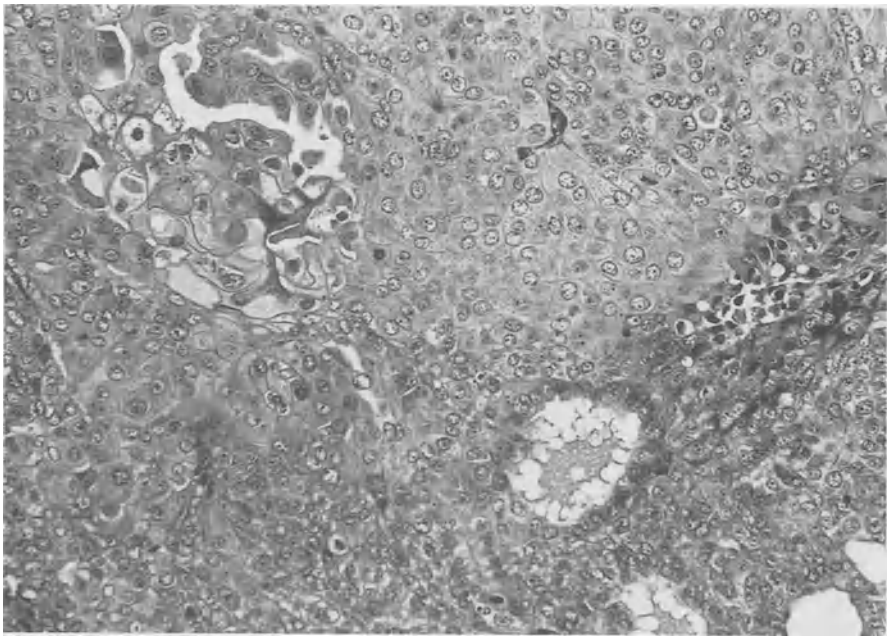

FIG. 17. Histological findings of the tumor obtained after transplantation of clone 1-D-9 cells, a clone of adenosquamous carcinoma cells (original magnification ×100)

differentiated clones. These findings indicate that even if the morphology of the cultured cells changes into that of the poorly differentiated type, they do not be become pure poorly differentiated adenocarcinoma cells.

Genesis of Endometrial Adenosquamous Carcinoma

The original tumor from which Ishikawa cells derived was a well-differentiated adenocarcinoma. A detailed review of the original tumor revealed the presence of squamous metaplasia, in which no signs of malignancy were detected. However, two clones of adenosquamous carcinoma were obtained in the present study. This may suggest that adenosquamous carcinoma of the endometrium could be generated by the ability of multipotential differentiation of single cells.

Cloning as a Method to Preserve the Characteristics of Cells

In general, when compared with the original tumor, the established cancer cells are highly homogeneous. The common belief is that they retain their morphological and physiologic characteristics despite repeated subcultures. However, it is necessary to reconsider this subject. Even when cells from the primary culture or the first transplanted tumor are well differentiated, after long-term subculture they invariably regress to the poorly differentiated stage [2].

It may be claimed correctly that, after approximately 10 passages, the cells undergo some morphological and physiological changes, especially in the case of well-

differentiated cells. Thus, cells of up to the 20th passage may be of more value as an experimental system than those that have undergone more than 100 passages.

We thought well-differentiated clones may be obtained by cloning Ishikawa cells. That is, we thought that clones with features close to those of the primary culture could be isolated from cell lines that have undergone many subcultures. Although we obtained various well-differentiated clones, we think these results only suggest that cells from the 25th passage maintained a certain degree of differentiation. Therefore, it is probable that well-differentiated cells cannot be isolated by this means after the stage wherein all the cells have regressed to the poorly differentiated stage.

Consequently, it may be claimed correctly that the most reliable way to retain the original characteristics of cells is to preserve large numbers of cells, distributed in small aliquots, in the frozen state and to use the same cells, after thawing an aliquot, each time. Moreover, it is important to avoid frequent passages when special characters of the cells are necessary for a given investigation.

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Part 2
Apoptosis, Proliferation, and Growth Factors

Endometrial Stress Neuropeptides: Paracrine Effects on Cell Proliferation and Apoptosis

Achille Gravanis[1] and Andrew N. Margioris[2]

Summary. Human endometrium should be considered as a neuroendocrine organ, in addition to its classical description as a relatively positive target of gonadal steroids. Indeed, the two major types of human endometrial cells, namely epithelial and stromal, produce, among other neuropeptides, the stress-associated peptides corticotropin-releasing hormone (CRH), endorphins, and dynorphins. Multiple lines of evidence suggest that the stress-associated neuropeptides of the human endometrium are under the endocrine control of gonadal steroids, as well as under autocrine/paracrine regulation by prostanoids and interleukins. Endometrial stress-associated neuropeptides appear to exert their biological effect locally (i.e., within the uterus) because human endometrium and myometrium also express the relevant receptors. More specifically, recent data suggest that CRH participates in the regulation of proliferation of tumoral endometrial cells. CRH stimulates the production of proapoptotic protein Fas ligand by endometrial stromal and decidual cells, inducing apoptosis of activated lymphocytes. Similarly, endometrial dynorphins may induce apoptosis of stromal cells through the induction of the proapoptotic Fas receptor. Thus, endometrial stress neuropeptides exert paracrine regulatory effects on uterine cell proliferation, apoptosis, and function, interacting with other local effectors. Dysregulation of these paracrine circuits results in uterine malfunction.

Key words. Apoptosis, corticotropin-releasing hormone, dynorphins, endometrium, proliferation

Introduction

Corticotropin-releasing hormone (CRH), a 41-amino acid neuropeptide, is the major endogenous regulator of endocrine, autonomic, immunologic, and behavioral adaptation to stress. In the central nervous system (CNS), CRH is synthesized principally within the parvocellular part of the paraventricular nucleus of the hypothalamus. Its role there is to regulate and coordinate the body's response to stress via its induction

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of proopiomelanocortin (POMC) gene expression in the corticotroph cells of the anterior pituitary and stimulation of adrenocorticotrophic hormone (ACTH) secretion [1–4]. Outside the CNS, the *CRH* gene is expressed in multiple peripheral tissues including the adrenal gland, immune cells, along the gastrointestinal tract, lung, and skin [5–10]. CRH is also present in the peripheral part of the reproductive tract, including human placenta, uterus, and the gonads of both sexes [11–14]. CRH exerts its many biological effects by binding to specific plasma membrane receptors that are coupled to G_s-proteins and adenylate cyclase. So far, two distinct CRH receptor genes have been identified [15,16]. The first, the CRH-R1, binds to CRH with high affinity, its K_d estimated to range between 0.95 and 2 nM. CRH-R1 was cloned from human and mouse pituitary and rat brain. Two splice variants of the second CRH receptor type (CRH-R2) have been isolated, differing only in their N-terminal domain: (*1*) CRH-R2α (K_d 7.2–22 nM), which was cloned from rat brain; and (*2*) CRH-R2β (K_d 10–29 nM), which was isolated from mouse heart. The CRH receptors are widely distributed in several tissues, including the central and peripheral nervous systems, the adrenals, retina, spleen, immune cells, heart, skeletal muscles, skin, gonads, placenta, endometrium, and myometrium, indicating the diverse and multiple roles that CRH appears to exert [17].

CRH is closely associated with the endogenous opioid peptides (EOP). EOP derive from three precursors of similar molecular size and sequence homology [18–20]. POMC is the precursor of ACTH, α-melanocyte-stimulating hormone (α-MSH), β-endorphin, and lipotropin. Proenkephalin is the precursors of enkephalins, whereas prodynorphin is the precursor of dynorphins, rimorphin, leumorphin, and neoendorphins. EOP exert their actions via specific opioid binding sites. A variety of opioid receptor types and subtypes are present throughout the body [21–27]. The main opioid receptor types are the μ, κ, δ, ε, and σ subtypes. Each EOP exhibits distinct binding activity toward each type of opioid receptor. Opioids are mainly localized within the CNS, including the hypothalamus and spinal cord, as well as in both lobes of the pituitary. Opioid receptors are also widely distributed in peripheral tissues, including the peripheral part of the sympathetic nervous system, the adrenal cortex, and the medulla, as well as throughout the gastrointestinal tract, immune cells, heart, and vasculature. However, as is the case with the CRH receptors, opioid receptors are heavily present in all reproductive tissues, including human gonads, placenta, and endometrium [28–39].

Expression and Regulation of Endometrial CRH

The CRH transcript and its peptide product are present in normal and tumoral human glandular endometrial cells [40,41], which is in contrast with the situation in rat placenta; however, endometrial CRH is expressed in both species [42]. The size of the endometrial CRH transcript is approximately 1.3 kb and it appears to be identical to that in human hypothalamus and placenta. Immunofluorescence experiments in normal human glandular endometrium have revealed a cytoplasm rich in granules positive for immunoreactive (IR) CRH. However, it should be noted IR CRH is localized mainly in glandular cells, whereas endometrial stroma expresses the *CRH* gene weakly [40]. Immunohistochemical data show that both epithelial and decidualized

stromal cells of early pregnant rat uterus contain IR CRH, suggesting that epithelial cells in the endometrium are the main source of intrauterine CRH in the nonpregnant uterus, whereas decidualized normal stromal cells strongly express the *CRH* gene, a phenomenon observed in both human and rodent uterus [40–42]. Furthermore, the CRH transcript and peptide product are abundant in human decidua and stroma, following decidualization in vitro by a mixture of progesterone, relaxin, and estrogens [43]. The physicochemical characteristics of IR CRH in normal and tumoral human endometrium and rat uterus extracts are those of synthetic 41-amino acid CRH peptide [40]. These data suggest that the cycling human endometrium possesses all the necessary enzymes for the posttranslational processing of preproCRH, giving rise to a fully bioactive end-product. The principal receptor for the CRH ligand, the CRH-R1 receptor, is also present in both epithelial and stromal cells of the human endometrium [44,45], as well as in human myometrium [29]. Interestingly, the affinity of the CRH receptors in the human myometrium increases during the later stages of pregnancy, resulting in a linear acceleration of cAMP production as pregnancy progresses, which acts as a potent myometrial relaxant [46]. Appropriately enough, at term, there is a marked reduction in the ability of CRH to stimulate cAMP in the myometrium, which is most probably due to a reduction in the number of G_s α-subunits caused by the rising biological activity of oxytocin. It should be noted that oxytocin activates protein kinase C, which, by phosphorylating the CRH receptor, leads to its severe desensitization toward CRH [47,48].

Three known inducers of hypothalamic CRH, 8-bromo-cAMP, forskolin, and epidermal growth factor (EGF), also stimulate the activity of a CRH promoter introduced into endometrial cells [49]. Indeed, it is widely recognized that cAMP is a major inducer of CRH expression in both the hypothalamus and placenta, as well as a stimulator of its secretion. It should be noted that all data available indicate that cAMP affects *CRH* gene expression rapidly, without the mediation of de novo synthesis of regulatory proteins, supporting the hypothesis that the effect of cAMP is associated with factors within the nuclear compartment, consistent with a direct effect on *CRH* gene transcription mediated via an effect on its promoter [49].

In contrast, estrogens, which suppress the activity of the CRH promoter in the hypothalamus and placenta [50], also suppress endometrial CRH [49]. Estradiol is not capable of inhibiting forskolin- or EGF-induced CRH promoter, acting directly on pairs of functional half-palindromic estrogen-responsive elements (ERE), present in the promoter region of the *CRH* gene. Glucocorticoids decrease the activity of the CRH promoter, although there is no consensus glucocorticoid-response element in the CRH promoter. The inhibition of forskolin- or EGF-induced CRH promoter points to an effect via the activation of cAMP- and EGF-dependent pathways. The inhibitory effect of glucocorticoids in the endometrium resembles that described for hypothalamus and is exactly opposite what has been found to take place in the human placenta, suggesting that the regulation of the transcription of the *CRH* gene is cell specific, depending on the presence or absence of certain specific transcription factors [49].

The cytokines interleukin (IL)-1 and IL-6 have been shown to stimulate the activity of the CRH promoter introduced into human endometrial cells [51]. This effect appears to be mediated via prostaglandins, in accordance with what has been described in the hypothalamus and placenta.

Expression and Regulation of Endometrial Opioid Peptides

It is intriguing that all three opioid peptide precursors are synthesized by human endometrial cells [52,53]. The size of the POMC transcript present in the epithelial cells of the human endometrium is approximately 1.2 kb (i.e., similar to that present in the pituitary gland), whereas the size of the prodynorphin gene transcript is approximately 2.3 kb (i.e., similar to that present in the CNS) [53]. Supporting transcript data are the physicochemical characteristics of IR β-endorphin and IR dynorphin present in the human endometrium. Indeed, both IR peptides have been shown to have similar or identical characteristics to those found for authentic β-endorphin and the 8 kDa dynorphin molecules [53]. Furthermore, endometrial cells express multiple types of opioid receptors, including the κ1, κ2 and κ3 variants [54].

The regulation of β-endorphin production from human endometrial cells is interesting. Estrogens and glucocorticoids suppress the secretion of endometrial β-endorphin, whereas progesterone, dihydrotestosterone, and gonadotrophin-releasing hormone (GnRH) do not appear to have any significant effect [51]. Interestingly, the antiglucocorticoid–antiprogestin RU486 exhibits agonist effects, possibly acting via glucocorticoid receptors. In contrast with β-endorphin, the secretion of endometrial dynorphin appears to be induced by GnRH but not affected by any steroid hormones. As a consequence, the regulation of endometrial opioids appears to have similarities to that reported for opioids in the arcuate nucleus in the hypothalamus and pituitary gland [20]. Indeed, estrogens suppress the secretion of β-endorphin and the transcription of the *POMC* gene in the rat hypothalamus, whereas GnRH induces the production of hypothalamic/pituitary dynorphins. The type-specific regulation of endometrial opioids suggests that each family of opioids possesses a quite distinct physiological role within the uterine cavity. The presence of high-affinity opioid-binding sites on its organ implies that local opioids exert mainly autocrine and/or paracrine effects without excluding the possibility of systemic actions. It is hypothesized that while endometrial dynorphins may exert their biological effects via κ1 opioid receptors, β-endorphin may affect principally κ2 opioid receptors.

Paracrine Effects of Endometrial Stress Neuropeptides on Cell Proliferation and Apoptosis

CRH Inhibits the Proliferation of Human Endometrial Adenocarcinoma Cells

Graziani et al. have shown that CRH inhibits the proliferation of Ishikawa human endometrial adenocarcinoma cells in a time- and dose-dependent manner [55]. A significant decrease in telomerase activity, which parallels tumoral cell growth inhibition, is observed in CRH-treated cells. This effect is blocked in a dose-dependent manner by CRH antagonists, implying the participation of the CRH-R1 receptor. Experiments with the protein kinase (PK) A inhibitor 14–22 amide and forskolin, as

well as measurements of intracellular cAMP, suggest the downstream involvement of the cAMP–PKA pathway in CRH-induced inhibition of Ishikawa cell growth. Based on these findings and other data, we hypothesize that the mitogenic effect of estrogens on human endometrium may be mediated by a local CRH circuit. Indeed, the expression of endometrial CRH is under the negative control of estrogens [49]. In Ishikawa cells transfected with the CRH promoter linked to the luciferase reporter, estrogen inhibited the activity of the promoter in a time- and dose-dependent manner. The inhibitory effect of estrogen was completely reversed by a molar excess of the antiestrogen tamoxifen, suggesting that the effect was mediated by estrogen receptors. Together, these findings suggest that estrogen may exert its mitogenic proliferative effects on endometrial cells by inhibiting the tonic inhibitory effect of endogenous CRH. Thus, it is possible that endometrial CRH and estrogen may create a local regulatory circuit, affecting endometrial cell proliferation (Fig. 1).

Uterine CRH Exerts Immune Actions, Modulating the Differentiation and Remodeling of Endometrial Stroma

It is now generally accepted that CRH exerts a proinflammatory/procytokine effect. Indeed, it now appears certain that the CRH neuropeptide is an integral part of the immune phenomena taking place during the initiation of the inflammatory response. Supportive of this hypothesis is the ubiquitous presence of CRH at the sites of inflammation in both humans and rodents, as well as the fact that the immunoneutralization of CRH appears to markedly attenuate the inflammatory response [56].

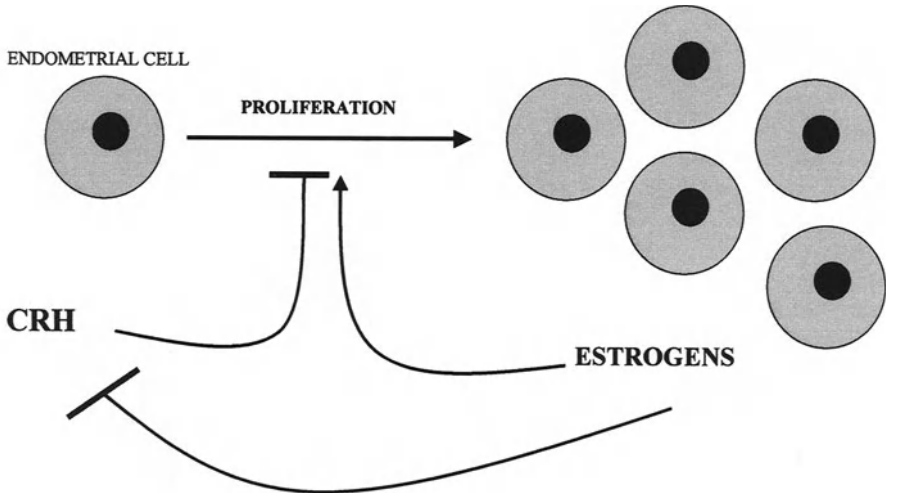

FIG. 1. Uterine corticotropin-releasing hormone (*CRH*) inhibits proliferation of endometrial cells. Estrogens may exert their mitogenic proliferative effects on endometrial cells and inhibit the tonic inhibitory effect of endogenous CRH. It is postulated that endometrial CRH and estrogens compose a local regulatory circuit within the uterine cavity, regulating cell proliferation

Interestingly, in human endometrium, a phenomenon with characteristics of an aseptic inflammatory reaction takes place during the differentiation of endometrial stroma to decidua. More specifically, during decidualization, the endometrial stroma is subjected to numerous functional changes, including an increase in its vascular permeability, remarkable cell growth, and remodeling of its extracellular matrix. It has been shown that CRH induces the decidualization of endometrial stroma [57] and that it potentiates the decidualizing effect of progesterone. Furthermore, progestins stimulate the expression of endometrial CRH in a cAMP-dependent manner [58]. Indeed, in stromal cells, CRH may mediate, via the CRH-R1 receptor, the cAMP-dependent part of the decidualizing effect of progesterone, an effect blocked by the cAMP inhibitor Rp-Br-cAMP. In addition to progesterone, several locally produced proinflammatory immune factors exert a decidualizing effect. Thus, prostaglandins and interleukins are prominent members of this category of modulators. It should also be stressed here that these types of local factors usually exert their effect in a paracrine manner. Indeed, endometrial stroma produces several inflammatory factors, including prostaglandin (PG) E_2, IL-1, and IL-6 [59]. In humans, PGE_2 enhances, whereas IL-1 inhibits, the decidualizing effect of progesterone [60–62]. It is interesting that PGE_2, IL-1 and IL-6 are also major inducers of endometrial CRH [51], suggesting that CRH interacts with these local factors during the decidualization process. Indeed, it has been shown that CRH inhibits the production of PGE_2

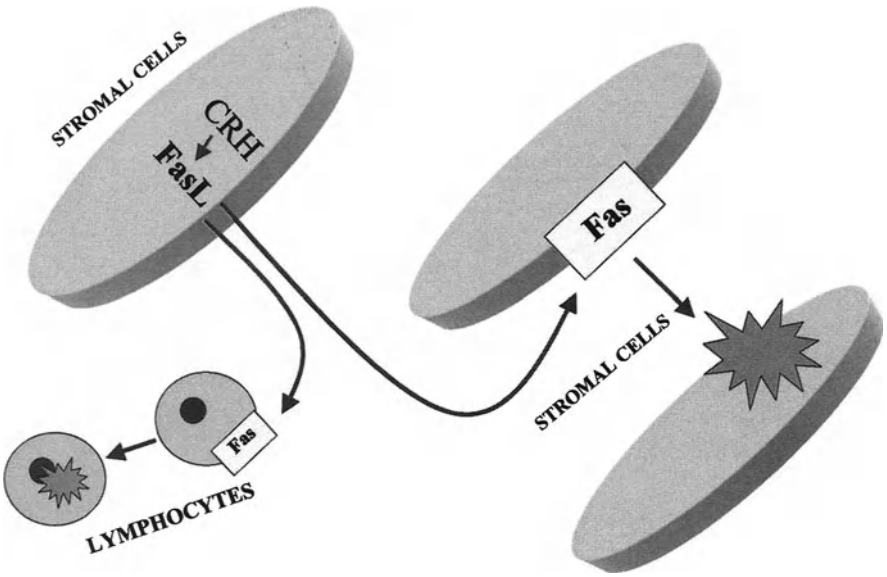

FIG. 2. Uterine corticotropin-releasing hormone (*CRH*) induces apoptosis of activated lymphocytes. CRH produced locally by stromal decidual cells and extravillous trophoblasts acts in an autocrine/paracrine fashion, through CRH-R1, to stimulate Fas ligand (*FasL*) expression and to potentiate the ability of these cells to cause apoptosis of local activated T lymphocytes (Fas receptor positive)

by human endometrial stromal cells [63]. Thus, it is possible that endometrial CRH, in addition to its direct decidualizing effect, may modulate the decidualizing effect of progesterone via an action on locally produced modulators, including PGE_2.

It should be noted that CRH stimulates the production of both IL-1 and IL-6 in human endometrial stromal cells [63] and that IL-1 is a major modulator of the decidualization process, blocking the differentiation of human endometrial stromal cells induced by ovarian steroids or cAMP [61]. The stimulatory effect of CRH on stromal IL-1 suggests that CRH exerts its decidualizing effect either as a principal regulator or as a modulator of progesterone, the classical decidualizing effector. It is interesting that progesterone per se induces the expression of the *CRH* gene in the stromal cells of the human endometrium [58]. Thus, it is possible that progesterone-regulated endometrial CRH may exert an inhibitory effect on decidualization through the induction of a local inhibitor, possibly IL-1, as part of a complex closed-loop feedback system fine-tuning the response of stromal cells to these factors. In conclusion, it now appears that a close interaction takes place within the human endometrium involving CRH, prostanoids, and cytokines. The following sequence of events may take place during decidualization: (*1*) progesterone, in addition to its strong decidualizing effect, also induces the production of endometrial CRH; (*2*) CRH participates in stromal decidualization, regulating the local modulators of this process (i.e., it inhibits the enhancer PGE_2, induces the inhibitor IL-1, and stimulates the inducer IL-6); and (*3*) subsequently, endometrial PGE_2, IL-1, and IL-6 exert a positive effect on the expression of endometrial CRH, completing this endometrial paracrine network.

Uterine CRH Induces Apoptosis of Activated Lymphocytes, Regulating Local Immune Surveillance and Early Maternal Tolerance

The human endometrium is one of the principal examples where local modulators of the inflammatory response serve a key physiologic function. Indeed, the classic modulators of inflammation appear to be part of the mechanism regulating the implantation of conceptus. In fact, in mice, blockade of the IL-1 effect by the specific antagonist IL-1ra inhibits implantation [58]. In vivo experiments in the mouse have shown that intraperitoneal injections of CRH antibodies at day 2 of pregnancy decrease the number of fetuses within the uterus by 60% [64]. This observation is further supported by experiments in rats using antalarmin, a CRH-R1-specific antagonist. Indeed, the administration of antalarmin to early pregnant rats (day 1 of pregnancy) results in a 70% reduction in the number of implantation sites [65]. Thus, blocking CRH results in an anti-nidation effect when it occurs at a very early stage of pregnancy. It is evident that both methods of blocking the effects of uterine CRH (antibodies or antalarmin) do not completely abolish nidation, suggesting the presence of other, redundant mechanisms supporting the implanted embryo. Recent experimental findings [65] show that CRH participates in the nidation of the fertilized egg by inhibiting the local maternal immune response to the implanted embryo (Fig. 2). Indeed, CRH stimulates the expression of the proapoptotic Fas ligand (FasL)

protein in stromal decidual and trophoblastic cells, thus potentiating their ability to induce apoptosis of the surrounding maternal T lymphocytes, activated by the presence of the embryo [65]. Expression of FasL by uterine cells can induce apoptosis of activated T lymphocytes expressing increasing numbers of the Fas membrane protein. Another role that is attributed to maternal and fetal FasL is that it limits the migration of fetal cytotrophoblast cells into maternal tissue and vice versa [66]. The intrauterine presence of CRH, in both maternal (stromal decidua) and fetal (trophoblast) sites, suggests that locally produced CRH regulates FasL production, thus affecting the invasion process through a local auto/paracrine regulatory loop of cytotrophoblast cells regulating their own apoptosis.

Certain types of cancer invade the proximal tissues in a way similar to the way the trophoblast invades the uterine wall. Thus, studying the CRH/FasL relationship may lead to clues that could help better understand the invasiveness of uterine tumors. It has also been suggested that CRH may contribute to tumorigenesis within the uterine cavity by inhibiting local immune surveillance, through induction of FasL and immune cell apoptosis. Research is in progress to study the relationship between CRH/FasL and endometrial and trophoblastic tumorigenesis.

Uterine Dynorphins Regulate Endometrial Stromal Cell Apoptosis

The human endometrium expresses specific κ-opioid binding sites and their endogenous ligands, the dynorphins [53,54]. Recent findings suggest that locally secreted κ-opioid peptides may play a role as paracrine modulators of endometrial stromal apoptosis. It should be noted that apoptosis plays a central role in endometrial physiology, including the decidualization process, the implantation of the fertilized egg, menstruation, and tumorigenesis. The expression of several factors involved in the regulation of apoptosis is detectable in human endometrial stroma. Thus, the Fas antigen, a member of tumor necrosis factor receptor (TNFR) family and a type I membrane protein [67] induces apoptosis via cross-linking to FasL [68]. Members of the Bcl-2 family, such as the apoptosis inhibiting proteins Bcl-2 and Bcl-xL and their apoptosis-promoting homologs Bax and Bak, have also been found in human endometrium [69–71]. κ-Opioid agonists stimulate apoptosis of endometrial stromal cells [72]. This effect is dose dependent and reversible by the general opioid antagonist naloxone, an observation indicating that this phenomenon is mediated via specific opioid receptors. The proapoptotic effects of κ-opioids are exerted through regulation of the pro- and antiapoptotic proteins, including the Fas and Bcl-2 families (Fig. 3). Indeed, κ-opioids cause a rapid but transient upregulation of the apoptotic protein Fas, suggesting that their effect on accelerating apoptosis of endometrial stromal cells is mediated by activation of the Fas/FasL proapoptosis pathway. Interestingly, κ-opioids stimulate the production of the antiapoptotic members of the Bcl-2 family of proteins, namely Bcl-2 and Bcl-xL, whereas they have no significant effect on the apoptosis-promoting homologs Bax, Bcl-xs, and Bak, implying a transient survival mechanism activated in stromal cells as a parallel rescue response to apoptosis-inducing factors.

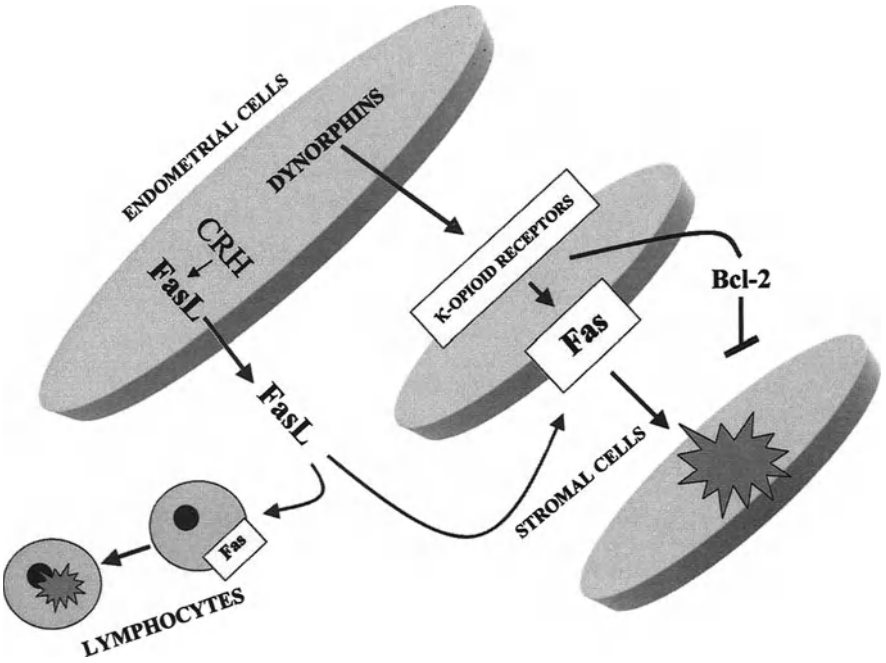

FIG. 3. Uterine dynorphins regulate endometrial cell apoptosis. Dynorphins, produced by endometrial cells, induce the expression of the proapoptotic Fas receptor on neighboring endometrial stromal cells, making them more vulnerable to apoptotic stimuli. Corticotropin-releasing hormone (*CRH*)-stimulated proapototic Fas ligand (*FasL*) may act on these cells, facilitating apoptosis. Dysregulation of these local circuits results in abnormal cell apoptosis and/or tumorigenesis

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Effect of Hepatocyte Growth Factor on the Expression of Matrix Metalloproteinases in the Invasion of Endometrial Cancer Cells in a Three-Dimensional Coculture System

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Summary. Matrix metalloproteinase (MMP)-2 and -9 are secreted and translocated from endometrial stromal cells to cancer cells in a steroid-dependent manner. We investigated the paracrine effect of hepatocyte growth factor (HGF) on the expression of MMPs and tissue inhibitor of metalloproteinases (TIMPs) in stromal and endometrial cancer cells, and correlated the results with cancer cell invasiveness in a three-dimensional (3D) coculture. The 3D coculture of endometrial stromal and cancer cell lines (HEC-1A, HEC-IB, or KLE) was maintained in the presence or absence of HGF. Invasion of the cancer cells was quantified by Boyden's chamber assay. Under the same conditions, the expression of MMP-2 and -9, membrane type-1 matrix metalloproteinase (MT1-MMP), and TIMP-1 and -2 were examined by zymography and reverse transcription–polymerase chain reaction. A significant increase in the invasiveness of all three cancer cells in the presence of HGF was observed by Boyden's chamber assay. HGF enhanced the activation of MMP-2 and -9 by zymography of MMPs. HGF strongly induced MMP-9 mRNA expression in stromal cells, but had little effect on MMP-2 mRNA. MT1-MMP mRNA was detected only in KLE and stromal cells, and was also increased by the presence of HGF. TIMP-1 and -2 mRNAs were ubiquitous, with no dependence on HGF.

The effect of HGF on the invasiveness of 3D cocultured endometrial cancer cells and stromal cells appears to be due to: (*1*) the induction of MMP-9 mRNA expression in stromal cells; and/or (*2*) increased activation of MMP-2 and -9 by proteolytic digestion.

Key words. Hepatocyte growth factor, matrix metalloproteinases, endometrial cancer, three-dimensional coculture

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Introduction

For cells to become invasive, there are several conditions that need to be met. Examples include the loss of adhesion molecules and the destruction of the basement membrane and extracellular matrix [1–3]. For these processes to occur successfully, type IV collagen, which is the principle component of the extracellular matrix, must be lysed [4,5]. Type IV collagen can be lysed with matrix metalloproteinase (MMP)-2 and -9, which are known to increase the metastasis of cancer cells [6]. We have shown previously that MMP-2 and -9 are secreted and translocated from endometrial stromal cells to cancer cells (HEC-1A cells) in a steroid-dependent manner [7].

Meanwhile, hepatocyte growth factor (HGF) is secreted by fibroblasts and is involved in the regulation of cell growth and proliferation, such as morphogenesis, mitogenesis, motogenesis, metastasis, and anti-apoptotic actions, in cancer cells [8]. HGF is composed of a 69-kDa α-chain and a 34-kDa β-chain. The α-chain contains four continuous kringle areas at the hairpin of the N-terminal, whereas the β-chain contains a serine-protease area [9]. The Met receptor of HGF is composed of 50-kDa α-chain and a 145-kDa β-chain, and exists on the cancer cell surface [10–14]. Invasiveness of endometrial cancer cells has been reported to correlate with HGF concentrations [15] and increased MMP activity is known to be implicated in this process [16].

We think that the culture conditions are crucial to clarify this process. When two-dimensional cell cultures were compared with three-dimensional (3D) cell cultures, there was a difference in the tyrosine phosphorylation of focal adhesion kinase (FAK), as well as in the expression of integrins, paxillins, and cytoskeletal components, which has been shown to affect intercellular interaction and cell activity [17]. Therefore, we adopted the 3D coculture of endometrial cancer cells and stromal cells to minimize morphological changes that influence intercellular interaction and also to reconstruct a functional environment that is as close as possible to in vivo conditions.

In the present study, we attempted to clarify the effect of HGF on the activation of MMPs and TIMPs expression, and also on the invasiveness of cancer cells by using 3D coculture of human endometrial cancer cells and stromal cells.

Methods

Cell Preparations

All three cancer cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). The HEC-1A cell line (ATCC HTB-112) is a grade II adenocarcinoma of the endometrium with *c-fos* oncogene, which was separated from endometrial carcinoma patients by Kuramoto et al. in 1968 [18]. The HEC-1B cell line (ATCC HTB-113) is also a subtype of a grade II adenocarcinoma cell line separated from a similar patient. The KLE cell line (ATCC CRL-1622) is an adenocarcinoma cell line obtained mainly from Caucasian patients. The carcinoma cells were maintained according to the provider's instructions. Cells were then adjusted to Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS; Gibco-BRL). Human endometrial tissues were isolated by curet-

tage of hysterectomy specimens from patients with benign conditions and no evidence of endometrial disease. Prior informed consent was obtained in all cases and all patients selected were confirmed to be of reproductive age and at the proliferative phase of their menstrual cycle. Nearly pure stromal cell fractions were separated as described previously [7].

3D Coculture

In order to simulate in vivo conditions, 3D artificial endometrial tissue was reconstructed according to the methods of Park et al. [7] as follows: a type IV collagen (Sigma, St. Louis, MO, USA) and matrigel (Sigma) solution (volume ratio 4:1) was mixed with approximately 2×10^6 stromal cells in a 24-well culture plate. This gel was then solidified in a 37°C humidified incubator containing 5% CO_2 for 20 min. After gelation, the stromal cells in the gel (also called 'artificial tissue') were cultured in DMEM plus 10% FBS for another 24h. The artificial tissue was washed several times with DMEM containing 0.3% growth factor-reduced human serum albumin (HAS; Gibco-BRL) before being cultured in DMEM containing 0.3% HAS. Approximately 2×10^5 HEC-1A, HEC-1B, or KLE cells were inoculated onto the 3D reconstructed tissue directly or in a culture insert with a layer of a 0.45 μm pore size membrane coated with a collagen I/matrigel mixture, and cocultured in the presence or absence of 10 ng/mL human recombinant HGF (Sigma) until the reverse transcription–polymerase chain reaction (RT-PCR) or the invasion assay was performed. Using a membranous culture insert enabled us to separate stromal cells from endometrial cancer cells while soluble factor-mediated cellular communications were preserved.

RT-PCR

Total RNA was extracted from stromal cells and cancer cells using RNAsol B (Biotech Laboratories, Houston, TX, USA) according to the manufacturer's instructions. RNAs of 1.9 or higher in the absorption ratio at 260–280 nm were used for cDNA synthesis. A 4–5 μg total RNA sample was used for cDNA synthesis with 18-base oligo dT (GenoTech, Seoul, Korea) and reverse transcriptase EX (Takara Shuzo, Ghiga, Japan). The primer sequences and sizes expected in the PCR are as follows. For MMP-2, forward 22mer 5′-ATGCTTCCAAACTTCACGCTCT-3′, reverse 21mer 5′-CACAG CCAACTACGATGACGA-3′, and a 828-bp product; for MMP-9, forward 22mer 5′-CACACCACAACATCACCTATTG-3′, reverse 20mer 5′-CAGGGTTTCCCATCAGCATT-3′, and a 515-bp product; for TIMP-1, forward 21mer 5′-CCAGAGAGACACCAGAG AACC-3′, reverse 20mer 5′-GAAAGATGGGAGTGGGAACA-3′, and a 711-bp product; for TIMP-2, forward 21mer 5′-GCACATCACCCTCTGTGACTT-3′, reverse 20mer 5′-GTCGAGAAACTCCTGCTTGG-3′, and a 282-bp product; for membrane type-1 matrix metalloproteinase (MT1-MMP), forward 21mer 5′-GTCATCTGCTCCTTTTCCATC-3′, reverse 21mer 5′-GTGCCTTCTCTGCTCCTTTCT-3′, and a 782-bp product. All primers were custom synthesized and purified (Bioneer, Daejon, Korea). The PCR was conducted with EX Taq polymerase (Takara, Shuzo, Japan). Each PCR cycle consisted of one cycle of 3 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 57°C, and 1 min at 72°C; and one cycle of 5 min at 72°C for 1 min.

Gelatin Substrate Zymography

For the assessment of MMP activity, zymography was conducted using gelatin as a substrate [19]. Nonreduced and standardized aliquots of serum-free medium conditioned with cancer cells and stromal cells were collected and copolymerized with 10% standard polyacrylamide gel containing 1% gelatin [20]. After electrophoresis, sodium dodecyl sulfate (SDS) was washed by 2.5% Triton X-100 twice for 1 h, followed by incubation in a Tris-based buffer (pH7.5) overnight. Gels were stained with 2.5% Coomassie brilliant blue (Sigma) and destained. Gelatinolytic activity was detected as clear bands on a background of uniform staining.

Invasion Assay

In order to quantitatively determine the degree of invasiveness of HEC-1A, HEC-1B, and KLE cell lines in the coculture system, Boyden's chamber assay was performed using 8-μm pore transwell polycarbonate membrane inserts (Nalge Nunc, New York, NY, USA). The membranous insert was soaked in 100 μL of 1:8 diluted matrigel in cold DMEM (Biocoat, Bedford, England) and dried under laminar flow overnight. Approximately 1×10^5 HEC-1A, HEC-1B, or KLE cells were inoculated into each insert and cocultured with stromal cells. The culture medium was changed every day. Three days after the coculture, the inserts were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 3 h at 25°C. After being washed with PBS, fixed cells were treated briefly with ice-chilled methanol. The permeable cells were then stained with Mayer's hematoxylin solution (Sigma) for 2 min and washed with PBS for 10 min. The transwell membrane was removed from the insert, placed on a glass slide upside down, immersed in a diluted glycerol solution (1:10 in PBS), and observed under a microscope. Cells trapped in the membrane pore were counted. The assay was repeated three times per cell line, and two separate observers determined the degree of invasion.

Statistical Analysis

One-way ANOVA was used to compare the invasion assay results between the different treatments. *P*-values less than 0.05 were considered to be of statistical significance.

Results

Expression of MMPs and TIMPs mRNAs in both endometrial cancer cells and stromal cells after 3D coculture was examined by RT-PCR to observe the paracrine effect of HGF.

In the absence of HGF, the expression level of MMP-2 mRNA (828 bp) was high in stromal cells, but undetectable in cancer cell lines (Fig. 1A), whereas the levels of MMP-9 mRNA (515 bp) expression were low in stromal cells and undetectable in the cancer cell lines (Fig. 1B). In the presence of 10 ng/mL HGF in the culture medium, neither MMP-2 nor MMP-9 mRNA was expressed in the cancer cells. However, expres-

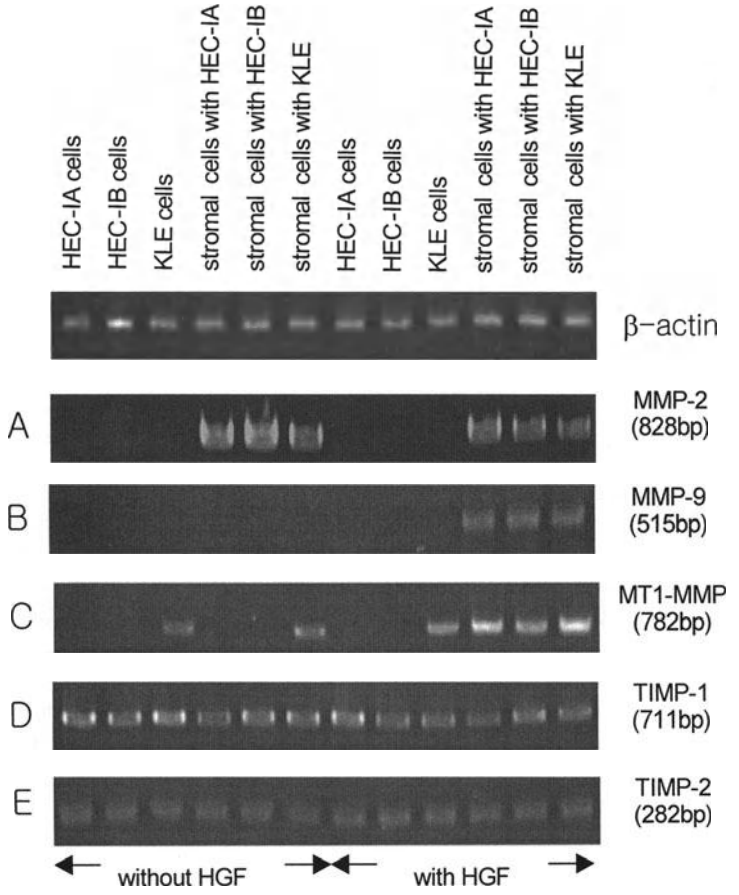

FIG. 1. Expression profiles of matrix metalloproteinase (*MMP*)-2, -9, membrane type-1 matrix metalloproteinase (*MT1-MMP*), tissue inhibitor of metalloproteinases (*TIMP*)-1, and -2 mRNA analyzed by reverse transcription–polymerase chain reaction (RT-PCR) in the absence (*lanes 1–6*) and presence (*lanes 7–12*) of hepatocyte growth factor (*HGF*). Three cancer cells were cocultured with the stromal cells pair wise in three-dimensional coculture in the absence of HGF as described in the Methods. RT-PCR was conducted with total RNAs isolated from HEC-1A cells (*lanes 1, 7*), HEC-1B cells (*lanes 2, 8*), KLE cells (*lanes 3, 9*), stromal cells cocultured with HEC-1A cells (*lanes 4, 10*), cocultured with HEC-1B cells (*lanes 5, 11*), or cocultured with KLE cells (*lanes 6, 12*). **A** PCR products were amplified with MMP-2 primers. **B** PCR products were amplified with MMP-9 primers. **C** PCR products were amplified with MT1-MMP primers. **D** PCR products were amplified with TIMP-1 primers. **E** PCR products were amplified with TIMP-2 primers

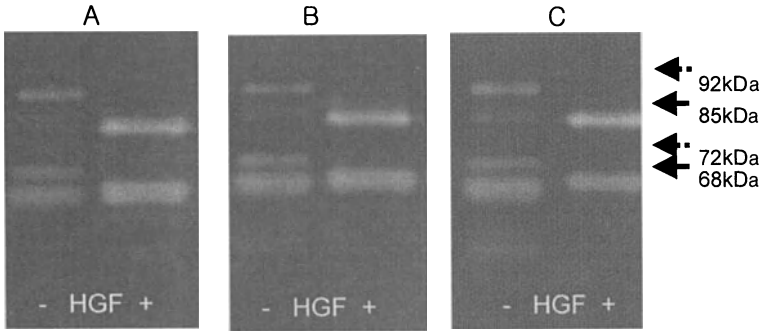

FIG. 2. Hepatocyte growth factor (*HGF*) induction of matrix metalloproteinase (MMP) activity. Cancer cells were cocultured with the stromal cells for 48 h and then the conditioned culture media were collected and subjected to zymography using gelatine as a substrate. Conditioned medium was collected from **A** HEC-1A/stromal cells, **B** HEC-1B/stromal cells, and **C** KLE/stromal cells cocultured in the absence (*left lanes*) and presence (*right lanes*) of 10 ng/mL HGF. *Arrows* at 85 and 68 kDa represent the active forms of MMP-9 and -2, respectively. *Arrows* at 92 and 72 kDa represent the proforms of MMP-9 and MMP-2, respectively

sion levels of MMP-2 and -9 mRNA were higher in stromal cells compared with values obtained in the absence of HGF; in particular, the expression of MMP-9 mRNA was strongly induced by HGF (Fig. 1A,B).

In the absence of HGF, MT1-MMP mRNA (782 bp) was detectable only in KLE cells and cocultured stromal cells. However, in the presence of HGF, significantly higher expression was observed in KLE cells and in all three cocultured stromal cells, which indicates that MT1-MMP mRNA was induced and upregulated by HGF (Fig. 1C).

Both TIMP-1 (711 bp) and TIMP-2 (282 bp) mRNA were expressed in all three cancer cells and stromal cells, but the expression level of TIMP-1 mRNA was approximately threefold higher than that of TIMP-2. The expression levels and patterns of both TIMP-1 and -2 mRNA were ubiquitous and similar irrespective of the presence of HGF (Fig. 1D,E).

Zymographic analysis was then conducted to investigate whether the expression levels of MMP mRNAs in cells reflected the presence of active MMPs in the media from the coculture. A significant proportion of MMP-2 and -9 existed as proforms (72 and 92 kDa, respectively) in the absence of HGF. However, most MMP-2 and -9 existed as active forms in the presence of 10 ng/mL HGF (68 and 85 kDa, respectively). This finding indicates that HGF is effective at enhancing the conversion of proforms of MMP-2 and -9 into their active forms (Fig. 2).

A quantitative invasion assay was performed to measure the invasiveness of the endometrial cancer cells in the 3D coculture. The presence of 10 ng/mL HGF in the coculture significantly promoted cancer cell invasion ($P < 0.05$) compared with control. Each assay was performed three times and data are presented as the mean ± SD number of invading cells (Fig. 3).

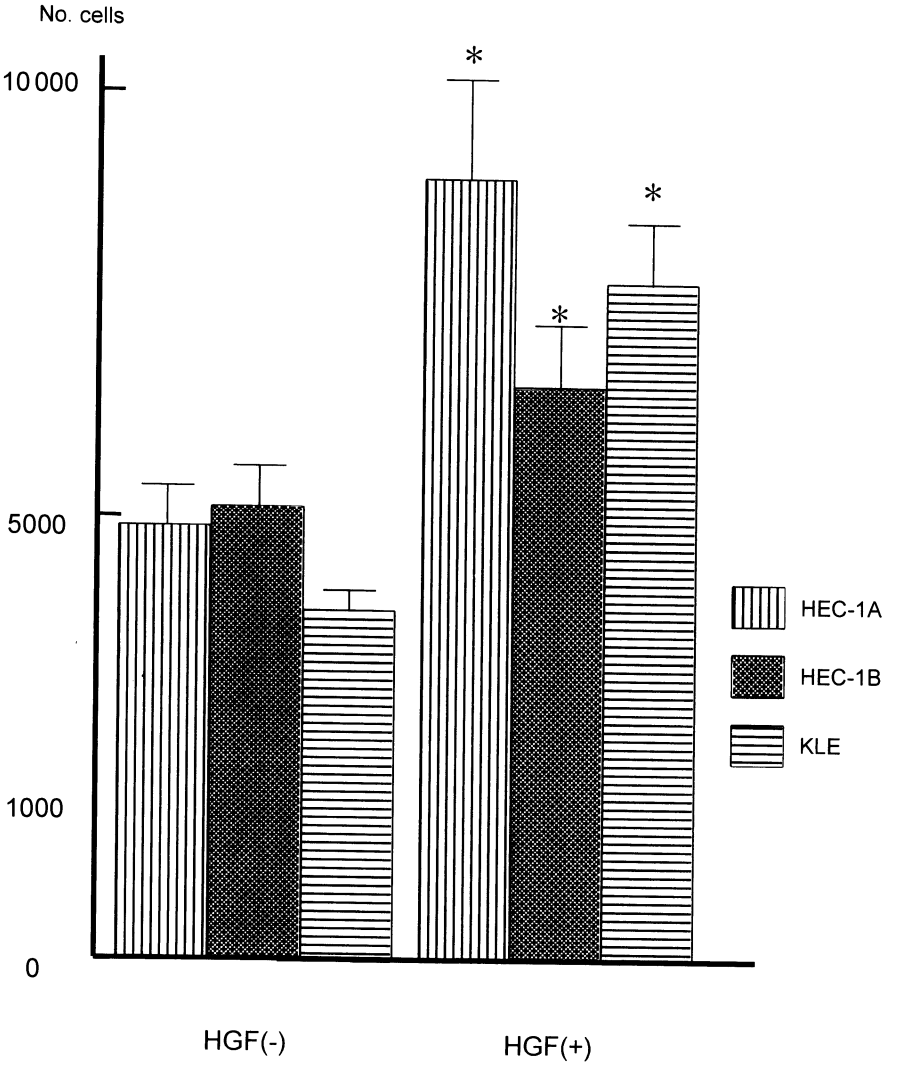

FIG. 3. Effects of hepatocyte growth factor (*HGF*) on the invasion of cancer cells in three-dimensional coculture. Cancer cells were cocultured with stromal cells pair wise in the absence (*left lanes*) and presence (*right lanes*) of HGF for 72 h. Invading cancer cells were counted microscopically using the Boyden's chamber assay. Results are expressed as the mean ± SD number of invading cells. $^*P < 0.05$ compared with control (without HGF)

Discussion

HGF has been known to enhance tumor growth, invasion, and metastasis [21–23]. In the present study, all three endometrial cancer cell lines revealed a significant increase in the degree of invasiveness determined in the invasion assay in the presence of HGF. Therefore, it is suggested that HGF enhances endometrial cancer invasion.

None of the three cancer cell lines under coculture conditions with stromal cells expressed MMP-2 or -9 mRNA, regardless of whether HGF was present or absent in the culture, whereas the expression levels of MMP-9 mRNA in stromal cells was significantly increased in the presence of HGF. The lack of MMP-2 or -9 mRNA expression in cancer cells is consistent with previous reports [7,24], suggesting that endometrial cancer cells utilize MMPs secreted from stromal cells in the invasion process. Furthermore, the addition of HGF to the coculture induced the expression of MMP-9 mRNA in stromal cells, but had little or no effect on MMP-2. The addition of HGF could increase the availability of MMPs by inducing the expression of MMP-9 mRNA. Thus, the net effect of HGF would be increased cancer cell invasion.

Because TIMP-1 and -2 mRNA were expressed ubiquitously in both cancer cells and stromal cells independently of HGF, it can be assumed that the effect of HGF on the invasion process of endometrial cancer cells is not related to TIMPs. However, it is interesting to note that the expression of MT1-MMP mRNA in HEC-1A and HEC-1B cells was also induced in the presence of HGF. MT1-MMP has been known to provide a binding site for TIMPs, thereby converting a proform MMP into a mature, active form [25–28]. The results of zymographic analysis support this hypothesis. In the presence of HGF, most MMP-2 and -9 in the culture media existed as active forms, which is in contrast with the significant amount of proforms observed in the absence of HGF. Collectively, it is concluded that HGF present in the 3D coculture induces the expression of MMP-9 and MT1-MMP mRNA in stromal cells, the former being translocated to the surface of the cancer cells and the latter promoting an enzymatic cleavage of a proform MMPs into an active form. Both may result in an increase in the invasiveness of cancer cells.

It has been reported previously that MT1-MMP is synthesized in moderately differentiated thyroid carcinoma cells [29] and, in the present study, we demonstrated that MT1-MMP is expressed weakly in KLE cells, as well as stromal cells, and that the expression of MT1-MMP mRNA was increased in the presence of HGF. This suggests that the availability of MT1-MMP on the surface of KLE and stromal cells may contribute to cancer invasion by promoting the MMP-2 or -9 translocation/utilization. The speculation would be that MMP-2 or -9 is synthesized and secreted from stromal cells before it has been processed enzymatically to gain full activity. The enzymatic cleavage takes place mainly on the surface of stromal cells, where MT1-MMP exists and acts as a catalyst in a membrane-bound form. However, for a small proportion of cells, the cleavage may take place on the surface of cancer cells, such as for KLE cells. This line of speculation has also been suggested previously [30] and is confirmed by our zymographic results. Our data clearly demonstrate that the quantity of active MMP-2 and -9 increases with a corresponding decrease in the quantity of MMP-2 and -9 proforms when HGF is present.

There were no significant changes in TIMP-1 and -2 mRNA expression levels induced by the addition of HGF, suggesting their constant expression in cancer cells

and stromal cells. Therefore, a disruption in the balance between available MMPs and TIMPs may be an initiation event of the cancer invasion process, and HGF acts primarily by shifting the balance in favor of invasion by enhancing the availability of MMP-2 and -9.

HGF has been known to increase cancer cell mobilization, invasion, and tubular formation in endothelial cells [31,32]. It has also been reported to increase the expression of MMP-9 in the proximal tubule cells of human kidneys [33] and in human epithermal cells [19]. HGF is also involved in the gene activation mechanisms for MMP-1 and -9 in oral squamous cell carcinomas by upregulation of the E1AF, a transcription factor for the *Ets* oncogene family [34]. In hepatocytes, HGF promotes MMP-9 expression and tumor necrosis factor-α activity, but has no effect on MMP-2 expression while suppressing the actions of TIMP-1 and -2 [35]. In bone stem cells, HGF increases the synthesis of MMP-2 and -9 by hydroxyapatites [36]. The results of the present study are, in general, in agreement with previous observations that an increase in the invasiveness of keratinocytes is accompanied by a concomitant induction of MMP-9 by HGF [19] and that the MMP-9/MMP-2 ratio was considerably higher in endometrial cancer cells compared with normal endometrial tissues [37]. The three endometrial cancer cell lines have c-Met receptors for HGF [9], so when the effect of HGF on endometrial cancer cells is minimal or absent, MMP-2 appears to be physiologically expressed as the main degrading enzyme. However, when HGF is added, the expression and activity of MMP-9 is increased, which is subsequently utilized in the invasion mechanism. Therefore, it may be suggested that most cancer cells, including endometrial cancer cells, have a common mechanism for their invasion processes.

In conclusion, the findings presented in this report demonstrate that, first, the interaction between endometrial cancer cells and stromal cells in vitro regulates the expression of MMP-2 and -9 in a paracrine fashion similar to that in vivo. Second, the invasion of endometrial cancer cells is closely related to the availability of MMP-2 and -9, which is greatly enhanced by HGF, either by inducing mRNA synthesis or by promoting conversion of proforms into active forms. Third, there is no change in TIMP-1 and TIMP-2 expression induced by HGF. Therefore, the balance between MMPs and TIMPs is altered in favor of MMPs in the presence of HGF and the altered balance could result in an increased proteolytic activity of the cancer cells.

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Part 3
Cell Cycle Regulators

Involvement of Cell Cycle Regulators in Steroid Hormone-Induced Growth of Endometrial Carcinoma

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Summary. The involvement of cell cycle regulators in steroid hormone-dependent growth and growth suppression was examined using cultured normal endometrial glandular cells and estrogen receptor (ER)/pregesterone receptor (PR)-positive endometrial carcinoma Ishikawa cells. The results indicated that the estradiol (E2)-induced upregulation of cyclin D1 may play a crucial role in the growth of normal endometrial glandular cells and is mediated by c-Jun via an activating protein (AP)-1-binding site-like sequence of the promoter. However, in ER-positive Ishikawa cells, its molecular mechanism is different from the normal counterpart. In progestin-induced growth suppression, accumulation of p27 protein plays an essential role in both normal and malignant endometrial cells, possibly via inhibition of the ubiquitin pathway of p27 degradation.

Key words. Endometrium, endometrial carcinoma, cyclin, AP-1, p27

Introduction

Normal endometrial glandular cells and a subset of endometrial carcinoma cells express estrogen receptors (ER) and progesterone receptors (PR). Growth of these ER-positive glandular cells is known to be stimulated by estrogen, whereas the growth of PR-positive cells is suppressed by progestins [1]. Various aspects of steroid hormone-induced proliferation and differentiation of the endometrium have been investigated, including up- and downregulation of steroid receptors [2,3], altered function of steroid-metabolizing enzymes [4], growth factors, and cytokines [5]. However, the molecular mechanisms of the steroid hormone-dependent growth control have not been fully elucidated. Recent studies have revealed that cell cycle regulators, such as cyclins (D1, E, A, and B1), cyclin-dependent kinases (cdk2, 4, and cdc2), tumor suppressors (p53, pRb), and cdk inhibitors (p16, p21, p27), are essential in cell cycle control. Cyclins are group of proteins that are expressed in a cell cycle-specific fashion. Cyclins form complexes with their respective cdks and the kinase activities generated

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by these complexes inactivate pRb, resulting in cell cycle progress [6,7]. In contrast, cdk inhibitors inactivate their respective cdks, leading to cell cycle arrest [8]. Therefore, analysis of the expression and interaction of these cell cycle-related molecules is important for understanding the growth mechanism of cells. The present study was undertaken in order to investigate the involvement of cell cycle regulators in steroid hormone-dependent growth control of the normal and neoplastic endometrium.

Immunohistochemical Expression of Cyclins and cdks in the Normal Endometrium

G1 Cyclins (Cyclin D1 and E)

The expression of cyclin D1 was observed sporadically in the nuclei of glandular cells during the proliferative phase. The expression of cdk4, which is a catalytic partner of cyclin D1, was also observed in the glandular cells during the proliferative phase. The cyclin D1-positive cells were also positive for cdk4 and the proliferation marker Ki-67. Expression of cyclin E was observed in glandular cells during the proliferative and secretory phases. The expression of cdk2, a partner of cyclin E, was also observed in glandular cells during the proliferative phase. The cyclin E-positive cells are often positive for cdk2. The cyclin E/cdk2-positive cells were positive for Ki-67 during the proliferative phase, but were negative during the secretory phase [9].

M Cyclins (Cyclin A and Cyclin B1)

The expression of cyclin A and B1 was observed sporadically in glandular cells during the proliferative phase. The cyclin A- and cyclin B1-positive cells were often positive for cdc2, a catalytic partner of cyclin A and cyclin B1. These cyclin A/cdc2- and cyclin B1/cdc2-positive cells were also positive for Ki-67. These findings suggest that cyclins and cdks are involved in the growth of normal endometrial glandular cells [9].

Molecular Mechanism of Estrogen-Induced Growth of Normal Endometrial Glandular Cells

To analyze the estrogen-induced growth mechanism, we prepared cultured normal endometrial glandular cells. These cells are positive for ERα, PR (A and B) and cytokeratin. Estradiol (E2)-induced an increase of ^{3}H-thymidine uptake and progesterone-induced suppression of uptake was observed [10]. Therefore, these cells were used in the following experiments.

Using these cells, we examined the expression of cyclins following E2 stimulation by Western blotting. The expression of cyclin D1 was first observed 4h after E2 stimulation and was serially followed by the expression of cyclins E, A, and B1. pRb phosphorylation was observed after 24h [11]. Because the sequential expression of the cyclins was the same as in other types of proliferating cells, the elevated expression of cyclin D1 is considered to be the first and most important event in E2-induced cell growth. Therefore, the mechanism of estrogen-induced cyclin D1 upregulation was investigated. In general, binding of the ER to the estrogen-responsive element (ERE)

of the target gene is necessary in estrogenic actions. However, because the cyclin D1 promoter lacks the ERE, a direct action of ER on cyclin D1 expression is unlikely. Instead, the cyclin D1 promoter contains an activating protein (AP)-1 binding site-like sequence. AP-1 is a group of early transcription factors, such as Jun and Fos. The original or consensus AP-1 binding site sequence is TGACTCA, but the AP-1-like sequence in the cyclin D1 promoter, located at nucleotides –953 to –947, is TGAGTCA [12]. In addition, promoters of Jun and Fos have functional EREs and estrogen-induced expression of Jun and Fos has already been reported [13]. Hence, the involvement of Jun and Fos in the expression of cyclin D1 was examined.

Immunostaining for AP-1 revealed that c-Jun expression is observed only during the proliferative phase, whereas c-Fos was observed during both the proliferative and secretory phases. Western blotting using E2-treated cultured endometrial glandular cells showed that c-Jun/c-Fos was expressed 2 h after E2 stimulation, which preceded cyclin D1 expression [11]. These findings suggest that E2-induced expression of cyclin D1 may be mediated by Jun/Fos via an AP-1-binding site.

In order to verify this hypothesis, the cyclin D1 transcription mechanism of normal endometrial glandular cells was analyzed with a luciferase assay using plasmids containing various sized deletion constructs (ranging from –1749 to –78 of the promoter) of the cyclin D1 promoter [14]. The results indicated that, when E2 was absent, transcriptional activity was low in most promoter constructs. In contrast, when E2 was added, only two promoter constructs (the longest and the second longest inserted constructs), both having an AP-1-like sequence, showed elevated activities [11]. These findings suggest that the E2-induced expression of cyclin D1 may be mediated by an AP-1-like sequence. However, the difference in size between the second and third longest constructs is approximately 320 bp. Thus, there is the possibility that DNA other than that for the AP-1-like sequence in this area could contribute to the transcription and that proteins other than AP-1 could bind to the DNA. Therefore, in order to evaluate the specificity of the binding between the AP-1-binding site-like sequence and the AP-1 protein, a gel shift assay and a competition assay were performed. In that study, three types of radio labeled DNA probes were used (i.e., probe D1: promoter DNA containing an AP-1-like sequence; probe M: a mutant of probe D1 in the AP-1-like sequence; and probe C: promoter DNA containing a consensus AP-1-binding sequence). The results of the gel shift assay indicated that a specific band shift was observed when E2-treated nuclear extract was treated with probes D1 and C. The competition assay indicated that an excessive amount of cold probe D1 and probe C eradicated this specific band. In addition, a band supershift was observed when anti-c-Jun antibody was added. These findings suggest that c-Jun, binds specifically to the AP-1-like sequence of the promoter. To further examine the relationship between cyclin D1 expression and c-Jun, a luciferase assay was performed using the same sets of plasmids with cotransfection of the c-Jun or c-Fos expression plasmid [15] without E2. The results showed that transcriptional activity in promoters only containing an AP-1-like site increased only when c-Jun was cotransfected. Collectively the results indicate that E2-induced expression of cyclin D1 in normal endometrial glandular cells is mediated by c-Jun through an AP-1-like sequence.

Molecular Mechanism of Estrogen-Induced Growth of ER-Positive Endometrial Carcinoma Ishikawa Cells

The effect of E2 on the expression of cyclins in ER-positive endometrial carcinoma Ishikawa cells was examined by Western blotting. The expression of cyclin D1 was noted before E2 stimulation and cyclin D1 expression showed only a slight increase after E2 stimulation. The expression of cyclin E increased after 6 h of E2 stimulation, showing a pattern distinct from that of normal endometrial glandular cells. The transcriptional activity of the cyclin D1 promoter in Ishikawa cells was examined using a luciferase assay. The results revealed that strong activity was noted from various sites of the promoter, even in the absence of E2. When E2 was added, increased activity was most marked in a promoter that contained the E2F (E2 promoter binding factor)-binding site at the 5′ end of the inserted construct, suggesting that E2 stimulates the cyclin D1 transcription through the E2F-binding site. These findings suggest that the mechanism of E2 stimulation seems to be different from that of normal endometrial glands [11].

Molecular Mechanisms of Progestin-Induced Growth Suppression of Normal Endometrial Glandular Cells and PR-Positive Ishikawa Cells

Involvement of cdk inhibitors in progestin-induced growth suppression was investigated. Of several inhibitors tested, only p27, a cyclin E/cdk2 inhibitor, showed elevated expression during the secretory phase, which was in contrast with the expression pattern of Ki-67. Because p27 was observed during the secretory phase, the effect of progestin on p27 expression in patients with endometrial hyperplasia treated with medroxyprogesterone acetate (MPA) was examined. The expression of p27 was nearly negative before treatment but, after MPA treatment, p27 expression increased markedly [16]. These findings suggest that p27 can be induced by progestins. To clarify the relationship between progestins and p27 expression in vitro, the effect of progesterone (P4) on the expression of cell cycle regulators was evaluated by Western blotting using cultured endometrial glandular cells. The results indicated that the expression of p27 increased in a dose-dependent fashion, whereas the expression of cyclin D1, E, and cdk4 was reduced. Then, the effect of MPA on the growth suppression of PR-positive Ishikawa cells was examined. MPA caused growth inhibition in a dose-dependent manner and p27 expression was observed to increase in a dose-dependent manner [10]. These findings suggest that p27 expression was induced by progestins in both normal and malignant endometrial cells.

In order to investigate the mechanism of p27 expression, the expression of *p27* mRNA was examined by Northern blotting. In contrast with the p27 protein, *p27* mRNA did not show any marked changes either during the menstrual phases of the normal endometrium or in MPA-treated Ishikawa cells, suggesting that the amount of p27 protein is not controlled by the transcription level [10]. The effect of progestins on p27 protein degradation was then investigated. In both normal glandular cells and Ishikawa cells, cycloheximide, a protein synthesis inhibitor, was added first and the

amount of p27 protein was compared in cells with and without progestins. The results showed that in both types of cells, p27 protein was more abundant in the progestin-treated groups. These findings suggest that progestins inhibit p27 protein degradation and that the accumulation of p27 protein is due to the prolonged half-life as a result of post-translational mechanisms [10]. Because the amount of p27 protein was controlled by protein degradation, the involvement of the ubiquitin–proteasome pathway was then examined. Recent studies have revealed that p27 is poly-ubiquitinated by the SCF (Skp1-Cul1-F box protein) complex, which is composed of molecules including Skp1, 2, and cullin 1, and the ubiquitinated p27 protein is finally degradated by the proteasome [17]. Thus, the expression of Skp1, 2, and cullin 1 in the normal endometrium was examined by immunostaining. Expression of Skp2 and cullin 1 was observed in glandular cells of the proliferative phase, showing a contrast with p27 expression. In addition, P4 treatment reduced the expression of Skp2 by cultured normal endometrial glands. These findings suggest that the progestin-induced suppression of Skp2 may be involved in p27 accumulation. To confirm the functional involvement of p27, the p27 expression plasmid [18] was transfected to normal glandular cells. The growth of normal glandular cells was reduced significantly, without any effect on the expression of cyclin E and cdk2 [10]. These findings suggest that p27 may be involved functionally in the growth suppression of endometrial glandular cells.

Conclusion

In conclusion, these data show the essential roles of cell cycle regulators in steroid hormone-induced growth stimulation and suppression. E2-induced upregulation of cyclin D1 may play an important role in the growth of normal endometrial glandular cells and the activation of cyclin D1 is mediated, possibly, by binding of c-Jun to the AP-1 site of the promoter. However, in endometrial carcinoma cells, the molecular mechanism is different from the normal counterpart. In P4-induced growth suppression, accumulation of p27 protein plays an essential role in both normal and malignant endomtrial cells, possibly via inhibition of the ubiquitin pathway of p27 degradation.

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Expression of Cell Cycle Regulators in Endometrial Adenocarcinoma

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Summary. Abnormal expressions of cell cycle regulators, such as cyclin-dependent kinases (cdk), cyclins, and cyclin kinase inhibitors, are supposed to play an important role in the tumorigenesis and progression of carcinoma. The aim of the present study was to investigate the correlation of the expression of these cell cycle regulators with proliferative activity and clinicopathological parameters in endometrial adenocarcinoma, and the regulation of these proteins by sex steroid hormones in endometrial cancer cells. Tissue samples of 127 endometrial adenocarcinomas (endometrioid type) were used in the present study. Immunohistochemical staining of cycle regulators was performed according to the labeled streptavidin–biotin method. Ishikawa cells, in which estrogen receptor (ER) cDNA was transfected by the SuperFect method, and HEC-265 cells positive for the progesterone receptor were used in an in vitro study. Quantitative analysis of proteins was performed by immunoblotting. Expression of cyclin D1, cyclin E, cyclin A, p53, and p27 was positively correlated with Ki-67 expression. Expression of cdk2, cyclin D1, cyclin E, cyclin A, p53, and p27 was positively associated with histological grade. Expression of cyclin E was significantly correlated with lymphovascular space involvement (LVSI) and myometrial invasion, and expression of cyclin A was correlated with LVSI and group (coexistence with or without hyperplasia). p53 was related with stage, LVSI, myometrial invasion, and group; whereas p27 was correlated with stage, LVSI, and lymph node metastasis. Estradiol (E2) revealed a stimulatory effect on the growth of ER-transfected Ishikawa cells, in addition to enhancing their expression of cyclin A and cyclin E. In contrast, medroxyprogesterone acetate (MPA) suppressed HEC-265 cell growth after its accumulation of p27 in the cells. In conclusion, the expression of cell cycle regulators was significantly associated with cell proliferation, histological grade, and some clinicopathological parameters in endometrial adenocarcinoma. A cell line stably sensitive to E2 was established after ER cDNA was transfected into Ishikawa cells, and cyclin A and E may be involved in the enhanced cell growth induced by E2. p27 accumulation

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induced by MPA may be involved in the progesterone-induced growth suppression of endometrial cancer cells.

Key words. Cyclin, p27, estrogen, progesterone, endometrial cancer

Introduction

The cell cycle is divided into the G1, S, G2, and M phases, which are controlled and coordinated by cell cycle regulators such as cyclin-dependent kinases (cdk), cyclins, and cyclin-dependent kinase inhibitors (Fig. 1). The cell cycle can be compared with an automobile. Cdk acts as an engine, whereas cyclin acts as an accelerator due to periodic increases and decreases in its activity; the Cdk inhibitor plays the role of a brake. The cell cycle is regulated strictly by the phase-specific function of these cell cycle regulators. The level of cdk expression is almost regular during any phase of the cell cycle. Cdk is activated, like an engine, by cyclins that act as an accelerator and the cell cycle progresses. In the G1–S phase, cdk2, cdk4, cyclin D, and cyclin E act specifically and advance the cell cycle to the S-phase. In the S–G2–M phase, cdk2, cdc2, cyclin A, and cyclin B act specifically and progress the cycle to the M-phase. In contrast, cdk inhibitors inhibit the progression of the cell cycle by suppressing the kinase activity of specific cyclin/cdk complexes. The cdk inhibitors are classified into two major families: (*1*) the INK4 family, such as p15, 16, 18, and 19; and (*2*) the CIP/KIP family, such as p21 and p27. The former group inhibits specifically the cyclin D/cdk4

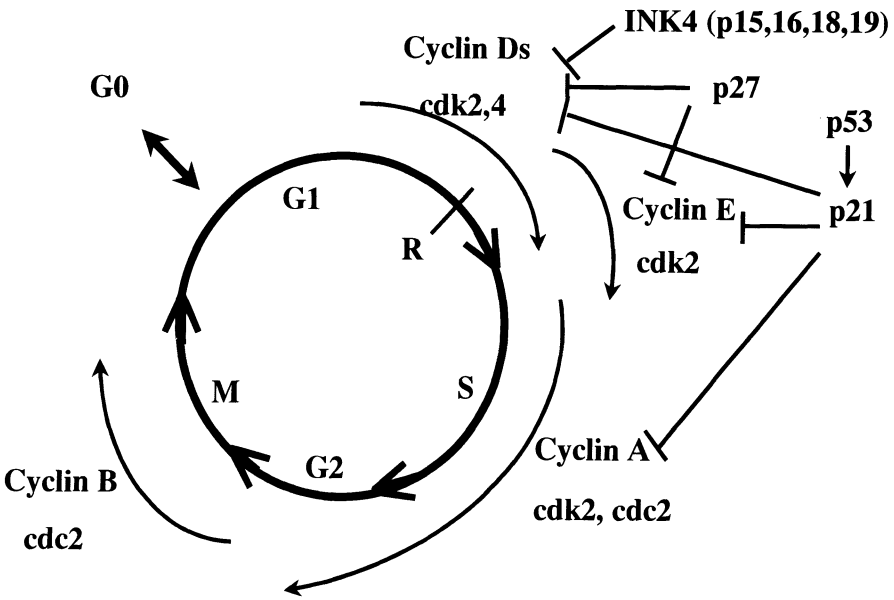

FIG. 1. Cell cycle regulation. *cdk*, cyclin-dependent kinase

complex. p21 inhibits the cyclin D/cdk2 or cdk4 complex, the cyclin E/cdk2 complex, and the cyclin A/cdk2 or cdc2 complex. p27 inhibits the cyclin D/cdk2 or cdk4 complex and the cyclin E/cdk2 complex.

Abnormal expression of cell cycle regulators is supposed to play an important role in tumorigenesis and cancer progression [1]. In fact, overexpression of cdk and cyclins [2,3], *p53* gene mutation [4], and decreased p27 expression [5,6] in cancers have been reported. Cyclin D1 is reported to be frequently amplified in breast cancers [7]. It can activate the estrogen receptor (ER) through direct binding to a cyclin-dependent kinase partner. Cyclin D1 results in an increased binding to the estrogen-responsive element and upregulates ER-mediated transcription [8]. The relationship between carcinogenesis in a hormone-responsive organ, such as the endometrium, and abnormal expression of cell cycle regulators has not been clarified in detail and it is not yet known how sex steroid hormone regulates the expression of these proteins in endometrial carcinoma.

The aim of the present study was to investigate: (*1*) the correlation between protein expression and both proliferative activity and clinicopathological parameters in endometrial adenocarcinoma, endometrioid type; (*2*) the regulation of cyclin expression by estrogen; and (*3*) the regulation of a cell cycle inhibitor by progestin in endometrial cancer cells.

Expression of Cell Cycle Regulators in Endometrial Endometrioid Adenocarcinoma

Cdk2, as the main engine of the cell cycle, cyclin D1, cyclin E, and cyclin A as accelerators of cdk2, and p53 and p27 as brakes of the cell cycle were examined immunohistochemically. Ki-67 was used as a proliferative marker.

Methods

Tissue samples of 127 cases of endometrial adenocarcinoma, endometrioid type, consisting of 73 well-differentiated (G1), 26 moderately differentiated (G2), and 28 poorly differentiated (G3) adenocarcinomas were obtained surgically with informed consent at Kitasato University Hospital between 1983 and 2000. Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded tissues using the labeled streptavidin–biotin (LSAB) method (DAKO, Kyoto, Japan). Primary antibodies are listed in Table 1. The labeling index (LI), which was calculated as the percentage of positive nuclei in at least 1200 cells, was used to interpret the staining. All staining was specifically localized in the nuclei.

Correlation of Cell Cycle Regulator Expressions with Ki-67 Expression

The LIs of cyclin D1, E, A, p53, and p27, but not cdk2, were significantly correlated with the LI of Ki-67. Statistical analysis was performed using Spearman's rank correlation test (Table 2).

TABLE 1. Antibodies and dilution

Primary antibodies	Dilution
Ki-67 (rabbit polyclonal; DAKO)	1:50
cdk2 (rabbit polyclonal; Santa Cruz)	1:2000
Cyclin D1 (mouse monoclonal; Oncogene)	1:80
Cyclin E (mouse monoclonal; Novocastra)	1:40
Cyclin A (mouse monoclonal; Novocastra)	1:100
P53 (mouse monoclonal; Novocastra)	1:80
P27 (mouse monoclonal; Novocastra)	1:200

DAKO, Kyoto, Japan; *Sauta Cruz*, Sauta Cruz, CA, USA

TABLE 2. Correlation of cell cycle regulator expression with Ki-67 expression

	cdk2	Cyclin D1	Cyclin E	Cyclin A	p53	p27
r_s	0.184	0.266	0.398	0.416	0.286	0.226
P-value	0.0574	0.0031	<0.0001	<0.0001	0.0031	0.0192

$P < 0.01$, $P < 0.05$ (Spearman's rank correlation test)

TABLE 3. Correlation of cell cycle regulator expression with histological grade

			cdk2	Cyclin D1	cyclin E	Cyclin A		
	n	Ki-67	Labeling index (mean ± SD)				p53	p27
G1	73	49.4 ± 24.0*	10.7 ± 14.3**	12.5 ± 23.9*	31.5 ± 33.3*	16.3 ± 6.9*	11.7 ± 12.4*	55.1 ± 25.0*
G2	26	52.2 ± 21.6	17.3 ± 16.3	14.4 ± 26.6	37.8 ± 32.0	18.3 ± 8.8	25.6 ± 26.7	64.4 ± 15.1
G3	28	72.4 ± 15.9*	20.7 ± 19.7**	15.8 ± 18.9*	51.0 ± 30.8*	30.2 ± 11.8*	50.9 ± 29.4*	75.0 ± 10.7*

*$P < 0.01$, **$P < 0.05$ (Mann–Whitney *U*-test)

Correlation of the Expression of Cell Cycle Regulators with Histological Grade

The LIs of cdk2, cyclin D1, E, A, p53, and p27, as well as Ki-67, in grade G1 carcinomas increased significantly, more than the LIs in samples with a higher histological grade (Table 3). Statistical analysis was performed with the Mann–Whitney *U*-test.

Correlation of the Expression of Cell Cycle Regulators with Clinicopathological Parameters

Factors of endometrial adenocarcinomas, such as stage, histological grade, coexisting with or without endometrial hyperplasia [9], myometrial invasion, lymphovascular space involvement (LVSI), lymph node metastasis, ER [10], and progesterone receptor (PR) [11] are useful for identifying whether patients are at high or low risk for relapse of endometrial carcinoma.

The FIGO stage was correlated with p53 and p27 expression. The LVSI was correlated with cyclin E, A, p53, and p27. Lymph node metastasis was correlated with p27.

TABLE 4. Correlation of cell cycle regulator expression with clinicopathological parameters

Clinicopathological parameters		n	cdk2	Cyclin D1	cyclin E	cyclin A	p53	p27
			Labeling index % (mean ± SD)					
Stage	FIGO	85	13.1 ± 16.4	14.8 ± 25.9	39.7 ± 34.8	19.2 ± 11.6	19.2 ± 20.3†	60.7 ± 21.8*
	FIGO	12	21.2 ± 18.5	12.3 ± 26.6	40.4 ± 28.1	16.6 ± 8.5	23.9 ± 28.6†	57.9 ± 24.1*
	FIGO	27	13.0 ± 15.5	10.2 ± 12.0	28.5 ± 28.3	15.9 ± 7.9	27.8 ± 29.8†	61.1 ± 19.8*
	FIGO	3	11.4 ± 12.6	16.2 ± 14.2	29.3 ± 41.0	39.5 ± 0.7	90.7 ± 2.3†	77.4 ± 4.9*
Lymphovascular involvement	Negative	87	12.5 ± 15.6	13.0 ± 24.1	32.0 ± 33.7†	16.7 ± 10.4*	16.7 ± 19.0†	56.6 ± 23.2†
	Positive	30	18.2 ± 18.8	15.3 ± 24.4	55.5 ± 29.1†	22.3 ± 10.0*	37.2 ± 33.6†	72.2 ± 13.3†
Lymphonode metastasis	Negative	100	13.9 ± 17.1	14.6 ± 25.6	37.9 ± 34.4	18.0 ± 11.2	16.7 ± 17.7	57.7 ± 22.8†
	Positive	13	16.0 ± 11.4	12.9 ± 12.4	42.2 ± 27.4	21.4 ± 8.5	38.5 ± 34.5	75.8 ± 13.2†
Myometrial invasion	<1/3	64	14.5 ± 15.5	5.8 ± 8.2	16.3 ± 22.4*	16.3 ± 8.8	9.5 ± 11.3*	59.2 ± 24.4
	≧1/3	54	13.8 ± 16.7	14.4 ± 25.0	40.0 ± 34.1*	19.3 ± 9.8	24.1 ± 25.5*	62.6 ± 18.0

† $P < 0.01$, * $P < 0.05$ (Mann–Whitney U-test)

TABLE 5. Correlation of cell cycle regulator expression with clinicopathological parameters

Clinicopathological parameters		n	cdk2	CyclinD1	cyclinE	cyclinA	p53	p27
			Labeling index % (mean ± SD)					
Group	1	56	11.9 ± 14.6	13.3 ± 22.7	33.0 ± 31.5	15.4 ± 8.7†	16.7 ± 27.0*	61.4 ± 19.7
	2	52	15.9 ± 18.6	14.8 ± 27.0	38.1 ± 34.5	21.2 ± 11.6†	25.8 ± 31.0*	60.4 ± 21.7
	3	17	15.5 ± 15.0	8.0 ± 10.0	45.0 ± 36.8	18.7 ± 12.1	24.1 ± 30.2	57.8 ± 27.9
ER	Positive	56	14.5 ± 16.6	13.6 ± 25.0	41.1 ± 33.8	19.2 ± 10.9	19.6 ± 21.7	61.1 ± 23.0
	Negative	42	13.8 ± 17.1	13.5 ± 22.3	30.2 ± 31.4	17.5 ± 11.1	24.5 ± 28.7	65.3 ± 17.9
PR	Positive	50	13.9 ± 15.8	12.3 ± 24.6	38.6 ± 34.6	19.3 ± 10.5	17.8 ± 20.9	63.2 ± 21.2
	Negative	48	14.1 ± 17.8	14.8 ± 23.0	35.5 ± 31.3	17.4 ± 11.8	25.9 ± 27.8	63.1 ± 18.8
Menopause	Pre	36	14.3 ± 15.4	8.3 ± 14.7	25.1 ± 32.4	17.2 ± 11.3	61.5 ± 24.6	61.5 ± 24.6
	Post	89	14.2 ± 16.8	14.8 ± 24.4	41.4 ± 32.9	19.3 ± 10.9	60.7 ± 20.1	60.7 ± 20.1
Recurrence	–	108	15.2 ± 17.0	14.2 ± 23.8	37.2 ± 33.2	18.1 ± 10.6	23.6 ± 30.0	62.1 ± 20.9
	+	8	7.9 ± 8.9	15.5 ± 32.0	45.9 ± 33.1	17.2 ± 8.7	30.3 ± 39.6	65.0 ± 20.1

† $P < 0.01$, * $P < 0.05$ (Mann–Whitney U-test)

ER, estrogen receptor; *PR*, progesterone receptor

Myometrial invasion was correlated with the expression of cyclin E and p53 (Table 4). With regard to hormone-related factors, the expression of cyclin A and p53 was higher in group 2 (i.e., coexisting with normal endometrium without hyperplasia) than in group 1 (i.e., coexisting with hyperplasia). No other factors were associated with the expression of cell cycle regulators (Table 5).

Discussion

Previous reports have showed that increased cyclin E expression is linked with poor histological grade in 28 cases of endometrial adenocarcinoma (endometrioid type) [12] and in breast cancer [13]. Staining for cyclin A expression is significantly

more frequently positive in poorly differentiated adenocarcinoma than in well-differentiated adenocarcinoma [3,14]. Kallakury et al. reported that cyclin A was expressed in 64% of endometrioid subtypes and 71% of serous subtypes of endometrial carcinoma [15].

Mutation of the *p53* gene is related to the progress of the disease and metastasis, and a prognostic influence for accumulated nuclear mutant type p53 protein has been demonstrated [4].

It has been generally accepted that decreased p27 expression is related to poor prognosis in tumors of different histogenesis, including lung [5], esophageal [16], colon [17], breast [18], ovarian [19], and prostate cancers [20]. Surprisingly, p27 expression in endometrioid adenocarcinoma of the uterine corpus increased significantly in the higher histological grades in our study [21]. In contrast, two reports on endometrial adenocarcinoma demonstrated that decreased p27 expression was correlated with higher histological grade [22,23]. However, a trend associated with increased p27 staining with advanced grades of endometrial carcinoma was reported recently [24]. A possible mechanism for the abnormal expression of p27 could be considered as follow. First, the functional abnormality may be due to gene mutation. However, no detectable cancer-specific mutations were found in a total of 147 human tumors [25]. Although polymorphism as a nucleotide substitution of guanine for thymine (GTC $\rightarrow$ GGC) at codon 109 was found in endometrial, uterine cervical, and ovarian cancers, this polymorphism is also detected in normal cells [26]. Deletions of the *p27* gene have only been detected in B-immunoblastic non-Hodgkin's lymphomas and adult T cell leukemias/lymphomas [27]. Second, there may be a quantitative or structural abnormality of the cyclin E/cdk2 complex. There are two possibilities in this respect. One may be an excessive amount of the complex beyond the inhibitory action of p27. The other may be due to p27 that may act, controversially, as an assembly factor to stabilize the complex. Third, consumption of p27 may be trapped by other factors, such as cyclin D1 and D3, which suppresses the formation of the complex [28]. Fourth, p27 degradation by the ubiquitin–proteasome pathway, in which skp2 is implicated, may be disordered [29]. However, clarification of the precise mechanism of these possibilities requires further studies. Moreover, p27 may be overexpressed by a homeostatic feedback mechanism in cases of overexpressed p27, because high levels of expression of cyclin E and cdk2 are observed in these cases [5].

Our results show that cyclin E, A, and p27, as in the case of p53, may be markers for more proliferative and malignant features and may be added to the list of biomarkers used to characterize subsets of patients with endometrial adenocarcinoma.

Regulation of Cyclins by Estrogen in Endometrial Cancer Cells

A variety of clinical and experimental observations has established that stimulation of estrogens without an effect of progesterone is the leading cause of the development of endometrial hyperplasia and adenocarcinoma [30]. The understanding of cell cycle regulation has been advanced recently and molecular mechanisms through which estrogens regulate the cell cycle in cultured breast cancer cells have been reviewed

[31]. However, regulation of cyclins in endometrial cancer cells by estrogen has not yet been examined.

Methods

An ER transfectant of the Ishikawa cell line was used. Original Ishikawa cells were ER-positive and sensitive to estrogen, but less sensitive recently [32]. The HEGO (human ERα cDNA; wild type) was used as the ERα gene. pSG5-HEGO was used as the expression vector [33]. Genes were transfected using the SuperFect method (QIAGEN, Tokyo, Japan). Stable transfectants were selected by neomycin. The expression of the *ER* gene was analyzed by Western blotting and immunohistochemistry. Western blotting signals were detected with the enhanced chemiluminescence (ECL) method (Amersham, Buckinghamshire, UK). Immunohistochemical staining was performed using the LSAB method (DAKO). Estrogen responsiveness was analyzed by calculating growth curves. The medium used consisted of Dulbecco's modified Eagle's medium (DMEM) and F12 mixed in a ratio of 1 : 1 (ICN Biomedicals, Auroa, OH, USA) without phenol red and with 1% charcoal-treated fetal bovine serum. The ER was expressed strongly in ER-transfectants, Ishikawa-8, and -10 cells.

Effect of E2 on the Growth of ER-Transfected Ishikawa Cells

The dose-dependent stimulatory effect on cell growth of E2 on Ishikawa-8 cells at day 8 was analyzed by counting cells (Fig. 2). A maximal effect on cellular growth of 160% of control was observed with a concentration of 10^{-8} M E2. At 10^{-8} M, E2 significantly and time-dependently enhanced the growth of Ishikawa-8 cells.

Expression of Cell Cycle Regulators in ER-Transfected Ishikawa Cells

The LI for Ki-67 in Ishikawa-8 cells 24 h after the addition of E2 was 100%, the same as that without E2. The LI for cyclin E was 20.9% and 28.3% in the absence and presence of E2, respectively. The LI for cyclin E was increased by 33% in the presence of E2 compared with control. The LI for cyclin A was 26% and 34.9% in the absence and presence of E2, respectively. The LI of cyclin A was increased by 35% in the presence of E2 compared with control.

Discussion

Transfection experiments with wild-type and mutant ER cDNA transfected into different mammalian cell lines have been performed to reestablish hormone control over hormone-independent cells. Paradoxically, it has been reported that introducing exogenous ER into ER-negative cells leads to growth inhibition by estrogen rather than growth promotion [33]. The mechanism of the inhibitory effect of estrogen on the growth of these transfectants is not clear. The reason why our transfectants could respond to estrogen may be because we used cells that responded to estrogen originally and the post-ER signal transduction system has been preserved in these cells.

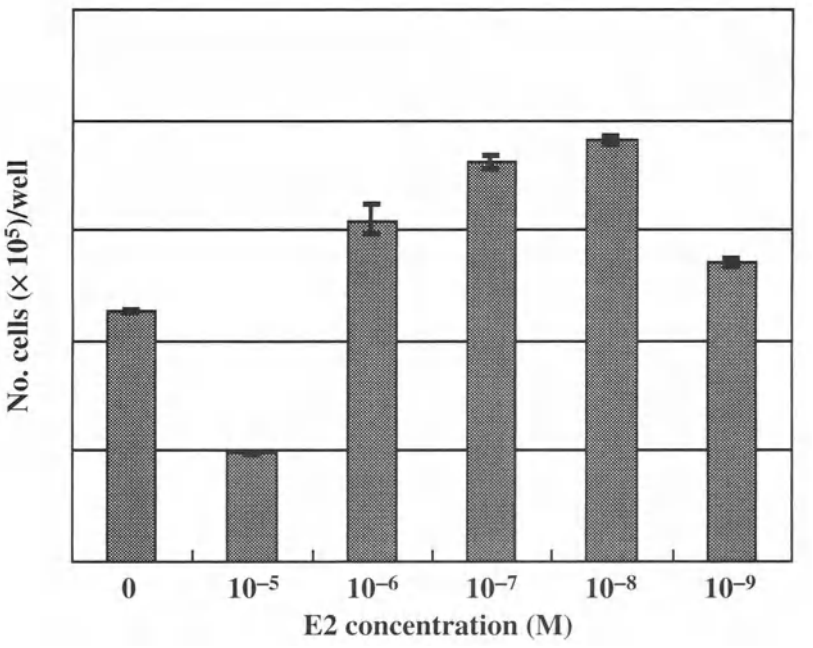

FIG. 2. Dose-dependent stimulatory effect of cell growth by estradiol (*E2*) on Ishikawa-8 cells at day 8

Altucci et al. demonstrated that E2 could activate cyclin D1 promoter–reporter constructs that contained imperfect partial estrogen-response elements in breast cancer cells and that the addition of E2 to G0/G1-arrested cells resulted in a three- to five-fold increase in cyclin D1 protein within 3–6 h [34]. Cyclin D1 plays the role of an upstream sensor of signals that are responsible for regulating the cyclin E/cdk2 complex through the formation of a complex with cdk4 and cdk-inhibitor sequestration [35]. E2–ER complexes regulate cell cycle parameters beyond cyclin E–cdk2, which is fully activated at 8–10 h after E2 treatment in breast cancer cells [36]. Stimulated cyclin E/cdk2 complex activity following the addition of E2 is the result of a minor fraction of the cyclin E/cdk2 complex, which is free of cdk inhibitors, such as p21, and is, therefore, active [37]. In the present study, cyclin A was activated by E2. Although the direct correlation between E2 and cyclin A is not clear, our results suggest that cyclins induced by estrogen may be involved in the estrogen-induced growth enhancement of human endometrial cancer cells.

Regulation of a Cell Cycle Inhibitor (p27) by Progestin in Endometrial Cancer Cells

Progesterone promotes the progression of the proliferative-phase endometrium to the secretory phase, suppressing cell growth and inducing differentiation. Progestin is also reported to suppress the growth of some endometrial carcinomas and its admin-

istration has been established as a progestin therapy [38]. However, the detailed mechanism controlled by progestin has not yet been established. Clinically, it is necessary to follow up patients for a long time in order to assess the effect of progestin by frequent cytological and histological examinations, which can add to the suffering of the patients.

It was recently reported that progestin induced p27 protein levels in breast cancer cells [39], but there is no report yet on the effects of progestin in endometrial cancer cells. Therefore, the aim of the present study was to investigate whether p27 protein is accumulated by medroxyprogesterone acetate (MPA) in relation to cell growth inhibition, using human endometrial cancer cell lines established in our laboratory.

Methods

HEC-265 and HEC-50B were used as human endometrial cancer cells in the present study. HEC-265 is positive for the PR, whereas HEC-50B is negative for the PR. MPA was used as progestin. MPA-responsiveness of the cells was examined by constructing growth curves. The expression of the p27 protein was analyzed by Western blotting. The p27 protein level was measured by a National Institutes of Health (NIH) image analyzer and standardized against the β-actin level.

Effect of MPA on the Growth of HEC-265 Cells

MPA produced dose-dependent inhibitory effects on the growth of HEC-265 cells. The number of cells was suppressed to 69% of control in the presence of 10^{-6} M MPA on day 6. In the presence of 10^{-5} M MPA, most cells were severely damaged (Fig. 3).

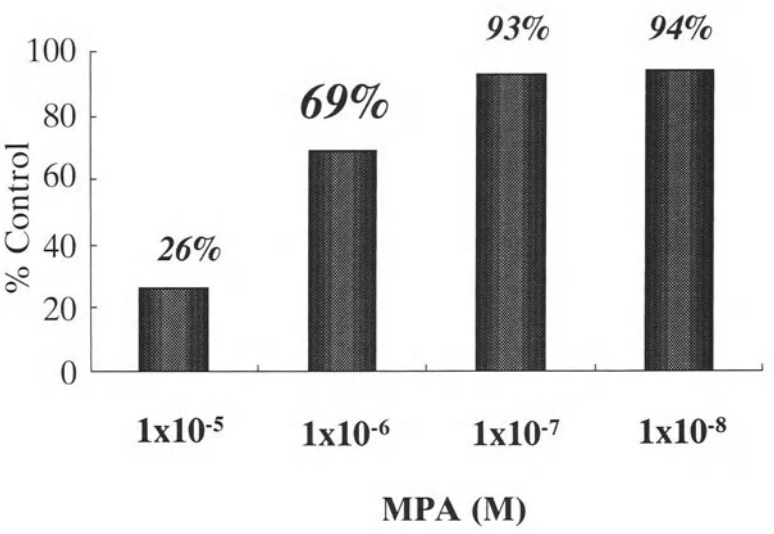

FIG. 3. Dose-dependent inhibitory effect of medroxyprogesterone acetate (*MPA*) on cell growth of HEC-265 cells at day 6

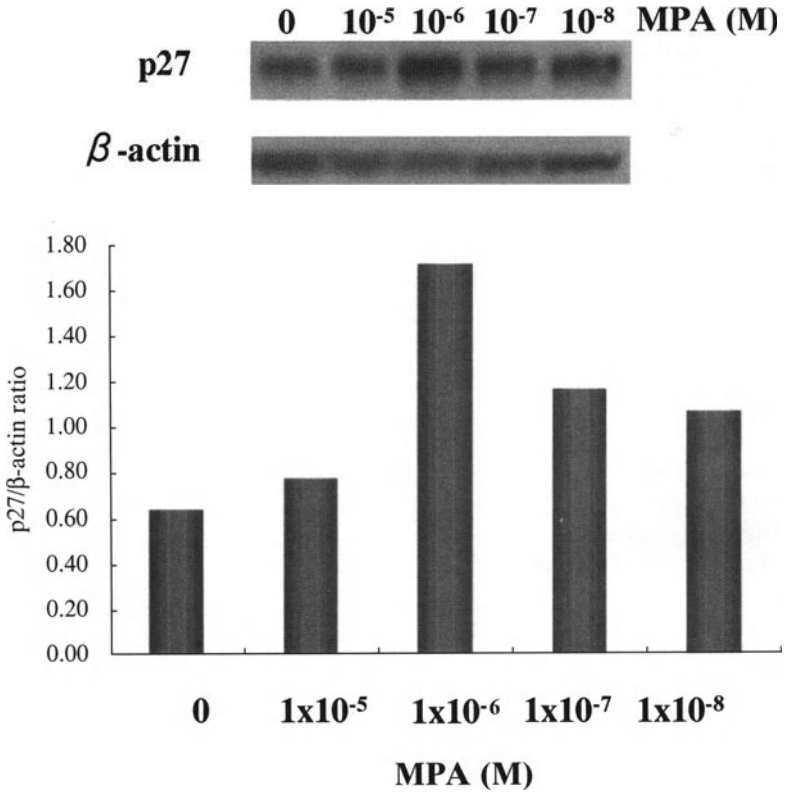

FIG. 4. Dose-dependent stimulatory effect of medroxyprogesterone acetate (*MPA*) on the accumulation of p27 by HEC-265 cells 24 h after the addition of MPA

Effect of MPA on p27 Accumulation in HEC-265 Cells

The maximal effect on p27 accumulation by HEC-265 cells occurred 24 h after the addition of 10^{-6} M MPA (Fig. 4). The accumulation of p27 in HEC-265 cells was stimulated in a time-dependent manner by MPA and was maximal (1.56-fold compared with control) 24 h after the administration of 10^{-6} M MPA (Fig. 5). In contrast, MPA did not stimulate the accumulation of p27 in HEC-50B cells.

Discussion

p27 is markedly increased in the secretory phase human endometrium [21]. p27 expression in hyperplastic epithelia of the endometrium before MPA treatment was negligible, whereas it was greatly increased after the administration of MPA [40]. It was recently reported that progestin induced p27 protein levels in breast cancer cells [39] and that progesterone-induced growth inhibition was preceded by decreased cyclin and cdk activity due to increased amounts of p27 [41]. Our results suggest that MPA-induced accumulation of p27 may be involved in the progesterone-induced

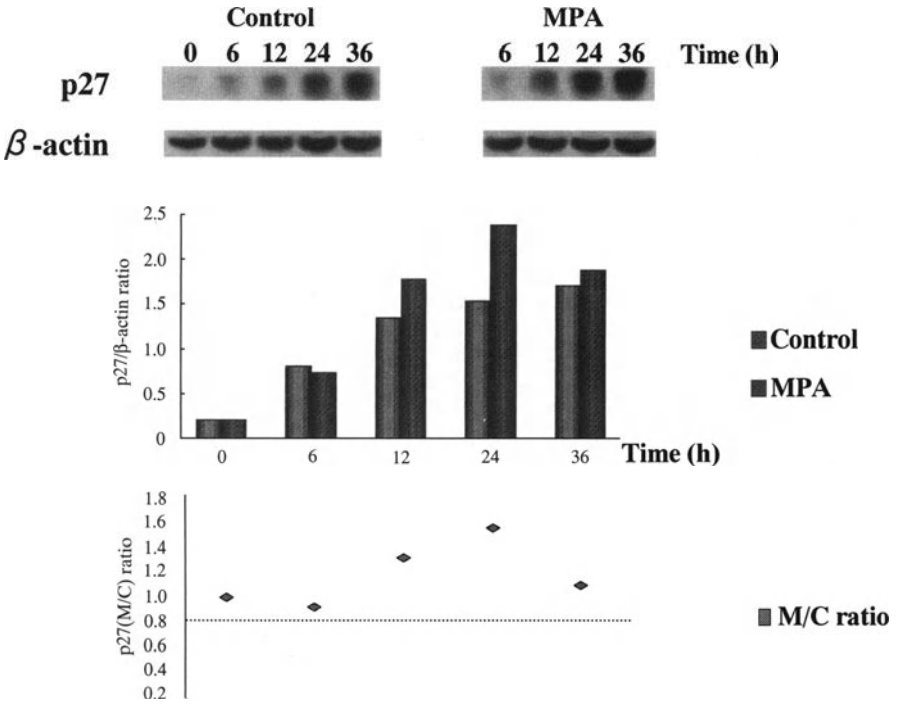

FIG. 5. Time-dependent stimulatory effect of of medroxyprogesterone acetate (*MPA*; 10^{-6} M) on the accumulation of p27 accumulation by HEC-265 cells

growth suppression of PR-positive human endometrial cancer cells. p27 could be a useful marker in evaluating the effectiveness of MPA therapy in patients with endometrial carcinoma because the accumulation of p27 may occur at an early stage of therapy and may play a role in predicting whether therapy will be effective or not.

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Part 4
Signaling Pathways

Estrogen Receptor Signaling and Crosstalk with the Ah Receptor in Endometrial Cancer Cells

Stephen Safe, Mark Wormke, Kelcey Walker, Richard Dickerson, and Emely Castro-Rivera

Summary. Common risk factors for the development of endometrial and breast cancer include early menarche, late menopause, null parity, and later age at first birth, indicating that "lifetime" exposure to estrogens increases the incidence of both tumors. In contrast, smoking protects against the development of endometrial cancer, whereas the role of smoking in breast cancer incidence is equivocal and may be dependent on the timing and duration of smoking. Constituents of cigarette smoke bind and activate the aryl hydrocarbon receptor (AhR), and research in this laboratory has focused on characterizing the inhibitory AhR–estrogen receptor (ER) α crosstalk in endometrial and breast cancer cell lines. Both Ishikawa and ECC1 endometrial cancer cells express the AhR and ERα proteins by Western blot analysis. Moreover, AhR ligands such as benzo[a]pyrene (BaP) and/or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induce CYP1A1-dependent activity or reporter gene activity in cells transfected with constructs containing dioxin-responsive elements as promoters. Estrogen responsiveness was also confirmed in these cells, as evidenced by gene/reporter gene assay and the induction of cell proliferation by 17β-estradiol (E2). Inhibitory AhR–ERα crosstalk studies have shown that TCDD and/or BaP inhibit E2-induced growth of endometrial cancer cells and also block hormone-activated reporter gene/gene responses. Although there are several possible mechanisms for the interaction between AhR and ERα signaling pathways, the role of AhR-mediated downregulation of ERα will be discussed as one possible mechanism. In addition, selective AhR modulators have been developed for the treatment of breast and endometrial cancer and the potential use of these compounds alone or in combination with tamoxifen will be outlined.

Key words. Endometrial cancer, aryl hydrocarbon receptor (AhR), inhibitory crosstalk

Introduction

Endometrial cancer is the most common gynecological cancer in the United States and it is the fourth leading cause of cancer in women after carcinomas of the breast, lung, and colon. In 1998, there were more than 36 000 cases of endometrial cancer in

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the United States and 6300 deaths from this disease [1–3]. Exposure to unopposed estrogens are risk factors for both endometrial and breast cancer, and these include early age at menarche, late age of menopause, null parity, obesity, and hormone-replacement therapy [2,4–7]. Increased risk for endometrial cancer is also associated with prolonged use of the selective estrogen receptor modulator (SERM) tamoxifen for the treatment of breast cancer [8–11]. This differential effect of tamoxifen on two hormone-dependent tumors is linked to the tissue-specific estrogen receptor (ER) agonist and antagonist activity in the breast and endometrium, respectively, and new SERMs with minimal ER agonist activity in the uterus have been reported [12,13]. Cigarette smoking has been linked to an increased incidence of several diseases; however, there is considerable evidence that suggests cigarette smoking is antiestrogenic [14]. For example, smoking decreases the age of menopause, increases the incidence of osteoporosis, and decreases the risk of benign breast cancer, endometrial cancer, and other estrogen-linked uterine diseases. Although the antiestrogenic effects of cigarette smoking in breast cancer are equivocal [15–17], several studies have reported a lower incidence of endometrial cancer in smokers [14,18–23]. Cigarette smoking does not alter serum levels of steroid hormones; however, increased urinary levels of 2-hydroxyestradiol have been reported [23,24].

The protective effects of cigarette smoking against the development of endometrial cancer increase in heavy smokers (i.e., the protective effect is dose dependent) and relative risks can be 0.5 or lower. The biologically active constituents of cigarette smoke that protect against endometrial cancer have not been determined; however, some of the combustion-derived polynuclear aromatic hydrocarbons (PAHs) identified in smoke exhibit antiestrogenic activity [25,26]. Studies in this laboratory have been investigating the mechanisms associated with inhibitory aryl hydrocarbon receptor (AhR)–ER crosstalk in breast and endometrial cancer cell lines. Selective AhR modulators (SAhRMs), such as 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) and substituted diindolylmethanes (DIMs) (Fig. 1), are also being developed for the treatment of hormone-dependent tumors [27–31]. This paper will outline results of in vitro studies in endometrial cancer cell lines and in vivo experiments in the female rodent uterus confirming functional inhibitory AhR–ERα crosstalk in both systems. These results not only indicate that AhR-active components in cigarette smoke may be responsible for decreased endometrial cancer in smokers, but also suggests that

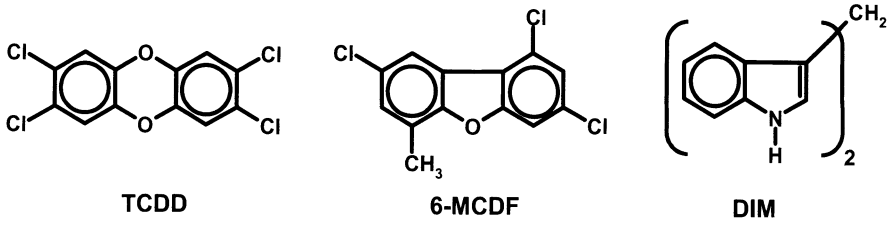

FIG. 1. Structures of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (*TCDD*) and the selective aryl hydrocarbon receptor modulators 6-methyl-1,3,8-trichlorodibenzofuran (*6-MCDF*) and diindolylmethane (*DIM*)

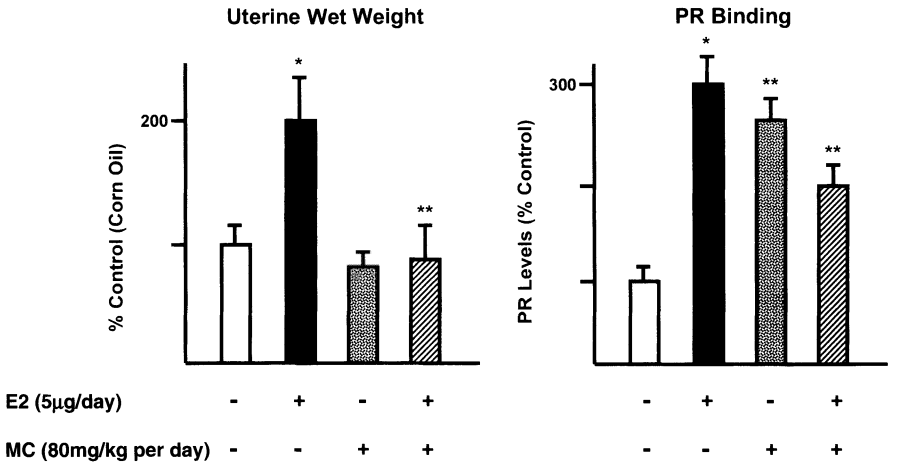

FIG. 2. Antiestrogenic activity of 3-methylcholanthrene (*MC*) in the rodent uterus. Immature 21-day-old female Sprague-Dawley rats were treated with estradiol (*E2*; 5 μg/day), MC (80 mg/kg per day), or their combination for 3 days. Uterine wet weight and progesterone receptor (*PR*) binding were determined 24 h after the last treatment. Significant ($P < 0.05$) induction (*) or inhibition (**) is indicated

SAhRMs may be useful mechanism-based drugs for the clinical treatment of endometrial and breast cancer [27–31].

Inhibitory AhR–ERα Crosstalk in the Rodent Uterus

The immature or ovariectomized rat and mouse uterus is highly estrogen responsive and, after exposure to 17β-estradiol (E2), there is a rapid increase in uterine wet weight and DNA synthesis, which is accompanied by increased expression of multiple E2-regulated genes. Initial studies showed that the E2-induced uterine wet weight increase in immature female rats was inhibited in animals cotreated with E2 and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an environmental toxicant that exhibits high affinity for the AhR [32–34]. Subsequent studies showed that TCDD and structurally related AhR agonists inhibited multiple E2-induced uterine responses, including progesterone receptor (PR) binding, peroxidase activity, epidermal growth factor receptor (EGFR) binding and mRNA levels, and c-fos mRNA levels [32–37]. We also investigated the antiestrogenic activity of the PAH 3-methylcholanthrene (MC), which is a prototypical AhR agonist [38]. The results demonstrated that MC (80 mg/kg per day for 3 days) alone inhibited uterine wet weight and also inhibited the E2-induced increase in uterine wet weight, as reported previously for TCDD (Fig. 2). Moreover, like TCDD, MC also induced hepatic CYP1A1-dependent ethoxyresorufin *O*-deethylase (EROD) activity in rats and induction of this response is a marker of AhR-mediated toxicities. Inhibition of E2-induced uterine responses by MC appears to

be mediated through the AhR and provides an explanation for the antiestrogenic activity of cigarette smoking, which can be mediated, in part, by AhR-active PAH combustion products.

Estrogen-Responsiveness of Endometrial Cancer Cells

Introduction

Endometrial cancer cells have been used extensively as in vitro cell culture models for investigating the mitogenic and antimitogenic activities of hormones, polypeptide growth factors, and other growth-regulatory factors [39]. Ishikawa cells are a well-differentiated endometrial adenocarcinoma cell line used for studying endometrial carcinogenesis [40]. Ishikawa cells are E2 responsive and express primarily ERα, with only low levels of ERβ detected [41]. E2 induces proliferation of Ishikawa cells and this is accompanied by induction of alkaline phosphatase, transforming growth factor (TGF) α, insulin-like growth factor 1 (IGF-1), and PR mRNA and/or protein levels [42–48]. In addition, TGFα and IGF-1 were also mitogenic in this line; however, the effects of polypeptide growth factors on uterine and endometrial cancer cell responses are both ER dependent and independent [45,48,49]. ECC1 endometrial cancer cells are also a well-differentiated E2-responsive cell line that expresses the ER and higher levels of PR [50,51]. The hormone responsiveness of HEC1A endometrial adenocarcinoma cells is less well defined. For example, in one study, it was reported that E2 activated the migration potential of HEC1A (and Ishikawa) cells through a basement membrane and 4′-hydroxytamoxifen (4OHT) antagonized this estrogenic response [52]. In contrast, Nguyen et al. [51] reported that HEC1A cells were negative for the ER (1.9 fmol/mg). Research in this laboratory has used Ishikawa, ECC1, and HEC1A endometrial cancer cells to investigate their E2 responsiveness, as well as their response to the SERM, 4OHT, TCDD, and related SAhRMs [53–55].

E2- and Tamoxifen-Responsiveness of Ishikawa, ECC1, and HEC1A Endometrial Cancer Cells

Research in this laboratory has identified the expression of ERα protein in ECC1, Ishikawa, and HEC1A cells [51–55]. E2 induced proliferation of all three cell lines and 10 nM E2 induced a greater than sixfold increase in the proliferation of ECC1 cells (Fig. 3). The mitogenic activity of E2 and 4OHT in Ishikawa cells is dependent on serum growth conditions, as reported previously [42–45]. Proliferation was observed only after cell growth in serum-free media, whereas E2 and 4OHT did not affect the proliferation of cells cultured in medium containing serum- or charcoal-stripped serum (data not shown). Moreover, in ECC1 and HEC1A cells, 4OHT acts primarily as an ER antagonist and exhibits minimal mitogenic activity only at some concentrations in HEC1A cells [53]. In parallel studies, the "pure" steroidal antiestrogen ICI 182,780 acts as an ER antagonist and inhibits growth of solvent (control)- and E2-treated endometrial cancer cells. These results suggest that, in contrast with some previous data, 4OHT exhibits primarily ER antagonist activity with respect to the hormone-dependent growth of endometrial cancer cells.

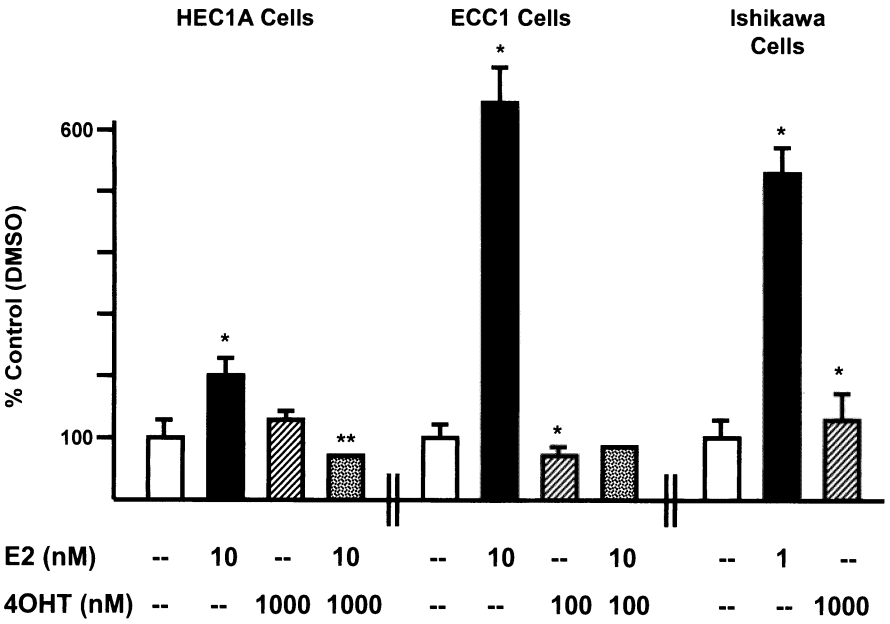

FIG. 3. Estradiol (*E2*)-responsiveness of endometrial cancer cells. Cells were essentially cultured as described previously [53–55] and treated with E2, 4′-hydroxytamoxifen (*4OHT*), and their combination. Significant ($P < 0.05$) cell growth (*) or inhibition (**) of E2-induced cell growth is indicated. *DMSO*, dimethylsulfoxide

In vitro studies with 4OHT showed that the ERα agonist activity in some cell lines was dependent on activation function 1 (AF1) of ERα, where 4OHT activated transcription in cells (e.g. HepG2) transfected with ERα-AF1 and an E2-responsive pC3 promoter reporter construct containing a human complement C3 promoter insert [56,57]. ERα-AF1 contains D538N, E542Q, and D545N mutations in helix 12 of ERα and, thereby, prevents AF2/ligand-dependent interactions with coactivators. We have also investigated the comparative activation of genes and/or reporter gene constructs by E2 and 4OHT in endometrial, breast, bone, and liver cancer cell lines cotransfected with wild-type ERα, ERα-AF1, and ERα-AF2, in which the AF1 (A/B domain) of ERα has been deleted [58–62]. The results, illustrated in Fig. 4, summarize the responses observed for E2 (10 nM) and 4OHT (1 μM) in ECC1 and HEC1A cells transfected with constructs containing E2-responsive rat creatine kinase B (2.9 kb/pCKB) and human complement C3 (−1807 to +58; pC3) gene promoter inserts. These data show that E2 induced transactivation in cells transfected with ERα and pC3 or pCKB, whereas 4OHT activated pC3 in both cell lines, but pCKB was activated by 4OHT only in ECC1 cells. 4OHT only exhibited AF1-dependent activity in ECC1 cells cotransfected with ERα-AF1/pCKB and did not activate pCKB or pC3 in cells transfected with ERα-AF2. In parallel studies, we also used an E2-responsive construct (pCATHD) containing a *cathepsin D* gene promoter insert (2.4 kb) that is activated by E2 (and weakly activated

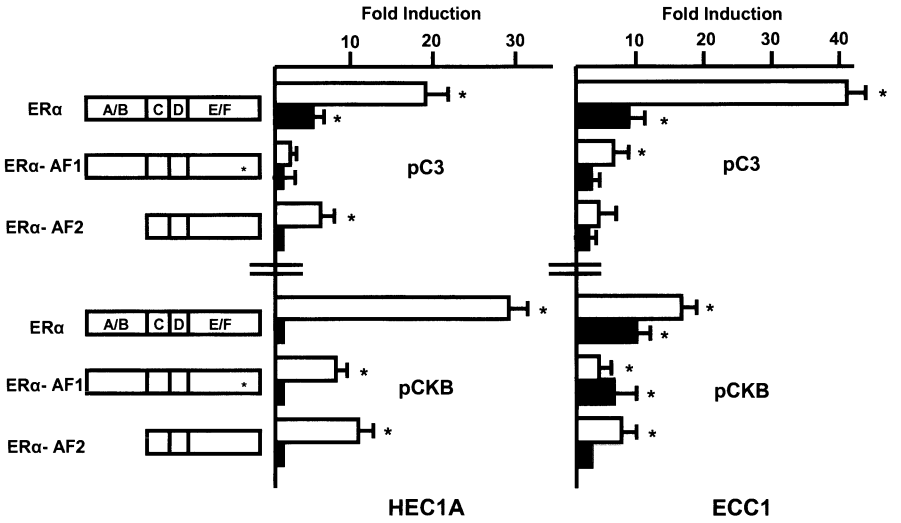

FIG. 4. Comparative estradiol (E2)- and 4′-hydroxytamoxifen (4OHT)-induced transactivation in endometrial cancer cells transfected with pC3 or pCKB and wild-type or variant estrogen receptor α (*ERα*). HEC1A (*left*) or ECC1 (*right*) endometrial cancer cells were transfected with pCKB or pC3 and wild-type or variant ERα, treated with 10 nM E2 or 1 μM 4OHT, and reporter gene activity was determined as described previously [58–62]. Significant ($P < 0.05$) induction is indicated with an asterisk. *AF1*, *AF2*, activation function 1 and 2, respectively

by 4OHT) in both endometrial cancer cell lines only after cotransfection with ERα, but not ERα-AF1 or ERα-AF2 [62]. These data demonstrate that the ERα agonist activity of 4OHT in HEC1A and ECC1 endometrial cancer cells was highly variable and dependent on cell context, gene promoter, and cotransfected wild-type/variant ERα. The AF1-dependent activity was observed only in ECC1 cells transfected with pCKB and studies in HepG2 (liver), MDA-MB-231 (breast), ZR-75 (breast), and U2 (osteogenic sarcoma) cancer cell lines showed that 4OHT exhibited activity with ERα-AF1 only in HepG2 cells transfected with pC3. 4OHT did not activate pCKB, pC3, or pCATHD in endometrial or other cancer cells transfected with ERα-AF2, suggesting that both domains of ERα are required for 4OHT-dependent transactivation.

Research in this laboratory has also observed significant differences in hormone-induced gene expression between E2-responsive endometrial cancer cell lines. For example, E2 induced *cathepsin D* gene expression in MCF-7 cells and reporter gene activity in cells transfected with constructs containing *cathepsin D* gene promoter inserts [63–65]. Cathepsin D was not responsive to E2 in ER-positive Ishikawa endometrial cancer cells [66], whereas studies in this laboratory showed that E2 induced *cathepsin D* mRNA levels in ECC1 cells and reporter gene activity in cells transfected with pCATHD constructs [54]. Moreover, the direct-acting antiestrogens ICI 182,780 and 4OHT did not induce *cathepsin D* mRNA levels in ECC1 cells. Differences in hormone responsiveness between Ishikawa and HEC1A endometrial cancer

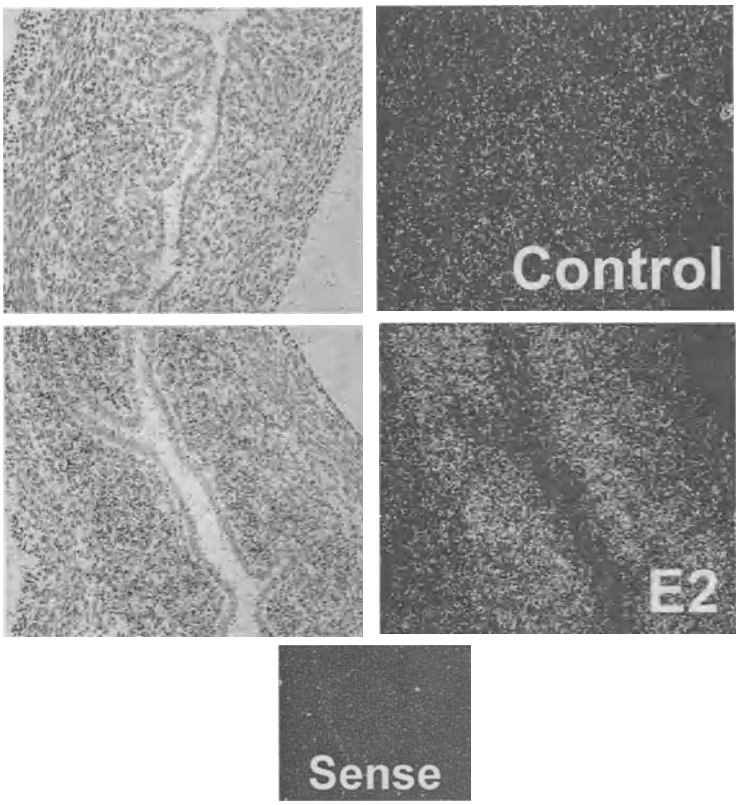

FIG. 5. Induction of vascular endothelial growth factor (VEGF) by estradiol (*E2*) in the mouse uterus. Twenty-five-day-old female B6C3F1 mice were treated with 200 ng 17β-estradiol or corn oil vehicle ($n = 4$ per treatment). One hour after treatment, the uteri were removed and fixed. Sections of the uteri were stained with ^{35}S-labeled cRNA antisense and sense probes for VEGF, washed, covered with emulsion, and developed. The stained sections were viewed under light and dark field microscopy (field width = 360 μm). A representative section of each treatment is shown

cells has also been observed for the induction of vascular endothelial growth factor (VEGF), which plays a critical role in angiogenesis. In vivo studies in 21-day-old female mice show that E2 induces VEGF gene expression as determined by in situ hybridization (Fig. 5). E2 induced VEGF expression in Ishikawa cells and analysis of the VEGF promoter identified a distal estrogen-response element (ERE) required for this response [67]. In contrast, E2 decreased VEGF mRNA levels in HEC1A cells and this response was dependent on ERα/Sp3 interactions with proximal GC-rich VEGF promoter elements [68]. These results demonstrate that estrogen and antiestrogen regulation of growth and gene expression in endometrial cancer cells is complex and dependent on ligand, cell context, and gene promoter.

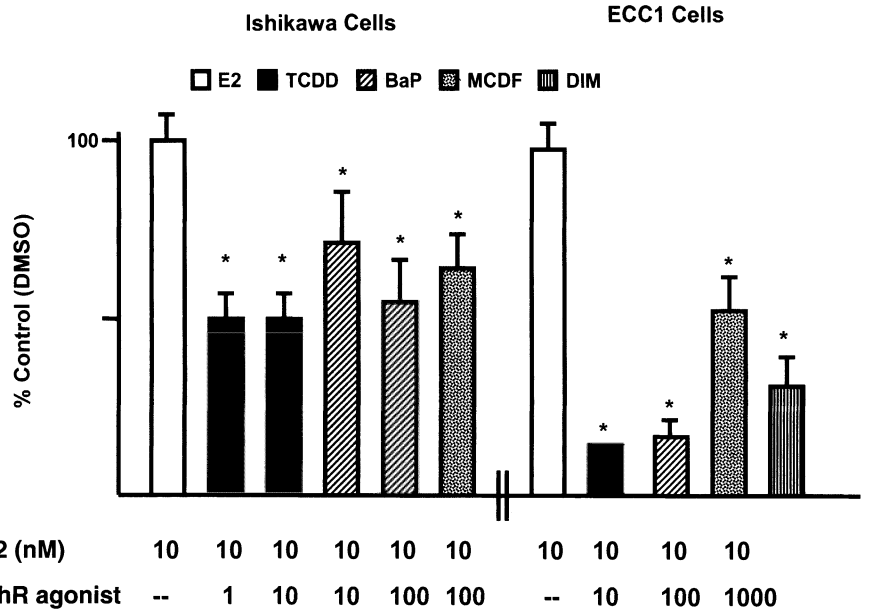

FIG. 6. Inhibitory aryl hydrocarbon receptor (*AhR*)–estrogen receptor α effects on hormone-induced endometrial cancer cell proliferation. The effects of 10 nM estradiol (*E2*) alone or in combination with AhR agonists was determined in ECC1 and Ishikawa cells as described previously [54,55]. Significant ($P < 0.05$) inhibition of E2-induced proliferation is indicated with an asterisk. *TCDD*, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; *BaP*, benzo[a]pyrene; *MCDF*, 6-methyl-1,3,8-trichlorodibenzofuran; *DIM*, diindolylmethane

AhR-ERα Crosstalk in Endometrial Cancer Cells

Previous studies in the rodent uterus and breast cancer cells demonstrated that TCDD and other AhR agonists block E2-induced growth and gene expression, and interactions between AhR and ER signaling pathways has also been investigated in human endometrial cancer cells [54,55]. Results showed that the AhR receptor was expressed in these cell lines and treatment with TCDD induced CYP1A1-dependent EROD activity, which is highly characteristic of Ah responsiveness. Inhibitory AhR-ERα crosstalk was also investigated in Ishikawa and ECC1 cells using TCDD, benzo[a]pyrene (BaP; a prototypical PAH), DIM, and 6-MCDF, a SAhRM that is relatively non-toxic but inhibits hormone-dependent tumor/cell growth and gene expression. The results in Fig. 6 show that TCDD, BaP, DIM, and 6-MCDF inhibit proliferation of Ishikawa and ECC1 cells. TCDD also inhibits E2-induced gene/reporter gene activity in both endometrial cancer cell lines and this includes *cathepsin D* mRNA levels in ECC1 cells. Thus, inhibitory AhR-ERα crosstalk is observed in endometrial cancer cells and parallels interactions between the signaling pathways previously observed in breast cancer cell lines [27–31]. Recent studies in this laboratory have demonstrated that

TCDD and other AhR agonists (including 6-MCDF) induce proteasome-dependent degradation of ERα in breast cancer cells and that these effects are reversed by proteasome (MG132) but not by protease (calpain II) inhibitors [69,70]. Moreover, in cells cotreated with E2 plus AhR agonists, the decreased ERα levels become limiting and result in decreased activation of genes/reporter genes. Using a similar approach, results of preliminary studies indicate that a similar mechanism contributes to inhibitory AhR-ERα crosstalk in endometrial cancer cells (data not shown).

Potential Applications of SAhRMs for the Treatment of Endometrial Cancer

Studies in this laboratory initially developed a series of alternate-substituted (1,3,6,8- and 2,4,6,8-) polychlorinated dibenzofurans (PCDFs) containing one or more alkyl substituents as AhR antagonists. These compounds, typified by 6-MCDF, bound to the AhR and induced formation of nuclear AhR complexes, but only weakly induced CYP1A1 and other AhR-mediated toxic responses [35,71–75]. Moreover, in cells and rodents cotreated with TCDD plus 6-MCDF, there was a significant inhibition of TCDD-induced CYP1A1, immunotoxicity, teratogenicity, and porphyria, demonstrating that 6-MCDF was an AhR antagonist [35,71–75]. Surprisingly, like TCDD, 6-MCDF inhibits estrogenic responses in the rodent uterus, breast, and endometrial cancer cell lines, and this inhibitory AhR-ERα crosstalk is accompanied by minimal AhR-mediated CYP1A1 induction or toxicities [35,38,54,55,75,76]. We have also characterized DIM and ring-substituted DIMs as a second group of SAhRMs that also exhibit minimal AhR-mediated toxicity but inhibit E2-induced estrogenic responses [77,78]. Like TCDD, SAhRMs activate inhibitory AhR-ERα crosstalk in the rodent uterus, breast, and endometrial cancer cell lines and inhibit mammary tumor growth in carcinogen-induced female Sprague-Dawley rats [70,78–81]. Although we have not investigated the direct effects of SAhRMs on endometrial tumor formation and growth in vivo, the inhibitory responses observed in the uterus and endometrial cancer cells (Fig. 6) suggest that these compounds may be useful for the clinical treatment of endometrial cancer. A recent study examined the effects of tamoxifen, 6-MCDF, and their combination for the treatment of carcinogen-induced mammary tumor growth in female Sprague-Dawley rats [70]. The results show that significant inhibition of tumor growth was observed at doses of 100 and 50 μg/kg per day for tamoxifen and 6-MCDF, respectively, whereas no significant inhibition was observed at lower doses (50 and 25 μg/kg per day for tamoxifen and 25 μg/kg per day for 6-MCDF). In contrast, cotreatment with tamoxifen plus 6-MCDF at the lowest dose (25 μg/kg per day) completely blocked tumor growth, and these interactions were synergistic [70] (Fig. 7). In parallel studies, it was shown that 6-MCDF blocked tamoxifen-induced uterine (but not bone) ER agonist activities, suggesting that combined tamoxifen–SAhRM treatment may provide enhanced endocrine therapy for the treatment of breast cancer and protection from tamoxifen-induced endometrial cancer. Research in this laboratory is investigating the mechanisms of inhibitory AhR–ERα crosstalk and further applications of SAhRMs for treating ER-positive and -negative breast and endometrial cancers.

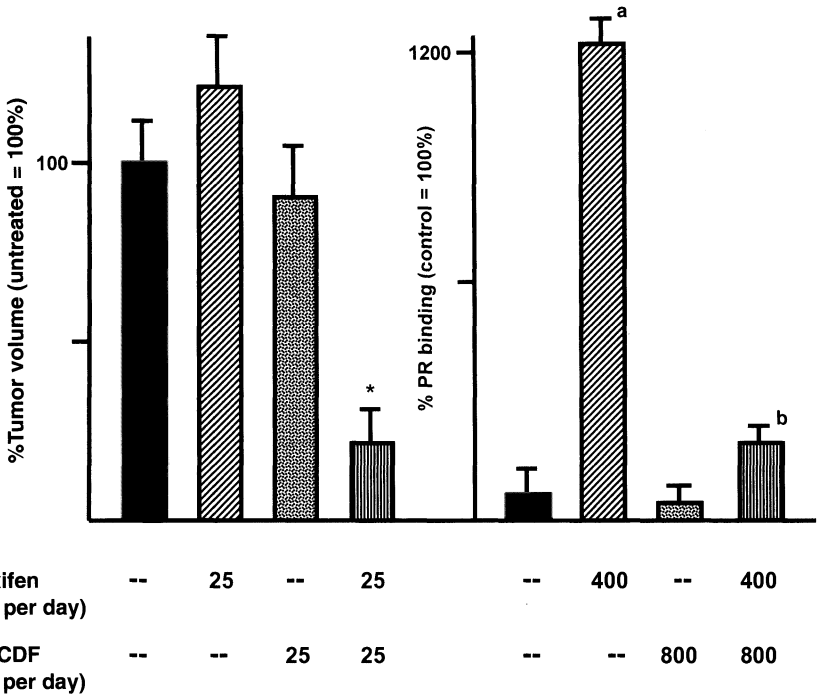

FIG. 7. Antiestrogenic and antitumorigenic activity of 6-methyl-1,3,8-trichlorodibenzofuran (*MCDF*) in female Sprague-Dawley rats. Ovariectomized (*right*) or carcinogen-induced (*left*) Sprague-Dawley rats were treated with different doses of tamoxifen, 6-MCDF or their combinations as described previously [70]. Significant ($P < 0.05$) inhibition of tumor growth (^{a}) or tamoxifen-induced progesterone receptor (*PR*) binding (^{b}) are indicated

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Expression of Cyclooxygenase (COX)-2 in Human Endometrial Adenocarcinoma Cell Line HEC-1B: An In Vitro Model of the Expression of COX-2 by Platelet-Activating Factor, Human Chorionic Gonadotropin and Prostaglandin E_2, and the Possible Signaling Pathways Involved

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Summary. Cyclooxygenase (COX) is the rate-limiting enzyme that converts arachidonic acid to prostaglandins (PGs). COX exists in two isoforms, COX-1 and COX-2. Most PGs in the human endometrium are secreted from epithelial cells and play a role in blastocyst implantation and stromal decidualization. Disruption of the COX-2 gene impairs fertility due to impairment of blastocyst implantation and interference with the decidualization process. During implantation of the blastocyst, COX-2 is expressed in the endometrium. Extracellular stimulation raises levels of calcium, inositol phosphates, and cAMP in uterine cells. These second messengers then activate protein kinase (PK) A and PKC and the extracellular signal-regulated kinases ERK-1 and ERK-2, prototype members of the mitogen-activated protein kinase (MAPK) family. MAPKs are the most attractive candidates to mediate COX-2 expression and thereby increase PG secretion in endometrial cells. MAPKs also play important roles in the regulation of cell growth and differentiation in response to growth factors, hormones, and cytokines in endometrial cells. Platelet-activating factor (PAF) and human chorionic gonadotropin (hCG) released during the secretory phase of the endometrium and in early pregnancy may activate MAPK in endometrial epithelial cells. We describe herein the activation of MAPK by PAF and hCG and the role of MAPKs in the induction of COX-2 in HEC-1B cells. We also discuss autoregulation of COX-2 expression by PGE_2 and its potential signaling pathways.

Key words. Cyclooxygenase-2, platelet-activating factor, human chorionic gonadotropin, prostaglandin E_2, HEC-1B

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Introduction

Cyclooxygenase (COX) catalyses a committed step in the formation of prostaglandins (PGs) and thromboxanes. COX, also called prostaglandin endoperoxide H synthase, exists as isozymes COX-1 and COX-2, which are encoded by separate genes. COX-1 and COX-2 are structurally similar and 60% identical in amino acid sequence. Both proteins catalyse the conversion of arachidonic acid to PGH_2. Distinct synthases act on PGH_2 and convert it to PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2. COX-1 is constitutively expressed in various tissues and its expression is regulated developmentally. In contrast, COX-2 is not expressed in most cells, but is induced by stimulation with growth factors, cytokines, tumor promoters, or chorionic gonadotropins [1]. Although both enzymes are associated with the endoplasmic reticulum and nuclear envelope, COX-2 is more abundant than COX-1 in the nuclear envelope [1], where it may catalyse the synthesis of prostanoids. A potential role for COX-2 in the nuclear envelope is supported by observations that various fatty acids and PGs function as proliferators that activate receptors in the nuclear peroxisome [2].

PGs also play an important role during blastocyst implantation in the endometrium and during decidualization of endometrial stromal cells. Implantation is analogous to an inflammatory-like response and is associated with expansion of extracellular fluid volume, increased vascular permeability, and vasodilatation. Implantation is inhibited by the COX inhibitor indomethacin and that inhibition is overcome by administration of PGs. Furthermore, PG synthesis during embryo implantation in the rodent has been demonstrated [3]. PGs are also implicated in adhesion of the ovine trophoblast to the endometrium [4], regulation of local immune responses, and the expression of endometrial plasminogen activator and maternal tissue inhibitors of metalloproteinase enzymes.

The expression of COX-2 is of note due to the synthesis of PGs in the endometrium. PGs are induced through COX-2 expression by many ligands present in the endometrium. In addition, the COX-1 knockout mouse does not exhibit a reproductive phenotype, whereas COX-2 knockout mice show impairment of implantation and defective decidualization [5].

The present review summarizes the current knowledge about the mechanism of COX-2 expression by platelet-activating factor (PAF), human chorionic gonadotropin (hCG), and PGE_2. We also describe the mechanisms by which COX-2 expression is accomplished in endometrial epithelial cells. Epithelial cells not only interact with the implanted blastocyst, but also communicate with underlying stromal cells. In this context, the epithelial PGs may send a decidualizing signal to stromal cells.

COX-2 in the Endometrium and Endometrial Epithelial Cells

The constitutively expressed *COX*-1 gene encodes an enzyme serving housekeeping functions in various cells in the endometrium, whereas the inducible COX-2 is expressed in specific cell types where its expression is regulated by intra- and extracellular stimuli. In the sheep endometrium, Charpigny et al. [6] showed that the COX-2 enzyme was strictly localized to lumenal cells and the outer glands of the

endometrium, where it is expressed primarily in epithelial cells. As the estrous cycle advanced, the outer glands were strongly stained with COX-2 antibody, whereas the deeper glands remained negative. In contrast, no changes were observed in COX-1 expression, which is localized to both epithelial and stromal cells, as well as to outer and deeper glands. In mouse, COX-2 was also localized to uterine stroma in regions of blastocyst attachment. Furthermore, Chakraborty et al. [7] showed that COX-1 is expressed before implantation in the mouse uterine lumenal epithelium and subepithelial stromal cells. In contrast, uterine COX-2 expression was restricted to the region surrounding the implanting blastocyst at the time of embryo attachment. The coincidence of implantation and COX-2 expression in uterine and/or trophoblastic tissue has been reported in the ovine, bovine, and human uterus [8]. COX-2 was found in endometrial glands, as well as in decidual tissues, of the human uterus [8,9]. Likewise, the COX-2 signal was detected during a late phase of gestation in mouse decidual cells [7].

Both interleukin (IL)-1β and tumor necrosis factor (TNF)-α induced PGF_2 production and COX-2 mRNA expression in rat uterine stromal cells in vitro [10]. Oxytocin increases COX-2 mRNA levels in bovine endometrial epithelial cells [11]. Interferon (IFN)-τ, a secretory product of the conceptus, stimulates PGE_2 production and COX-2 gene expression in the bovine endometrium in vitro [12].

Diversity of Signaling Pathways Involved in COX-2 Expression

cAMP Pathway

cAMP plays important physiological roles in endometrial epithelial cells by activating gene transcription and/or facilitating ionic conductance across the cell membrane. An accumulation of cAMP, which is correlated with COX-2 expression, occurs in endometrial stromal cells after stimulation with hCG [13]. In contrast, elevation of cAMP levels in endometrial epithelial cells is not correlated with the expression of COX-2 [14].

Calcium Pathway

The role of calcium signaling in COX-2 expression has not been documented in endometrial cells. In mesangial cells, a significant role for endothelin (ET)-1-mediated elevations in cytosolic calcium stimulating COX-2 expression has been demonstrated [15]. The ET-1-induced COX-2 expression was inhibited by pretreatment with inhibitors for calcium/calmodulin-dependent protein kinases. In addition, pretreatment with tyrosine kinase inhibitors also abolishes the ET-1-mediated COX-2 expression [15]. These results suggest that tyrosine kinase-dependent cascades, including phospholipase (PL) Cγ, underlie the COX-2 expression as upstream signals of calcium/calmodulin-dependent protein kinases. Likewise, the activation of protein kinase (PK) C through PLCγ activation is involved in platelet-derived growth factor (PDGF)-induced COX-2 expression in mesangial cells [16]. In bovine endometrial epithelial cells, Asselin et al. [11] examined the effect of oxytocin on the expression

of COX-2. Oxytocin possibly binds to a G-protein-coupled receptor, thereby increasing both inositol phosphates and the calcium concentration in epithelial cells. The oxytocin-induced calcium signal may account for both PGE_2 production and *COX*-2 gene expression.

Tyrosine Kinase Pathway

Tyrosine kinase-dependent mechanisms involved in COX-2 expression are not clearly understood. Epidermal growth factor (EGF), the PKC-activating phorbol ester TPA, and the protein phosphatase inhibitor okadaic acid stimulate COX activity as well as the level *COX*-2 mRNA, without affecting the level of *COX*-1 mRNA, in primary cultures of human amnion cells [17]. Herbimycin A, a tyrosine kinase inhibitor, suppressed the stimulation of COX-2 expression by all three stimulants. In human pulmonary epithelial cells, the induction COX-2 elicited by IL-1β is also mediated by tyrosine kinase [18]. However, any specific tyrosine kinases involved in COX-2 expression are not defined in these cells.

MAPK Activation

Binding of growth factors to receptor tyrosine kinases results in the activation of the GTP-binding protein $p21^{ras}$ (RAS) via the adapter molecule (Grb-2) and a guanine nucleotide exchange factor (mSos). $p21^{ras}$ activates Raf-1, which phosphorylates MAPK kinase (MEK), resulting in phosphorylation and activation of MAPK. The MAPK cascade activates a set of regulatory molecules, which, in turn, initiate both cell proliferation and differentiation.

In addition to MAPK activation by growth factors, many members of the superfamily of G-protein-coupled receptors also activate MAPK [19]. Individual G-protein-coupled receptors activate one or more of the G-protein subtypes, including G_s (which activates adenylate cyclase), G_i (which inhibits adenylate cyclase), and $G_{q/11}$ (which activates PLC and, subsequently, PKC) [19]. For example, cytokines and hormones activating heterotrimeric G-protein-coupled receptors increase intracellular calcium and, in turn, activate MAPK signals. The MAPK signaling pathway through βγ subunits is also mediated through phosphatidylinositol 3-kinase (PI3-K)-γ activation, which requires a tyrosine kinase, most likely a Src family kinase, as an upstream kinase [20].

Although activation of MAPK occurs in many cells, the function of MAPK activation in endometrial epithelial or stromal cells is not understood. The cyclical proliferation and differentiation of endometrial cells suggests an involvement of MAPK in endometrium regeneration. Here, we focus on the regulation of MAPK activity by stimulation of HEC-1B cells with PAF and hCG.

Activation of MAPK by PAF in HEC-1B Cells

PAF, a potent lipid mediator, is produced by the embryo [21] and uterus, and is thought to be an important mediator of maternal recognition of pregnancy [22]. In humans, PAF is expressed in the luteal-phase endometrium [23]. Interaction of PAF with a spe-

cific PAF receptor activates heterotrimeric G-proteins [24] and PLC, thereby activating PKC and elevating intracellular calcium levels [25]. Stimulation of the PAF receptor is correlated with generation of inositol phosphate in endometrial tissue [26]. PAF activates MAPK in Chinese hamster ovary cells expressing the PAF receptor [27], as well as in blood cells [28,29]. In guinea pigs, PAF treatment significantly increases the PG output in the late luteal phase in vitro, a stimulation dependent on mobilization of intracellular calcium. However, there is no evidence for PAF-induced COX-2 expression in endometrial cells.

As shown in Fig. 1, we have shown that PAF-induced MAPK activation is completely inhibited by PD098059 [30], a selective inhibitor of MEK-1 and MEK-2 [31]. This result is consistent with observations that PAF induces MAPK activation following stimulation of HEC-1A cells with PAF [32]. Wortmannin, an inhibitor of PI3-K, also inhibits PAF-induced activation of MAPK [33]. This result is inconsistent with another report, in which PAF-induced MAPK activation was only partially inhibited by wortmannin [28]. In a study using the HEC-1B cell line, it was reported that estradiol upregulates PAF-induced PLD activity [34]. In addition, activation of PLD was suppressed by PI3-K inhibitors. These observations suggest that PI3-K is upstream of PLD in HEC-1B cells.

Activation of MAPK by hCG in HEC-1B Cells

hCG is a glycoprotein hormone synthesized by the placental trophoblast. Although the physiological relevance of hCG action in the endometrium is not well established, there is evidence that gonadotropins, including hCG, enhance stromal decidualization [13]. hCG also upregulates COX-2 expression during differentiation of human endometrial stromal cells into decidua. hCG is present in the plasma after initiation of implantation of the blastocyst and hCG receptors are present in endometrial cells [14]. Therefore, hCG may play an important role in the endometrium in early pregnancy, in addition to its role in maintaining steroidogenesis in the corpus luteum of the ovary.

Davis et al. [35] reported that hCG stimulates phosphoinositide hydrolysis in bovine isolated corpus luteum cells and suggested that hCG and luteinizing hormone (LH), in addition to promoting accumulation of cAMP, may also stimulate PLC and generate inositol phosphates as second messengers. Stimulation of luteal cells with LH increases intracellular calcium ions. However, the authors did not address whether the effect was a primary response to receptor stimulation or a secondary response following cAMP accumulation. Subsequent studies using cells transfected with LH receptors show that the murine LH receptor activates both adenylyl cyclase and PLC signaling [36].

LH is a potent activator of MAPK in porcine granulosa cells [37]. cAMP also activates MAPK in these cells, but the marked discrepancy in the time course between cAMP accumulation and MAPK activation suggests that the involvement of cAMP in LH-stimulated MAPK activation is indirect [37]. We demonstrated complete inhibition of hCG-induced MAPK activation by PD098059 treatment [30], suggesting that MEK is upstream of hCG-mediated MAPK activation. Unlike PAF, activation of MAPK by hCG does not change following wortmannin treatment. In contrast, cAMP-induced activation of MAPK and the ability of H-89, a potent inhibitor of PKA, to inhibit hCG-

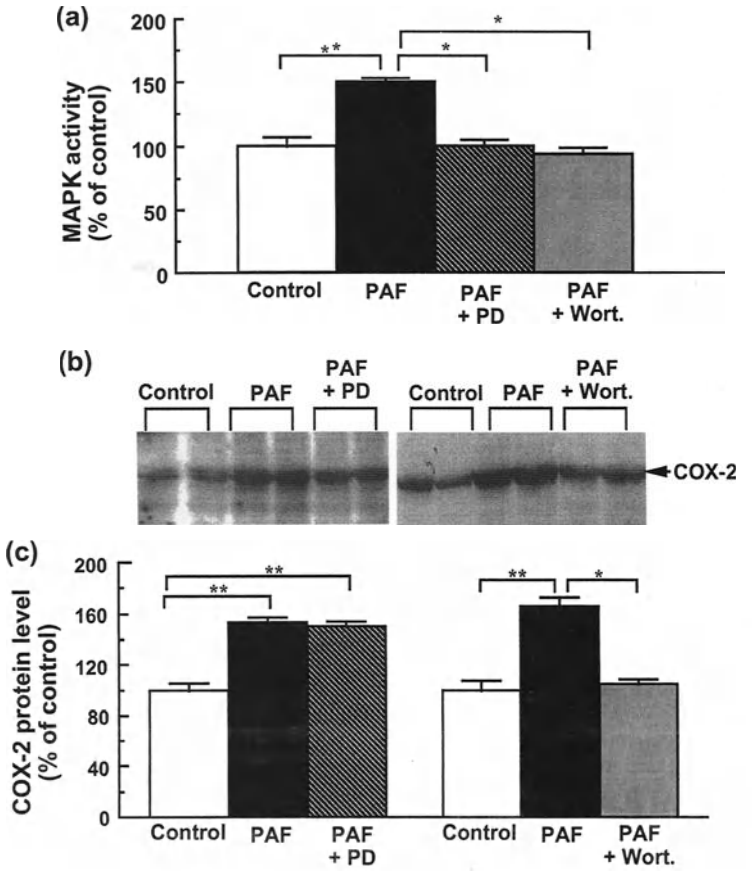

FIG. 1. Effects of PD098059 (*PD*) and wortmannin (*Wort.*) on platelet-activating factor (*PAF*)-induced mitogen-activated protein kinase (*MAPK*) activation and cyclooxygenase-2 (*COX*-2) expression in HEC-1B cells. **a** Effects of inhibitors on PAF-induced MAPK activation. Cells were preincubated for 1 h in KRH buffer. The inhibitors (75 μM PD098059 or 200 nM wortmannin) were added for the last 30 min of preincubation. Cells were then incubated for 10 min without (control) or with 1 μM PAF in the presence of the inhibitors, as indicated, in KRH buffer. Data are expressed as a percentage of the control value, which is defined as 100%, and are the mean ± SEM ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$. **b** A representative autoradiogram showing the effects of inhibitors on PAF-stimulated COX-2 expression. Cells were incubated for 12 h without (control) or with 1 μM PAF in the presence or absence of inhibitors. **c** COX-2 expression, with data (mean ± SEM; $n = 3$) expressed as a percentage of the control value, which is defined as 100%. $^*P < 0.05$, $^{**}P < 0.01$. From Munir et al. [30]

induced MAPK activation (Fig. 2) supports the contention that cAMP is upstream of the hCG-induced MAPK activation pathway.

Several investigators have analyzed the effects of cAMP on MAPK activation. Depending on the cell type, cAMP either stimulates or inhibits MAPK activity. In fibroblasts, cAMP inhibits growth factor-stimulated MAPK activation, probably through PKA-mediated Raf-1 phosphorylation [38]. However, in PC12 cells [39,40]

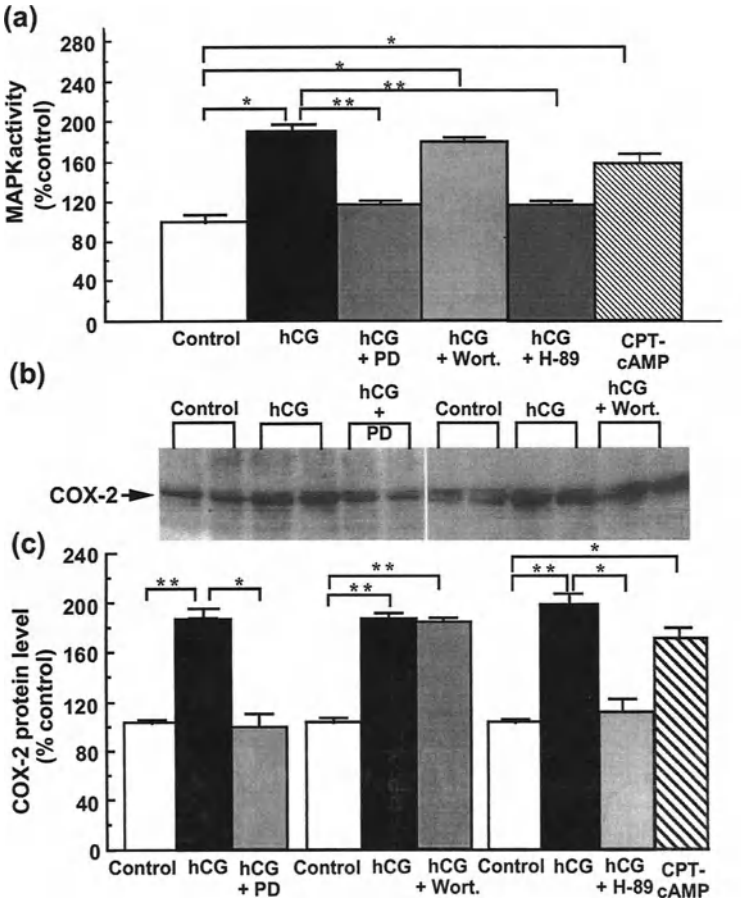

FIG. 2. Effects of PD098059 (*PD*), wortmannin (*Wort.*), and H-89 on human chorionic gonadotropin (*hCG*)-induced mitogen-activated protein kinase (*MAPK*) activation and cyclooxygenase-2 (*COX-2*) expression and the effect of CPT-cAMP in HEC-1B cells. **a** Effects of inhibitors on hCG-induced MAPK activation and the effect of CPT-cAMP on MAPK activity. Cells were preincubated for 1 h in KRH buffer. The inhibitors (75 μM PD098059 or 200 nM wortmannin) or 10 μM H-89) were added for the last 30 min of preincubation. Cells were then incubated for 10 min without (control) or with 0.15 IU/mL hCG in the presence of the inhibitors, as indicated. In the case of CPT-cAMP, after preincubation in KRH buffer, cells were incubated with 1 mM CPT-cAMP for 10 min. Data are expressed as a percentage of the control value, which is defined as 100%, and are the mean ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$. **b** A representative autoradiogram showing the effects of PD098059 and wortmannin on hCG-stimulated COX-2 expression. Cells were incubated for 12 h without (control) or with 0.15 IU/mL hCG in the presence or absence of the inhibitors. **c** Statistical analyses of the data shown in the autoradiogram and the effects of H-89 and CPT-cAMP on COX-2 expression. The effect of H-89 on hCG-induced COX-2 expression was analyzed in a manner similar to the other inhibitors. Cells were also incubated with 1 mM CPT-cAMP for 12 h. Data are expressed as a percentage of the control value, which is defined as 100%, and are the mean ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$. From Munir et al. [30]

cAMP induces MAPK activation. Vossler et al. [40] recently showed that the activation of MAPK by cAMP is largely independent of Ras but requires Rap 1, another small G-protein. They demonstrated that PKA stimulates Rap 1, which then selectively activates B-Raf and MAPK. Synergism between cAMP and activators of PKC in the activation of MAPK has also been reported [41].

Activation of MAPK by PGE_2 in HEC-1B Cells

We recently observed the activation of MAPK and PKB with concentrations of PGE_2 ranging from 10 nM to 1 μM in HEC-1B cells [42]. To determine the activation pathway of MAPK by stimulation with PGE_2, several PK inhibitors were tested. PD098059 and H-89 could completely inhibit activation of MAPK by PGE_2. In contrast, calphostin C, a selective inhibitor of PKC, failed to inhibit MAPK activation.

PKB Activation by PGE_2 in HEC-1B Cells

PKB, also known as Akt, is a serine/ threonine kinase. PKB activation is a widespread phenomenon occurring through intrinsic tyrosine kinase receptors for growth factor and insulin, associated tyrosine kinase receptors for IL-5 and IL-4 receptor, and G-protein-coupled serpentine receptors for PAF. PKB is a downstream target for PI3-K and has been shown to bind to and be activated by PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 signaling molecules generated by PI3-K after lipid phosphorylation [43]. We recently provided evidence of the activation of PKB by PGE_2 [42]. Wortmannin, a specific inhibitor of PI3-K at nanomolar concentrations, could completely inhibit the activation of PKB by PGE_2. This suggests the involvement of PI3-K in PKB activation in HEC-1B cells. The activated PKB influences metabolism through phosphorylation of glycogen synthase kinase-3 and phosphofructokinase, as well as transmission of a potent antiapoptotic signal [44]. The antiapoptotic signal may be mediated, in part, by phosphorylation and inactivation of Bad, a pro-apoptotic BCL-2 family member [44]. In the endometrium, there is expression of BCL-2 during the proliferative phase in endometrial glandular cells [45]. BCL-2 expression is elevated during the proliferative and menstrual phases in stromal cells [46]. The in vivo regulation of the expression of this gene is not hormonally driven [45] and it is possible that PGE_2, present in the endometrium regardless of the hormonal status, can take part in this process. In nonendometrial cells, PGE_2 was found to inhibit apoptosis, a process in which PKA was implicated [47].

Mechanism of COX-2 Expression

In vitro studies of COX-2 expression have been conducted in several cell types. Measurable changes in protein and mRNA levels of COX-2 occur in response to growth factors, tumor promoters, cytokines, hormones, and oncogene expression [48]. Different signaling pathways are likely to activate promoter and enhancer regions of the *COX-2* gene to modulate its transcription. Nuclear run-on experiments show that serum and phorbol ester stimulate COX-2 transcription [49]. Several recognition sequences in the COX-2 promoter have been identified as important for transcription.

In the murine COX-2 promoter [50], potential recognition sites for Sp-1 and C/EBP, as well as CRE sites and E-boxes, have been identified. The ATF family (CREB, ATF-2, ATF-3 etc.) of transcription factors can bind to CRE, whereas the E-box is recognized by members of the bHLH family of transcription factors, such as Myc, Max, MyoD, and USF. The putative CRE and E-box sequences bind members of these two transcription factor families [51]. The CRE sequence in the murine *COX*-2 gene is required for v-src to induce gene expression [51]. The CRE site of human COX-2 has been implicated in COX-2 expression in monocytic cells [52]. In the case of the rat *COX*-2 gene in ovarian granulosa cells, a C/EBP sequence was identified as a major regulatory sequence for induction by pituitary glycoprotein hormones [53]. In the chicken *COX*-2 gene, activating protein (AP)-1, SRE, nuclear factor (NF)-κB, Sp-1 and AP-2 sites occur in the promoter region [54]. Although COX-2 expression appears to be primarily regulated transcriptionally, the mRNA level may also change according to its stability. *COX*-2 mRNA is unstable compared with *COX*-1 mRNA, probably due to the multiple RNA instability sequences (AUUUA) present in the *COX*-2 3′-untranslated region (UTR). Factors that increase or decrease the half-life of *COX*-2 mRNA presumably affect its potency. For example, IL-1 appears to regulate COX-2 expression by such a mechanism: in the human endothelial cell line ECV204, IL-1 not only increases *COX*-2 gene transcription, but also *COX*-2 mRNA stability [55]. Conversely, treatment with dexamethasone inhibits transcription of COX-2 and reduces the stability of *COX*-2 mRNA.

Multiple signaling pathways through PKs affect COX-2 expression. In addition to PKA, PKC, and MAPK pathways, c-Jun N-terminal kinase (JNK) is also involved in COX-2 expression induced by v-src [56,57].

Role of Ovarian Steroids in COX-2 Expression

COX-1 protein is expressed at steady state levels in the sheep endometrium during the estrous cycle and comparable stages of pregnancy [58]. In contrast, COX-2 protein is highly and transiently expressed from days 12 to 15 of the estrous cycle and gradually declines to undetectable levels. Expression of endometrial COX-2 requires the activity of ovarian steroids. Treatment with progesterone alone induced high levels of endometrial COX-2, whereas endometria from ovariectomized ewes did not exhibit detectable levels of COX-2 [58]. Other support for the role of progesterone in the expression of endometrial COX-2 comes from the in situ hybridization study of Eggleston et al. [59]. These authors reported an eightfold increase in the level of *COX*-2 mRNA in ewe uterine tissue sections following progesterone administration. In the rat endometrium, Shoda et al. [60] observed that induction of COX-2 coincided with the peak of serum estradiol levels, suggesting an estradiol-dependent induction of COX-2. However, in the mouse, Chakraborty et al. [61] observed that steroid treatment had no effect on the induction of uterine COX-2. In our study, we found that both estrogen and progesterone treatment increased COX-2 expression in the endometrial cell line HEC-1B [30].

Induction of COX-2 by PAF and hCG in HEC-1B Cells

Most PGs in the endometrium are produced in glandular epithelial cells [62]. In the human endometrium, there are complex interactions between the epithelial, stromal, endothelial, and lymphoid cells. Because the uterine epithelial cells appear to mediate a decidualizing signal through their basal cell surface in response to the blastocyst, it has been hypothesized that PGs released from epithelial cells act on stromal cells. To analyze potential roles of PGs derived from epithelial cells, we examined PAF- and hCG-induced COX-2 expression in the endometrial epithelial cell line HEC-1B [30]. Stimulation of COX-2 expression by PAF and hCG required pretreatment of HEC-1B cells with a combination of estrogen and medroxyprogesterone acetate [30]. Although both agonists increased activation of MAPK and expression of COX-2, MAPK activation was not responsible for PAF-induced expression of COX-2, a conclusion based on the observation that PD098059 did not inhibit expression of COX-2 by PAF but did inhibit its expression by hCG [30]. Interestingly, wortmannin inhibited PAF-induced expression of COX-2. Although the result suggests that PI3-K accounts for the expression of COX-2 induced by PAF, the signaling cascade of PI3-K leading to upregulation of COX-2 is unknown.

Recent studies have provided evidence that COX-2 expression is regulated by MAPK. Assays of promoter deletions and site-directed mutagenesis of COX-2-driven luciferase reporter constructs show that MAPK is partially involved in v-src- [56], serum-, and PDGF-induced COX-2 expression in NIH 3T3 cells [57]. PD098059 also blocks stimulation of lysophosphatidic acid-mediated COX-2 expression in rat mesangial cells [63] and lipopolysaccharide-induced COX-2 expression in a murine macrophage cell line [64]. Contrary to these reports, PAF-induced COX-2 expression is not inhibited by addition of PD098059 [30]. However, wortmannin effectively blocked PAF-induced COX-2 expression. Given the low concentration of wortmannin used (200 nM), its effects could be exerted through a specific inhibition of PI3-K. PI3-K has been implicated in activation of JNK [65], NF-κB, and AP-1 [66], all of which are associated with stimulation of COX-2 expression. Furthermore, PKB (PKB/c-Akt) is a downstream target of PI3-K [67]. Wortmannin also inhibits protein synthesis by blocking translation [68]. We conclude that PAF-induced expression of COX-2 is probably mediated through the PI3-K signaling pathway, although the reactions downstream of the PI3-K pathway remain to be determined.

Consistent with previous findings, PD098059 inhibits hCG-mediated COX-2 expression, suggesting a role for MAPK in hCG-mediated COX-2 expression. We also found that CPT-cAMP directly stimulates COX-2 expression and that hCG-induced COX-2 expression is inhibited by H-89. H-89 also inhibits hCG-induced MAPK activation, indicating that MAPK is downstream of PKA. It was recently reported that micromolar concentrations of PD098059 inhibit COX-1 and COX-2 activity [69]. However, because we assayed changes in expression of COX-2 rather than COX-2 activity following inhibition of MAPK, we conclude that PD098059 treatment inhibits MAPK activation in our study [30].

In addition, we found an autoregulation of COX-2 expression by PGE_2 in HEC-1B cells. Both PI3-K and MAPK pathways underlie the molecular mechanisms of PGE_2-induced COX-2 expression (Fig. 3).

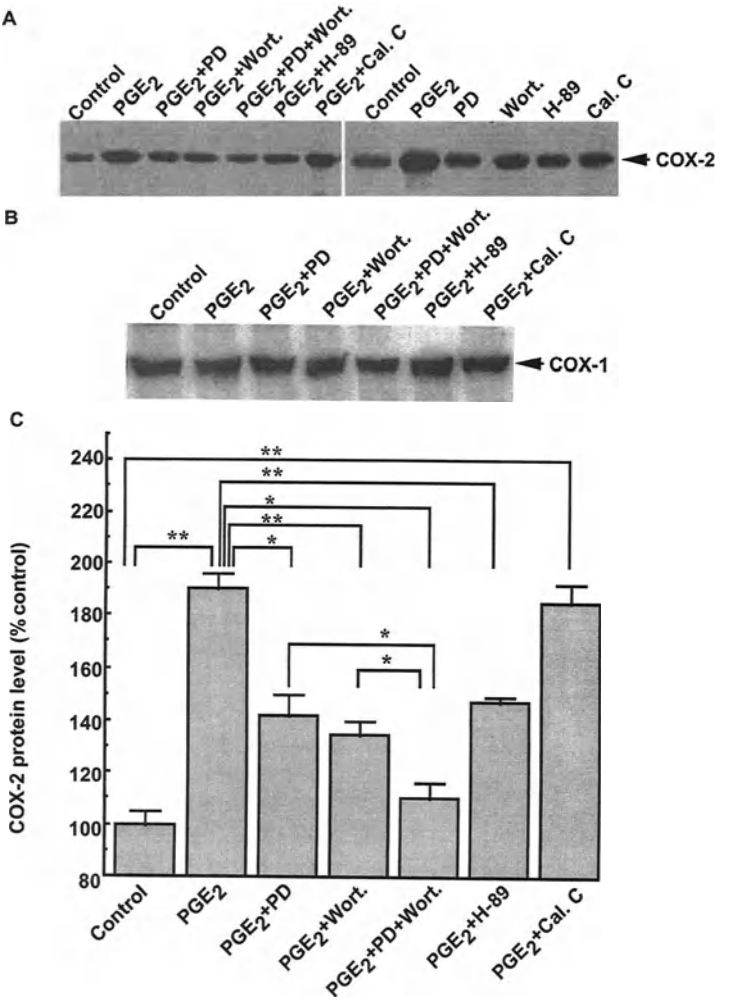

FIG. 3. Effects of inhibitors on prostaglandin E_2 (*PGE_2*)-induced cyclooxygenase-2 (*COX*-2) expression. **a** Representative autoradiogram of the effects of the inhibitors on PGE_2-stimulated COX-2 expression. Cells were preincubated for 30 min in the medium in the presence of nothing, 75 μM PD098059 (*PD*), 10 μM H-89, 500 nM calphostin C (*Cal. C*), or 200 nM wortmannin (*Wort.*). The cells were then incubated for 12 h without (control) or with 1 μM PGE_2 in the presence of the indicated inhibitors in the medium. **b** Immunoblotting with the COX-1 antibody. Cells were stimulated as described above and then immunoblotting was performed. **c** Summary of the effects of the inhibitors on PGE_2-stimulated COX-2 expression. Data are the mean ± SEM ($n = 3$) and are expressed as a percentage of the control value, which is taken as 100%. $^*P < 0.05$, $^{**}P < 0.01$. From Munir et al. [42]

Conclusion

Endometrial epithelial cells express COX-2 after stimulation with hormones and cytokines. Stimulation of the endometrium activates PKC, calcium, protein tyrosine kinase, and cAMP. These agents also potentially stimulate MAPK. In our study with HEC-1B cells, both PAF and hCG stimulated MAPK activity through different mechanisms, namely through a PI3-K-dependent pathway and a cAMP-dependent pathway, respectively. Diverse signaling pathways were also evident from the expression of COX-2 in HEC-1B cells, as shown in Fig. 4. The PI3-K-dependent and wortmannin-sensitive pathway was activated in PAF-induced upregulation of COX-2 expression. PKB may function downstream of PI3-K to regulate *COX*-2 mRNA translation through

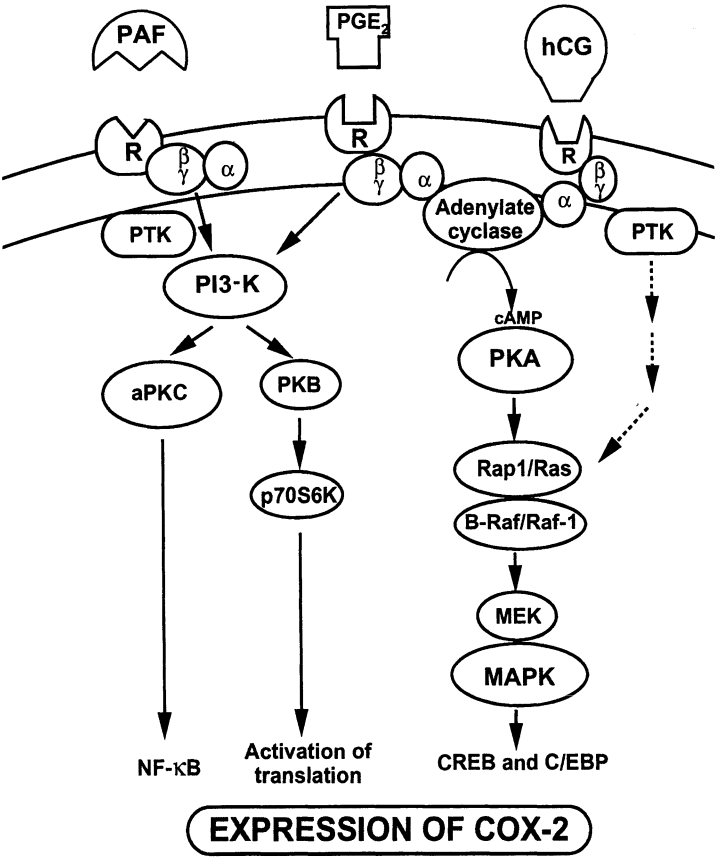

FIG. 4. Signaling pathways involved in cyclooxygenase-2 (*COX*-2) expression through platelet-activating factor (*PAF*), human chorionic gonadotropin (*hCG*), and prostaglandin E_2 (PGE_2) receptors in HEC-1B cells. *PTK*, protein tyrosine kinase; *PI3-K*, phosphatidylinositol 3-kinase; *PKA*, *PKB*, *PKC*, protein kinase A, B, and C, respectively; *NF-κB*, nuclear factor-κB; *MAPK*, mitogen-activated protein kinase; *MEK*, MAPK kinase; *CREB*, cAMP response element-binding protein

activation of p70 S6 kinase. The translational regulation may account for COX-2 expression through the phosphorylation of ribosomal S6 protein. In addition, activation of atypical PKC (aPKC) may stimulate the transcription of COX-2 mRNA through NF-κB. However, the exact mechanism has not been determined. In contrast, hCG-induced COX-2 expression was mainly through MAPK activation, where cAMP and PKA are upstream signals. In this context, PI3-K and MAPK are essential elements of PAF- and hCG-induced COX-2 expression, respectively, in endometrial epithelial cells.

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Inhibition of AKT Oncogenic Pathway in Endometrial Cancer Cells

Jiayuh Lin[1], Xiaohong Jin[1], Dana R. Gossett[1], and Shaomeng Wang[2]

Summary. The PTEN tumor suppressor, which is mutated in 40%–50% of endometrial cancers, is a negative regulator of AKT. In the present study, we examined the activation of AKT in a panel of endometrial cancer cell lines and tumor samples. Two endometrial cancer cell lines, namely Ishikawa and RL-95 cells, and several tumor samples showed elevated levels of phosphorylated AKT. The expression of PTEN significantly suppressed the growth of Ishikawa cancer cells. Next, we tested the inhibitory effect of a novel AKT-selective inhibitor, namely API-59, in endometrial cancer cells that have a PTEN mutation and high levels of phosphorylated AKT. API-59 inhibited AKT kinase activity in both Ishikawa and RL95-2 endometrial cancer cells that expressed high levels of AKT kinase activity. API-59 did not inhibit phosphorylation of AKT, phosphoinositide-dependent kinase-1, or extracellular signal-regulated kinase-1/2, suggesting that API-59 inhibits the AKT pathway at the AKT kinase level.

We next investigated whether API-59 induces apoptosis in these endometrial cancer cell lines. Exposure to API-59 induced significant apoptosis in both Ishikawa and RL95-2 cancer cell lines, which express elevated AKT kinase activity. Importantly, API-59 had only a minimal apoptosis-inducing capacity in HEC 1A and KLE cells, which express wild-type PTEN protein and lack AKT kinase activity. These data support the notion that API-59 is a highly selective inhibitor for AKT kinase in endometrial cancer cells. Together, these results indicate that the PTEN tumor suppressor and the novel AKT-selective inhibitor API-59 are potent therapeutic agents to target endometrial cancer cells with a high frequency of PTEN mutations and AKT activation.

Key words. PTEN, phosphoinositide-dependent kinase-1, AKT, endometrial cancer, cancer therapy

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Introduction

AKT is a serine/threonine kinase that contains a pleckstrin-homology (PH) domain and is activated in response to growth factor or cytokine treatment by a mechanism involving phosphatidylinositol 3-kinase (PI3-K) and phosphoinositide-dependent kinase-1 (PDK-1) [1–4]. The AKT protein has three isoforms: AKT1, AKT2, and AKT3 [5]. These three isoforms have greater than 85% sequence identity and have the same structural organization. AKT provides a survival signal that protects cells from apoptosis induced by various stresses [2,3,6]. The mechanisms by which AKT functions to promote survival are through the phosphorylation of Bad, glycogen synthase kinase-3 (GSK-3), forkhead transcription factor (FKHR), and caspase-9 [7–12] (Fig. 1). Phosphorylation of GSK-3, Bad, FKHR, and caspase-9 results in the inactivation of their apoptotic function. Bad is a member of the Bcl-2 family that promotes apoptosis through, at least in part, heterodimerization with the survival proteins Bcl-2 and Bcl-x_L [13]. AKT may also suppress apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of nuclear factor (NF)-κB [14–16] and phosphorylating the tuberous sclerosis complex-2 gene product tuberin to regulate cell growth in the insulin-signaling pathway [17,18]. Furthermore, AKT enhances the ubiquitination-promoting function of double minute chromosome (mdm2) by phosphorylating mdm2, which results in a reduction of the p53 tumor suppressor through ubiquitination [19,20].

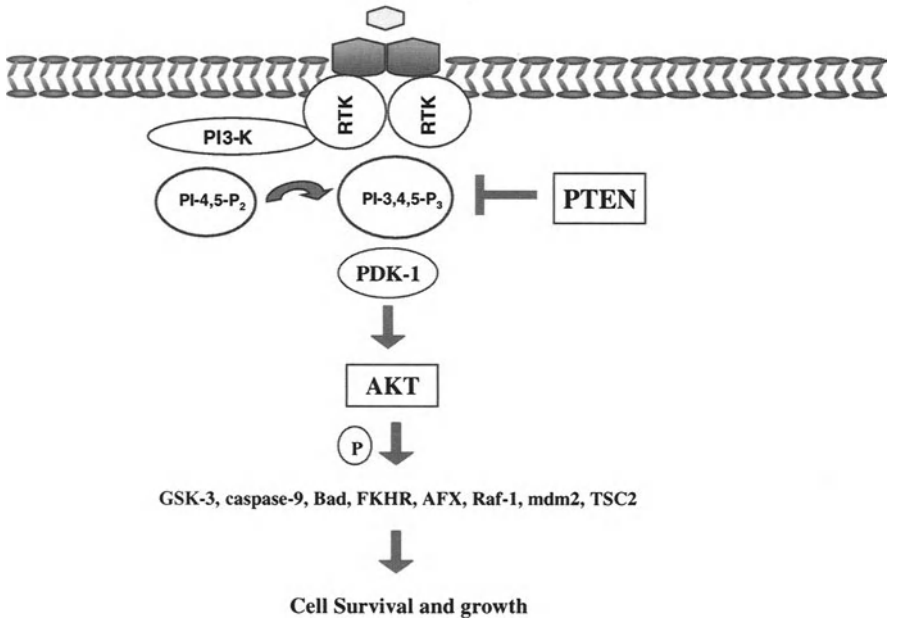

FIG. 1. The AKT signal transduction pathway. *PI3-K*, phosphatidylinositol 3-kinase; *RTK*, receptor tyrosine kinase; *PI*-4,5-P_2, phosphotidylinositol (4,5)-bisphosphate; *PI*-3,4,5-P_3, phosphatidylinositol (3,4,5)-trisphophate; *PDK*-1, phosphoinositide-dependent kinase-1; *GSK*-1, glycogen synthse kinase-1; *FKHR* and AFX, forkhead transcription factors; *TSC*2, tuberin

AKT2 oncogene amplification and high AKT kinase activity have been detected frequently in human carcinomas including breast, pancreatic, ovarian, thyroid, brain, prostate, and gastric adenocarcinomas [21–27]. Overexpression of AKT2 was able to transform NIH3T3 cells, as determined by growth in soft agar and tumor formation in nude mice [28]. Furthermore, the introduction of the *AKT* oncogene along with the c-*myc* or K-*ras* oncogenes in transgenic mice deficient for p53 was sufficient to induce ovarian tumor formation in a transgenic mouse model system [29], suggesting that the AKT pathway is likely to be involved in the oncogenesis of human cancer.

PTEN Functions

The recent discovery that the tumor suppressor PTEN is an antagonist of PI3-K and AKT kinase activity suggests that AKT is an important factor in the oncogenesis of cancer [63]. Characterization of the PTEN protein showed that it is a phosphatase that acts on proteins and on 3-phosphorylated phosphoinositides, including phosphatidylinositol (3,4,5)-trisphosphate, and that it can therefore modulate signal-transduction pathways that involve lipid second messengers [30,31] (Fig. 1). One of the functions of PTEN may inhibit eukaryotic cell polarity and chemotaxis that is mediated by PI3-K [32,33]. Overexpression of PTEN inhibited cell migration, which may be due to the decrease in phosphorylation of the focal adhesion kinase [34]. The lipid phosphatase activity of PTEN may be important for its tumor suppressor function [35], but may not be totally required to inhibit invasion of glioma cells [36]. The PTEN tumor suppressor is frequently mutated in many human carcinomas, including endometrial, brain, breast, thyroid, and prostate cancer [37–45]. In endometrial cancer, PTEN is frequently mutated in approximately 45% of cases [46–48]. This protein is thought to function as a tumor suppressor due to its ability to block G_1 cell cycle progression, induction of apoptosis, and its ability to negatively regulate the PI3-K/AKT pathway for cell survival [30,49]. Accordingly, in cancer cells that harbor a PTEN mutation or deletion, AKT activity is markedly elevated [30,41,44,50]. Therefore, the inactivation of the PTEN pathway through activation of AKT may play an important role in the oncogenesis of human cancer. In addition, expression of the wild-type *PTEN* gene inhibits cell growth by both induction of apoptosis and G_1 cell cycle growth arrest in glioblastoma, prostate, multiple myeloma, breast, and endometrial cancer cells harboring the PTEN mutation and AKT overactivity [38,40,42,51–57]. These reports suggest that reintroduction of wild-type PTEN into PTEN-deficient cancer cells may be a good therapeutic approach in the treatment of certain cancers.

Expression of PTEN Protein and Phosphorylated AKT in Endometrial Cancer Cell Lines

In order to study AKT activation in endometrial cancer, we examined tumor specimens from the University of Michigan Tumor Procurement Core. We demonstrated that tumor specimens of endometrial cancer frequently express elevated levels of phosphorylated AKT [51]. Normal endometrial scrapings contained only small amounts of phosphorylated AKT [51]. Next, we examined the expression of PTEN

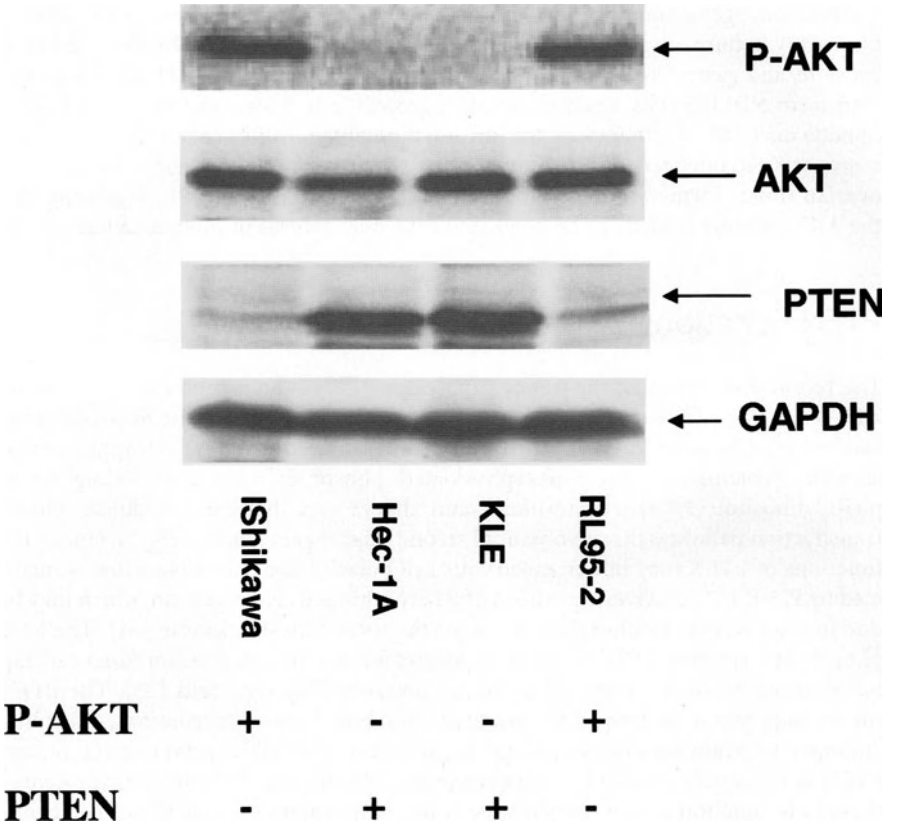

FIG. 2. Expression of PTEN protein and phosphorylated AKT (*P-AKT*) in human endometrial cancer cell lines. In these experiments, 100 μg total protein from endometrial cancer cell lysates were immunoblotted using either phospho-specific AKT (Ser-473; Cell Signaling Technologies, Beverly, MA, USA), phospho-independent AKT (Cell Signaling Technologies), or PTEN (Cell Signaling Technologies) antibodies. GADPH represents the internal protein control

protein and phosphorylated AKT protein in the human endometrial cancer cell lines RL95-2, Ishikawa, HEC 1A, and KLE. HEC 1A and KLE cancer cells express considerable amounts of the PTEN protein, whereas Ishikawa and RL95-2 cancer cells express little or no PTEN protein (Fig. 2). This is consistent with previous results showing that Ishikawa and RL95-2 cell lines express mutated PTEN, whereas HEC 1A and KLE cells express wild-type PTEN protein [58]. We also showed that Ishikawa and RL95-2 cell lines express high levels of phosphorylated AKT (Fig. 2) and have high AKT kinase activity (data not shown), whereas HEC 1A and KLE cells express little phosphorylated AKT (Fig. 2) and have undetectable AKT kinase activity (data not shown). Therefore, in these four endometrial cancer cell lines, there is perfect correlation between loss of PTEN expression and overactivation of AKT kinase activity. Other reports on clinical specimens of endometrial carcinomas also indicate a significant inverse cor-

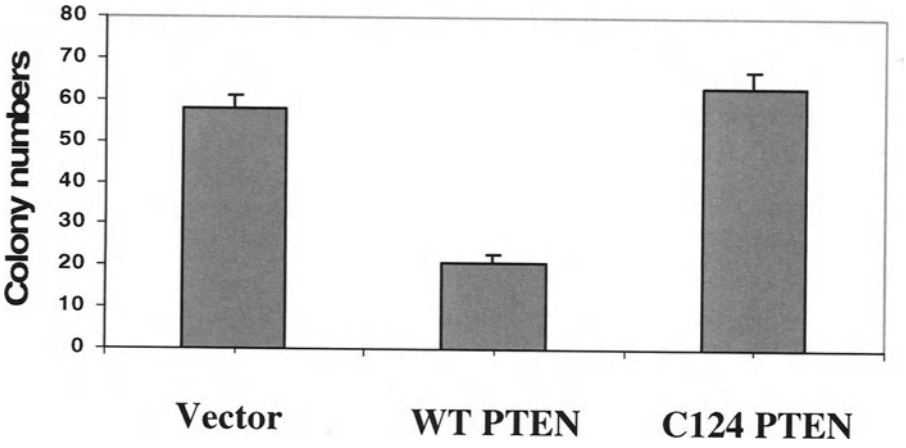

FIG. 3. Effect of PTEN on cell growth in Ishikawa endometrial cancer cells using the colony formation assay. Wild-type (*WT*) or C124 mutant PTEN cDNA in pTracer-CMV vector (Invitrogen, Carlsbad, CA, USA) was transfected into the Ishikawa endometrial cancer cell line using the Lipofectamine PLUS Reagent (Gibco/BRL, Rockville, MD, USA). These cells were allowed to grow without selection medium for 24 h, after which time they were replated onto a new plate with antibiotics (Zeocin; Invitrogen) for vector selection. Transfected cells were grown for 2–3 weeks on 50 μg/mL Zeocin. Zeocin-resistant colonies were stained with crystal violet solution for counting

relation between the expression of PTEN and phosphorylated Akt [50]. Furthermore, this study also indicated that the levels of phospho-Bad were greater in PTEN-negative cases, suggesting that Bad may be a target for AKT [50].

Inhibition of AKT by PTEN Tumor Suppressor

To evaluate the function of the *PTEN* gene as a tumor suppressor, adenovirus PTEN was transferred into Ishikawa and RL95-2 endometrial cancer cell lines with completely inactivated PTEN. The *PTEN* transgene significantly decreased levels of activated AKT and suppressed cell growth of Ishikawa (Fig. 3) and RL95-2 endometrial cancer cell lines in vitro through the induction of apoptosis [51–53]. The phosphatase-dead PTEN mutant C124S failed to inhibit growth of the Ishikawa endometrial cancer cell line [51], suggesting that the phosphatase activity of PTEN may be important for this function. Furthermore, the ex vivo tumor formation by Ishikawa cells was completely inhibited by the introduction of the wild-type *PTEN* gene [52]. However, neither regression nor progression was observed in inoculated tumors of either cell line following in vivo introduction of the wild-type *PTEN* gene [52]. These results suggest that the PTEN tumor suppressor may be a good candidate for gene therapy in patients with endometrial carcinoma. In addition to the induction of apoptosis, PTEN also induced a G_1 cell cycle arrest in endometrial carcinoma cells that lacked endogenous wild-type PTEN [53]. Growth arrest required a functional phosphatase domain, but not the PDZ interaction motif of PTEN. Growth arrest by PTEN may be

involved in the reduction of cyclin D3 levels and an associated increase in the amount of the inhibitor p27(KIP1) complexed with CDK2 [53]. These data suggest multiple mechanisms of PTEN-mediated growth inhibition, including induction of apoptosis and cell cycle arrest.

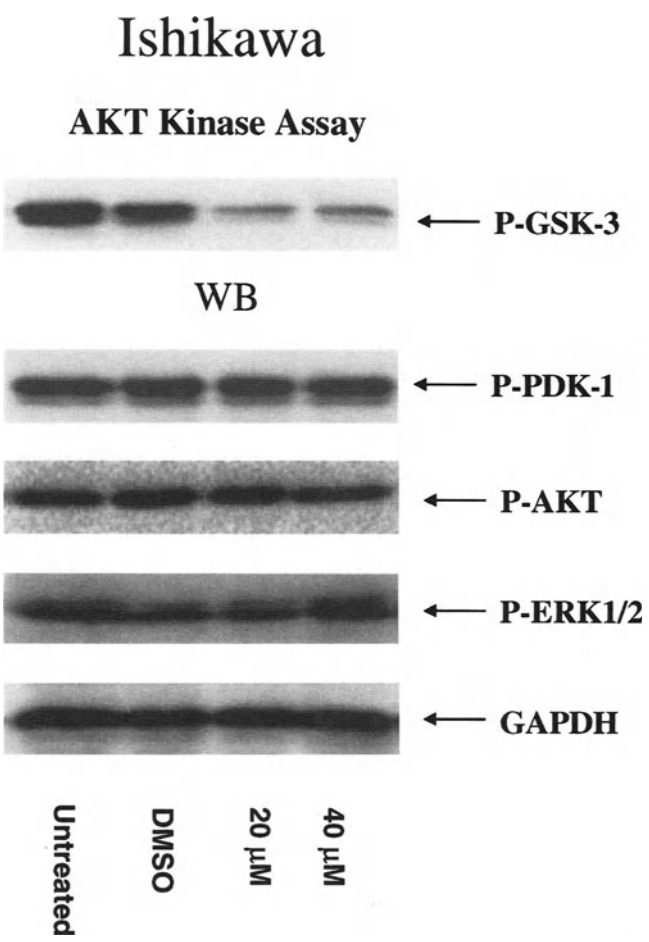

FIG. 4. Effect of API-59 on AKT kinase activity and phosphorylation (*P*) of phosphoinositide-dependent kinase-1 (*PDK-1*), AKT, and extracellular signal-regulated kinase 1/2 (*ERK*1/2) in Ishikawa endometrial cancer cells. Cells were seeded at a density of 1.2×10^6 cells in 100-mm dishes for 24 h before API-59 treatment. Cells were then exposed to API-59 at concentrations of 20 or 40 μM for 2 days. Cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Each gel was analyzed with phospho-specific PDK-1 (Ser 241), phospho-specific AKT (Ser 473), phospho-specific ERK1/2, and phospho-specific epidermal growth factor receptor (EGFR) (Tyr 1068; Cell Signaling Technologies, Beverley, MA, USA) antibodies. The same cell lysates were immunoprecipitated with AKT-specific monoclonal antibody and an AKT kinase assay was performed with an AKT kinase assay kit using GSK-3 as a substrate and phospho-specific glycogen synthse kinase-3 (*GSK-3*) α/β (Ser 21/9) for phosphorylated protein detection (Cell Signaling Technologies). *DMSO*, dimethylsulfoxide; *WB*, Western blots

Inhibition of AKT Kinase Activity in Human Endometrial Cancer Cell Lines

Because AKT activity is frequently activated in endometrial cancer and may provide a survival signal that protects cells from apoptosis induced by anti-cancer drugs, the development of potent and selective inhibitors targeting AKT is a novel therapeutic strategy for treating endometrial carcinoma. Although inhibitors that target the upstream regulators of AKT, namely PI3-K and PDK-1, have been reported [59,60], to date no selective AKT inhibitors have been developed. We tested a novel structure-based design small molecule inhibitor of AKT (termed API-59) that targets the ATP-binding site of AKT kinase in human endometrial cancer cells. Small molecule drugs have several advantages, including good delivery properties, good in vivo stability, a lack of possible immune response, and low cost [61,62]. We first examined whether the novel structure-based design AKT-selective inhibitor API-59 could inhibit AKT kinase activity in RL95-2 and Ishikawa endometrial cancer cell lines, which express high levels of AKT phosphorylation (Fig. 2). The addition of API-59 significantly inhibited AKT kinase activity when using GSK-3 as a substrate in Ishikawa endometrial cancer cells (Fig. 4). To demonstrate that API-59 selectively inhibits AKT kinase, and not other kinases, we used the same cell lysates to probe with antibodies against phospho-specific mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK)-1/2, phospho-specific AKT or phospho-specific PDK-1. As shown in Fig. 4, the lead compound API-59 did not inhibit those target protein activities. An equal loading of proteins was demonstrated by a reprobe of the same membrane with a GAPDH protein antibody. These data suggest that the lead compound API-59 only selectively inhibits AKT kinase activity. Importantly, API-59 does not affect the proteins that are either upstream of AKT (PDK-1) or in a distinct signal transduction pathway (ERK1/2) in Ishikawa endometrial cancer cells.

We next tested whether API-59 has any inhibitory effects in the RL95-2 endometrial cancer cell line, which also expresses elevated AKT kinase activity. The addition of API-59 inhibited AKT kinase activity in RL95–2 endometrial cancer cells (data not shown). However, API-59 did not inhibit the phosphorylation of ERK1/2, PDK-1, or even AKT itself in this cancer cell line (data not shown). Because API-59 did not inhibit AKT phosphorylation in either the RL95-2 or Ishikawa endometrial cancer cell lines, these results suggest that API-59 inhibits the AKT pathway at the AKT kinase level but that it does not affect the proteins that are immediately upstream of AKT, which may include PDK-1 and PI3-K.

Induction of Apoptosis in Human Endometrial Cancer Cell Lines Expressing Elevated Levels of AKT Activity

We examined further whether API-59 induced apoptosis in RL95-2 and Ishikawa endometrial cancer cell lines. Exposure of the cells to API-59 significantly induced apoptosis in both RL95-2 and Ishikawa cell lines, which express high levels of AKT kinase activity (Fig. 5). Importantly, API-59 had little capacity to induce apoptosis in the two independent human endometrial cancer cell lines, HEC 1A and KLE, that express wild-type PTEN and lack AKT kinase activity (Fig. 5). Therefore, these data

strongly support the contention that API-59 is a highly selective inhibitor of AKT kinase in these endometrial cancer cells. This is the first report of a selective inhibitor at the AKT kinase level in human endometrial cancer cell lines. Thus, API-59 represents a novel class of small molecule inhibitors capable of selectively inhibiting cell proliferation via modulating AKT function in cancer cells expressing high levels of AKT activity, but it has little effect on cancer cells lacking AKT activity. The inhibition of the high levels of AKT kinase activity using this AKT-selective small molecule inhibitor is a novel and attractive target-specific therapy for the treatment of endometrial cancer and deserves further investigation. In addition to endometrial cancer, the PTEN tumor suppressor is also frequently mutated in brain and prostate cancer [37,38,42,43]. Therefore, our studies using API-59 represent an important step toward developing small molecule therapy that selectively targets the AKT oncogenic pathway in endometrial, and possibly other, cancers.

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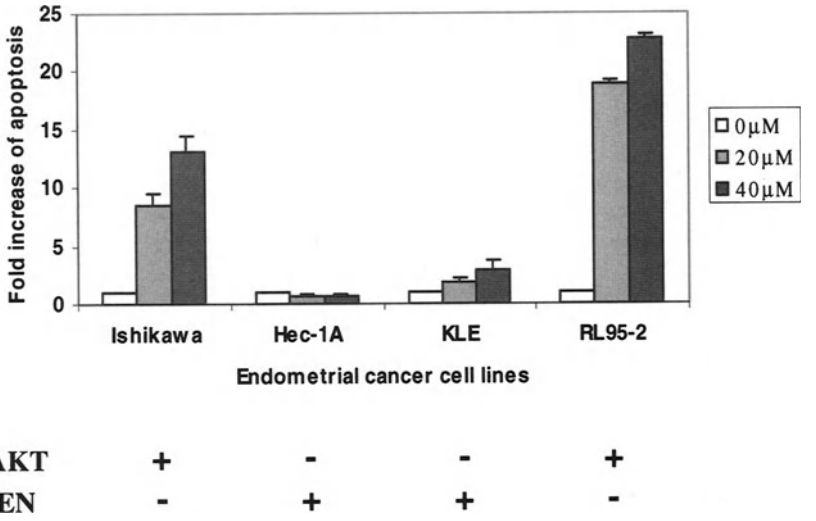

FIG. 5. API-59 induces apoptosis in RL95–2 and Ishikawa endometrial cancer cells expressing constitutively active AKT. Cells were seeded at a density of 1×10^5 cells in six-well plates for 24 h before treatment. Cells were treated with 20 or 40 μM API-59 or dimethylsulfoxide continuously until stained with propidium iodide (PI). Cells were stained with PI 3 days after treatment and were analyzed for a sub-G_1 peak using a FACScan flow cytometer. The fold increase in apoptosis is calculated as the percentage of apoptotic cells in the API-59-treated cell group/the percentage of apoptotic cells in the untreated group. The results are the mean ± SD of log PI from two to three independent experiments

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Part 5
Angiogenesis

A New Paradigm for Vascular Endothelial Cell Growth Factor Receptor Signaling in Adult Endothelium

Asif Ahmed and Peter Hewett

Summary. The primary role of the endometrium is the cyclical generation of a receptive uterine lining for the successful implantation of a fertilized ovum, a process that is dependent on the growth of new blood vessels. Outside of the female reproductive tract, the majority of the adult vasculature is quiescent and angiogenesis occurs only as part of the body's repair processes or in diseases including diabetic retinopathy, rheumatoid arthritis, atherosclerosis, and cancer. The cellular localization of several angiogenic factors has been described in the endometrium, although their precise biological roles in normal cycling endometrium and endometrial pathologies, such as endometriosis and endometrial cancer, has yet to be established. However, it is clear that vascular endothelial cell growth factor (VEGF) is central to the pathophysiological angiogenesis occurring in the endometrium. VEGF elicits its cellular responses via two high-affinity tyrosine kinase receptors, namely VEGFR-1 and VEGFR-2. Until recently, it was presumed that the majority of cellular effects induced by VEGF were mediated by VEGFR-2, whereas VEGFR-1 acted as a decoy. However, results from our laboratory and others have identified VEGFR-1 as an important mediator of VEGF signaling during angiogenesis in the adult. We have shown that activation of VEGFR-1 leads to the generation of nitric oxide, which acts as a cue for the negative regulation of VEGFR-2-dependent proliferation and promotes endothelial cell reorganization into three-dimensional vessel networks.

Key words. angiogenesis, endometrium, vascular endothelial cell growth factor, vascular endothelial cell growth factor receptor-1

Introduction

Blood vessels develop by the processes of vasculogenesis and angiogenesis. During angiogenesis, new blood vessels are derived from preexisting ones, whereas in vasculogenesis, vessels form following the in situ differentiation of endothelial cells from their precursors, a process recently recognized to persist in the adult [1–4]. In classi-

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cal sprouting angiogenesis, local vasodilation, vascular leakage, and basement membrane degradation leads to endothelial proliferation and migration into the surrounding extracellular matrix. The migratory endothelial cells form tubes that anastomose and are subsequently remodeled and associate with mesenchymal perivascular accessory cells (pericytes and vascular smooth muscle cells (VSMC)) to form differentiated vascular networks [3,4]. Complex vascular networks are also generated by intussusception, the division of vessels by longitudinal folding or invading tissue pillars, and elongation (endothelial cell rolling) [4–6].

Angiogenesis is regulated by the local balance of pro- and antiangiogenic factors, and the rate of endothelial cell division is extremely low in the adult due to the predominance of angiogenic inhibitors such as thrombospondin-1 [7] and the close association of endothelial and perivascular cells [8]. However, in the presence of sufficient concentrations of proangiogenic factors, endothelial turnover time can be reduced from years to a few days. Stimuli that tip the balance of angiogenic factors in favor of blood vessel growth are thought to represent "angiogenic switches" [9,10]. Physiological mediators, such as hypoxia, growth factors, and cytokines, the loss of a tumor suppressor gene (e.g., *p53*, *p16*), or the activation of oncogenes (H-*ras*, *src*, HER-2/neu) in malignancy may trigger the development of new vessels [11–13].

Angiogenesis in the Cycling Endometrium

Relatively little is known of the precise molecular cues governing neovascularization in the normal cycling endometrium or how they may be perturbed in diseases such as endometriosis and cancer. Endometrial angiogenesis occurs predominantly in the basalis layer during menstruation and in the subepithelial capillary plexus of the functionalis layer during the proliferative and early secretory phases of the cycle. This coincides with postmenstrual repair and endometrial thickening during the proliferative phase of the cycle [14]. Sprouting angiogenesis has not been detected in the endometrium and it appears that vessel elongation is the major mechanism of vessel growth post-menses [14,15]. Circulating endothelial progenitor cells (EPC) may home to the uterus and contribute to vessel development [16]. Understanding the mechanisms of EPC recruitment may provide new therapeutic opportunities for the treatment of endometrial disorders.

There is strong evidence to suggest that some endometrial-based disorders, including menorrhagia [17–21], endometriosis [22–25], and progestogen-related breakthrough bleeding in patients exposed to contraceptive sex steroids [26–31], are initiated or exacerbated by aberrant angiogenesis and vascular remodeling. These disturbances in angiogenesis may result from pathological changes in the endometrium or exposure to unopposed sex steroids, and manifest as changes in vascular morphology and function [20].

Angiogenesis and Endometrial Cancer

Endometrial carcinoma is the most prevalent gynecological cancer and, although it has a comparatively low mortality rate, it can be very aggressive. The incidence of endometrial carcinoma is highest among Western Caucasian women and is associ-

ated with early age at menarche, late age at menopause, and nulliparity [32,33]. Exposure to unopposed estrogen increases the risk of endometrial cancer, and hormone-replacement therapy [34] and polycystic ovarian syndrome are associated with a higher incidence of this disease. Other factors, such as obesity, diabetes, and hypertension, may increase the risk of endometrial cancer by indirectly promoting levels of circulating estrogen. Consistent with many malignancies, elevated angiogenesis, indicated by increases in microvessel density (MVD), is generally associated with poor prognosis in endometrial cancer [35,36]. Angiogenic activity tends to be greater in endometrial carcinomas occurring in atrophic endometrium compared with those derived from hyperplastic endometrium [36]. Tumor cells produce angiogenic factors that may act directly on the endothelium or indirectly to recruit and activate inflammatory cells [37] and stimulate stromal cells, such as myofibroblasts, to produce angiogenic factors [38]. Various factors, including VEGF, platelet-derived endothelial cell growth factor (PD-ECGF/thymidine phosphorylase), thrombospondin-1 and matrix metalloproteinases are associated with increased MVD [39–43]. The greatest angiogenic activity occurs at the tumor margins invading the underlying myometrium, where it has been reported to be four- to ten-fold higher than in the central tumor mass, and is significantly associated with poor prognosis, particularly within stage I endometrial disease [36,44].

Vascular Endothelial Growth Factor

Vascular endothelial cell growth factor (VEGF(-A)) was first identified as the potent vascular permeability inducing activity in tumors [45] and is now recognized as the most significant angiogenic factor eliciting a wide variety of responses predominantly in the endothelium. Six VEGF family members have been identified (VEGF-A, -B, -C, -D, -E, and placenta growth factor (PlGF)) that bind to one or more of three high-affinity tyrosine kinase receptors (VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and VEGFR-3 (Flt-4)) [46,47]. VEGF(-A) is produced in at least five different isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) through mRNA splicing and the isoforms show a progressive increase in their affinity for heparan sulfate with chain length that greatly affects their bioavailability [48–50]. Most activities induced by VEGF, such as endothelial cell proliferation, migration, and survival, appear to be mediated by VEGFR-2 [46,51,52]. VEGFR-1 activation upregulates urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 expression [53], and may regulate vessel permeability [54] in endothelial cells. During embryological development, VEGFR-1 appears to act as a decoy receptor, but recent evidence suggests that it plays an important role in VEGF signaling during pathological angiogenesis in the adult [55–58]. VEGFR-1 is also expressed by monocytes, which migrate and produce tissue factor in response to VEGF [59], and trophoblasts, where it mediates nitric oxide (NO) release [60]. VEGFR-1 is also produced as a soluble truncated extracellular domain splice variant (sVEGFR-1), which acts as a decoy receptor reducing VEGF activity by sequestration and dominant-negative inhibition [46,55]. Our laboratory has recently reported that VEGFR-1 negatively regulates VEGFR-2, promoting the reorganization of endothelial cells into capillary—like tube networks [56].

Expression of VEGF and its Receptors in Cycling Endometrium

VEGF expression is governed by many growth factors, cytokines, and tissue hypoxia, which induces VEGF transcription via the hypoxia-inducible factor-1α and increases VEGF mRNA stabilization [61–64]. The regulatory regions the *VEGF* gene contain estrogen-response elements [48] and patterns of VEGF expression correlate with estrogen levels in the mouse uterus [65]. Both estradiol and progestins stimulate VEGF expression in the rat uterus [66], and elevated levels of VEGF and increased MVD have been observed in the endometrium of women with implants containing the synthetic progestogen levonorgestrel.

The majority of endometrial VEGF appears to be produced by the glandular epithelium and, to a lesser extent, stromal cells [49,67,68]. Maximal VEGF expression occurs during the menstrual phase (days 1–3) of the cycle [31,68], induced by the hypoxia that ensues following the constriction of the spiral arterioles in conjunction with transforming growth factor-α and interleukin-1β [31,69]. Activated neutrophils release VEGF [70] and are associated with focal VEGF expression within microvessels undergoing elongation in the subepithelial capillary plexus and functionalis layers during the proliferative phase [71–73]. In addition, estrogen promotes the recruitment of monocytes to the endometrium and monocyte/macrophage VEGF expression [39,41].

VEGFR-1 and VEGFR-2 are largely restricted to the endothelium, but have also been identified in the VSMCs and glandular epithelium in the endometrium. Recent studies have detected maximal VEGF and VEGF receptor expression in the menstrual phase preceding increases in VEGFR-2 phosphorylation, endothelial cell proliferation [74], and endometrial blood vessel density [75] during the late menstrual/early proliferative phases [31,74–77]. Consistent with its role as an inhibitor of VEGF activity, sVEGFR-1 expression is inversely correlated with the degree of VEGFR-2 phosphorylation [31]. In addition, VEGF stimulates monocyte migration and infiltrating macrophages have been recognized as a significant source of VEGF and other angiogenic factors in many angiogenic settings [22].

VEGF and Endometrial Cancer

In common with many tumors, VEGF appears to be the major angiogenic factor in endometrial carcinomas and its expression has been reported to correlate with high MVD in many, but not all, studies [36,78,79]. PD-ECGF expression has been correlated with stage, tumor grade, and inflammatory infiltrate invasion of the myometrium [80]. Although PD-ECGF mRNA was reported to be associated with increased MVD in endometrial cancer [40], in a recent immunohistochemical study, PD-ECGF alone did not correlate with MVD [44]. However, high VEGF/PD-ECGF coexpression was found to be a marker of the potent angiogenic tumor phenotype, suggesting cooperation between the two factors [44].

Loss of steroid receptor responsiveness correlates with a more angiogenic endometrial cancer phenotype [81]. Several common genetic abnormalities occur in endometrial cancer, including microsatellite instability and PTEN (phosphatase and tensin homolog deleted on chromosome Ten), k-*ras* and β-catenin gene mutations in

endometrioid adenocarcinomas, and *p53* mutations and loss of heterozygosity in nonendometrioid carcinomas. The activation of oncogenes and loss of tumor suppressor genes, such as *p53*, and loss of heterozygosity, are often associated with increased VEGF expression [11–13,82]. Indeed, a recent paper showed that although VEGF was not an independent indicator of increased MVD in endometrial carcinoma, it was significantly correlated with *p53* expression [79]. We have shown that platelet-activating factor stimulates VEGF production in the HEC-1B endometrial cancer cell line [83]. Tumor-infiltrating macrophages represent an important source of VEGF and their presence is associated with increased MVD in endometrial cancer [37,84].

Role of VEGFR in Angiogenesis

VEGFR-2 is essential for vasculogenesis and blood vessels fail to develop in VEGFR-$2^{(-/-)}$ knockout mice [85]. VEGFR-$1^{(-/-)}$ null embryos undergo vasculogenesis, but are characterized by large numbers of endothelial cells that accumulate and form giant disorganized vascular channels filled with aggregates of hemangioblasts and endothelial cells [86]. This led Fong et al. to conclude that the primary role of VEGFR-1 during vasculogenesis is to limit the differentiation of hemangioblasts into endothelial cells [86,87]. In transgenic mice carrying signaling-incompetent VEGFR-1 that lacks the tyrosine kinase domains, vascular development proceeds with only minor disruptions, indicating that VEGFR-1 acts primarily as a decoy regulating the availability of VEGF during development [88]. These results are further supported by the fact that VEGF-$B^{(-/-)}$/$PlGF^{(-/-)}$ double knockout mice also develop normally [57]. However, this may be accounted for by the fact that most of the VEGFR-1 expressed during development is the soluble form (sVEGFR-1) of the receptor [57] and, therefore, does not preclude the possibility that VEGFR-1 signals in the adult.

In early studies VEGFR-1 was reported to be only weakly phosphorylated in response to VEGF compared with VEGFR-2 in endothelial cells. The weak activity of the VEGFR-1-specific ligand PlGF was presumed to be the result of the displacement of VEGF from VEGFR-1, thus increasing its availability to bind VEGFR-2 [51,89–91]. However, the apparent poor activation of VEGFR-1 may be due to difficulties in measuring VEGFR-1 phosphorylation, because it is expressed at lower (approximately 10-fold) levels than VEGFR-2 in primary endothelial cells. Indeed, recent studies utilizing chimeric VEGFRs have reported the degree of VEGFR-1 phosphorylation to be approximately equivalent to that of VEGFR-2 [52,92]. Activated VEGFR-1 associates with the *p85* subunit of phosphatidylinositol 3-kinase [89,93] and members of the Src family show increased phosphorylation following VEGF stimulation in $PAE_{VEGFR-1}$, but not $PAE_{VEGFR-2}$, cells [51].

The phenotypes of the VEGFR-1 knockout mice [85,86,94] are strikingly similar to our observations in both in vitro and in vivo angiogenesis assays following selective receptor activation or blockade [56]. In in vitro angiogenesis assays, selective VEGFR-2 activation prevented endothelial cell sprouting and capillary like tube formation, whereas inhibition of VEGFR-1 with neutralizing antibodies leads to a loss of endothelial tubule networks and the formation of large aneurysm-like structures [56]. Similarly, inhibition of VEGFR-1 in vivo resulted in the formation of massive blood vessels lined with aggregates of endothelial cells and unchecked proliferation of cultured endothelial cells [56]. It appears from these experiments that VEGFR-1 may

promote vascular connections within the capillary network (branching angiogenesis), whereas VEGFR-2 predominantly promotes tube length between branches. Our findings are supported by studies of fibroblasts expressing the activated cytoplasmic domain of the VEGFR-1, which spontaneously form tubular structures on matrix [95,96]. Further indirect supporting evidence comes from the JunB null mice, where failure of the fetal blood vessels to penetrate or branch into the labyrinth trophoblasts is associated with downregulation of VEGFR-1 [97]. This suggests that endothelial cells stimulated to proliferate and migrate via VEGFR-2 are unable to organize themselves into vascular structures without VEGFR-1. Thus, VEGFR-1 activation negatively regulates VEGFR-2 signaling to limit proliferation and promote reorganization of endothelial cells into three-dimensional tubular networks (Fig. 1).

VEGF Promotes the Release of NO via VEGFR-1 in Endothelial Cells

We and others have shown that VEGF stimulates the production of NO from human umbilical vein endothelial cells (HUVEC) [60] and from intact rabbit arterial strips [98]. VEGF-stimulated NO release induces angiogenesis both in vitro [99] and in vivo

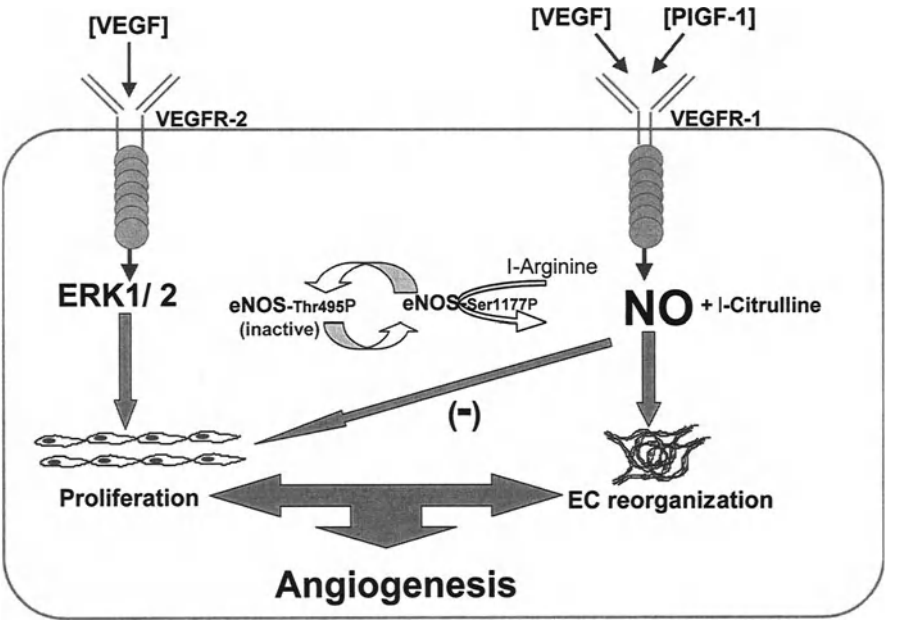

FIG. 1. Schematic diagram showing the regulatory pathways mediated by the vascular endothelial growth factor receptors (*VEGFR*)-1 and -2 in endothelial cells during angiogenesis. *VEGF*, vascular endothelial cell growth factor; *PlGF-1*, placenta growth factor-1; *ERK*, extracellular signal-regulated kinase; *eNOS*, endothelial nitric oxide synthase; *NO*, nitric oxide

[100]. Direct evidence for the requirement of NO in VEGF-mediated angiogenesis has been provided by studies using endothelial NO synthase $(eNOS)^{(-/-)}$ knockout mice, in which VEGF fails to induce angiogenesis [101–103]. However, it is not clear at what stage in the process of blood vessel formation that NO exerts its influence.

We have taken a number of approaches, including the use of blocking antibodies, selective ligands, and VEGFR-expressing cell lines, to identify the VEGFR responsible for VEGF-mediated NO release in endothelial cells. Porcine aortic endothelial cells (PAE) engineered to express VEGFR-1 ($PAE_{VEGFR\text{-}1}$) or VEGFR-2 ($PAE_{VEGFR\text{-}2}$) have served as a very useful model to define the activities of the VEGF receptors [51]. Basal levels of NO were found to be higher in $PAE_{VEGFR\text{-}2}$ compared with $PAE_{VEGFR\text{-}1}$. However, VEGF only produced a concentration-dependent increase of NO release in $PAE_{VEGFR\text{-}1}$ [56]. Consistent with these findings, stimulation of endothelial cells with $VEGF_{121}$, which only binds to VEGFR-2 [104], or blockade of VEGFR-2 with neutralizing antibodies, failed to affect VEGF-stimulated NO release. Furthermore, PlGF-1, which only binds to VEGFR-1, stimulates NO release in HUVEC. These findings also support our earlier observations that VEGF induces NO release via VEGFR-1 in human trophoblasts [60] and ECV304 cells [105]. In contrast, Murohara and colleagues have reported that VEGF increases permeability via NO and prostacyclin through VEGFR-2 activation on the basis of their observation that PlGF-2 fails to increase vascular permeability [101]. However, PlGF-2 contains a heparin-binding domain and, in addition to VEGFR-1, also binds to neuropilin [106], as well as heparan sulfate-containing proteoglycans, which may limit its availability for VEGFR-1. Similarly, we found that, unlike PlGF-1, PlGF-2 did not promote NO release in $PAE_{VEGFR\text{-}1}$ [56]. In addition, it appears that different VEGF family members can illicit distinct signaling responses from VEGFR-1 [107]. VEGF has been reported to upregulate eNOS in $PAE_{VEGFR\text{-}2}$ [108], but levels of NOS protein do not necessarily correlate with enzyme activity.

VEGFR-1 Modulates Endothelial Cell Proliferation

In our hands, the specific blockade of VEGFR-1 with neutralizing antibodies potentiates HUVEC proliferation in response to VEGF, indicating that VEGFR-1 transduces signals to negatively regulate VEGFR-2-dependent endothelial cell proliferation [56]. Similar findings were reported previously in trophoblasts [60] and in PAE transfected with chimeric VEGFRs [109]. NO may stimulate proliferation of endothelial cells at low concentrations, but inhibits their proliferation at higher concentrations by a ribonucleotide-dependent mechanism [110]. Use of the NO donor glyco-S-nitroso-*N*-acetylpenicillamine (SNAP)-1 or 8-bromo-cGMP attenuated VEGF-stimulated mitogenesis, indicating that NO activates cGMP to regulate endothelial cell proliferation (Fig. 1) [56]. Moreover, the inhibitory effect of the anti-VEGFR-1 antibody on endothelial cell proliferation was negated in the presence of the NO donor glyco-SNAP-1. Collectively, these data are in general agreement with a study demonstrating a role for NO in blocking endothelial cell proliferation and promoting their organization into capillary like tubules in response to basic fibroblast growth factor [111].

Zeng et al. have used chimeric VEGFRs expressed in HUVEC to show that VEGFR-1 downregulates VEGFR-2-mediated proliferation, but not migration, via the phos-

phatidylinositol 3-kinase (PI3K) pathway [52]. The inhibition of VEGFR-2 is mediated via CDC42 and, partially, by Rac-1, and appears to involve pertussis toxin-sensitive G-proteins and $G_{\beta\gamma}$ subunits [112]. The role of NO in this system is currently under investigation.

VEGFR-1 and Pathological Angiogenesis

Several recent studies have highlighted the therapeutic potential of VEGFR-1 inhibition in pathologies characterized by aberrant angiogenesis. The VEGFR-1-specific ligand PlGF is upregulated in several types of tumor [113–116]. Hiratsuka et al. proposed that VEGFR-1 acts as a positive regulator of pathological angiogenesis when levels of PlGF are elevated, because the growth of Lewis lung tumors overexpressing PlGF-2 was compromised in VEGFR-1 tyrosine kinase domain-deficient transgenic mice compared with wild-type mice [117]. In agreement with these findings, embryonic stem cell-derived tumors growing in $PlGF^{(-/-)}$ null mice were small and poorly vascularized compared with those in wild-type mice [57]. The inhibition of VEGFR-1 with blocking antibodies also suppresses angiogenesis occurring in ischemic retinas and inflammatory arthritis [58]. A significant amount of the antiangiogenic activity associated with VEGFR-1 inhibition may be due to a reduction in the differentiation and infiltration of myeloid progenitor cells [57,58]. Angiozyme, a novel ribozyme (catalytic RNA) targeted to VEGFR-1 is currently undergoing clinical trials, having exhibited good efficacy in animal models, producing greater tumor inhibition than ribozymes specific for VEGFR-2 [118].

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Sex Steroid-Dependent and -Independent Angiogenesis in Uterine Endometrial Cancers

Jiro Fujimoto, Ikumi Aoki, Hiroshi Toyoki, Sufia Khatun, Eriko Sato, and Teruhiko Tamaya

Summary. In general, tumors induce angiogenic factors specific to them, which leads to angiogenesis with advancement. However, angiogenesis in uterine endometrial cancers is complicated because hormone dependency in growth also modifies the angiogenic potential. Therefore, tumor-dormancy therapy against angiogenic potential in uterine endometrial cancers must be thoroughly considered. Upstream of the vascular endothelial growth factor (VEGF) gene conserves estrogen-responsive elements. Progesterone primed with estrogen induces thymidine phosphorylase (TP) in uterine endometrium. Sex steroid-dependent VEGF and TP are highly expressed in cases of early stage and well-differentiated uterine endometrial cancers, as is basic fibroblast growth factor (bFGF) in cases of advanced and poorly differentiated uterine endometrial cancers. A transcriptional factor for angiogenesis, namely ETS-1, is linked to VEGF in well-differentiated uterine endometrial cancers and to bFGF in poorly differentiated uterine endometrial cancers. Therefore, even if dedifferentiation and angiogenic switching occur due to advancement and long-term hormone therapy, the inhibition of ETS-1, along with main angiogenic factors, may be an effective strategy to suppress uterine endometrial cancers as a novel tumor-dormancy therapy.

Key words. Angiogenesis, ETS-1, sex steroids, uterine endometrial cancers

Introduction

In general, tumors induce angiogenic factors specific to them, which leads to angiogenesis with advancement. However, angiogenesis in uterine endometrial cancers is complicated because hormone dependency in growth also modifies the angiogenic potential. Therefore, tumor-dormancy therapy against angiogenic potential in uterine endometrial cancers must be thoroughly analyzed.

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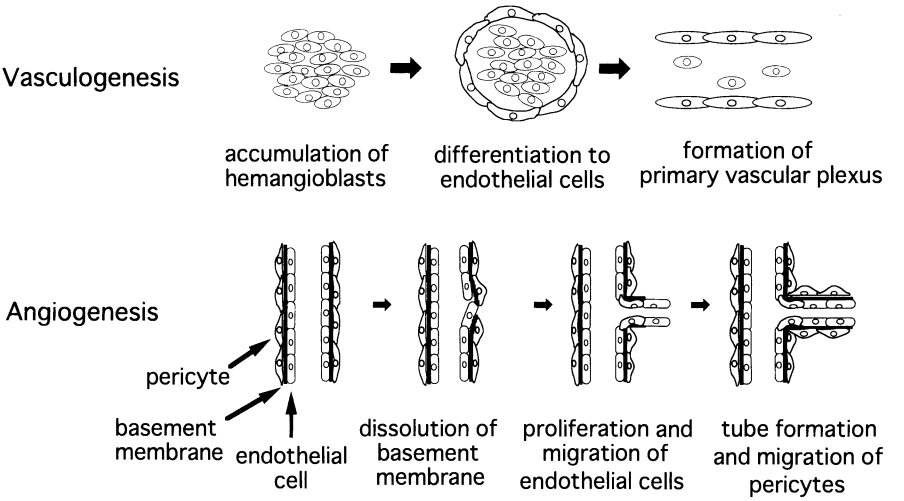

FIG. 1. Neovascularization. Vasculogenesis is the formation of a vascular plexus by differentiation from hemangioblasts to endothelial cells without any existing vascular system. Angiogenesis is the formation of new capillaries from existing capillaries

Tumor Angiogenesis

Neovascularization involves two concepts, as shown in Fig. 1. The two concepts are vasculogenesis and angiogenesis. Vasculogenesis is the formation of a vascular plexus by differentiation from hemangioblasts to endothelial cells without any existing vascular system. For example, a primary vascular plexus is formed from hemangioblasts in fetuses by the process of vasculogenesis. Angiogenesis is the formation of new capillaries from existing capillaries. Angiogenesis occurs in tumors and supports the growth and advancement of tumors.

The detailed process of angiogenesis in tumors can be seen in Fig. 2. Angiogenic factors from tumors induce and activate matrix metalloproteinase, plasminogen activator, collagenase, and other enzymes in endothelial cells. The enzymes dissolve the basement membrane of endothelial cells, after which the endothelial cells proliferate and migrate under the influence of angiogenic factors. Angiogenic factors induce the production of integrins in endothelial cells. The endothelial cells then form immature capillary tubes. In normal tissue, the capillary is matured upon being covered with pericytes. However, the covering pericyte layer of capillaries in tumors, consisting of fewer cells than normal with inferior function, is not very good. Therefore, cancer cells can easily migrate into and out of the immature capillary, as shown in Fig. 2.

When the cluster of cancer cells is smaller than 2 mm in diameter, angiogenesis does not occur. However, when the cluster size reaches 2 mm, angiogenesis occurs. Thereafter, the capillary network is formed rapidly to grow the tumors. Therefore, attaining a diameter of 2 mm is critical to the manner of tumor growth [1].

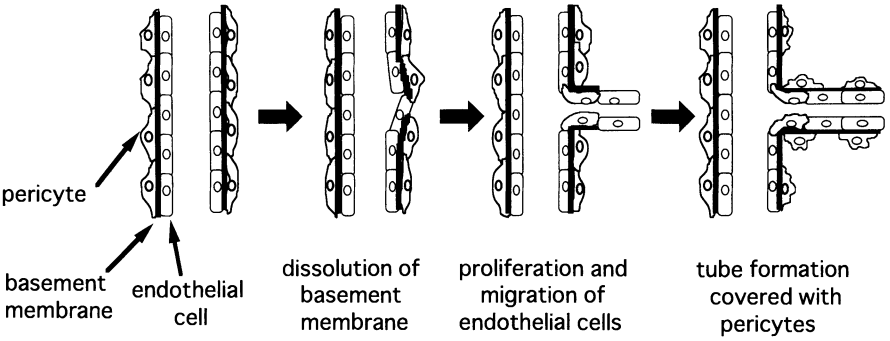

FIG. 2. The process of angiogenesis in tumors

Unfortunately, when a metastatic lesion has been discovered, its size is already larger than 2 mm in diameter. This means that angiogenesis in the metastatic lesion has already started. Therefore, although detachment and invasion of cancer cells can be suppressed, the growth of the previously formed metastatic lesions will not be suppressed. The growth of metastatic lesions must be suppressed to improve patient prognosis. The inhibition of angiogenesis should be an excellent strategy to suppress the growth of metastatic lesions.

Angiogenic Factors and a Transcription Factor

If there is a positive correlation between microvessel density in tumors and the target factor levels, it is plausible that the target factor is an angiogenic factor. The microvessel density is evaluated to count microvessels using immunohistochemical staining for CD31, CD34 or factor VIII-related antigen specific to the endothelial cells.

The angiogenic factors basic fibroblast growth factor (bFGF), thymidine phosphorylase (TP), vascular endothelial growth factor (VEGF), and interleukin (IL)-8 work on angiogenesis in uterine cancers [2–11]. bFGF is expressed in cancer and stromal cells, and works on basic angiogenesis. bFGF expression has been shown to increase with the advancement of clinical stage and with dedifferentiation [3], as shown in Fig. 3. Therefore, bFGF may be an excellent indicator for the advancement of endometrial cancers.

TP is expressed in stromal cells of cancers. TP in the uterine endometrium is regulated during the menstrual cycle, meaning that TP expression depends upon changes in sex steroid levels [12]. TP expression is significantly higher in well-differentiated uterine endometrial cancers (G1) than in moderately (G2) and poorly differentiated uterine endometrial cancers (G3) [7], as shown in Fig. 3.

VEGF is the most sensitive angiogenic factor and it is expressed in cancer cells. Isomers $VEGF_{165}$ and $VEGF_{121}$ move rapidly and bind to the receptors on endothelial cells. In G1 cells, VEGF expression is transiently increased by estradiol and this estradiol-induced VEGF expression is partially suppressed by additional progesterone

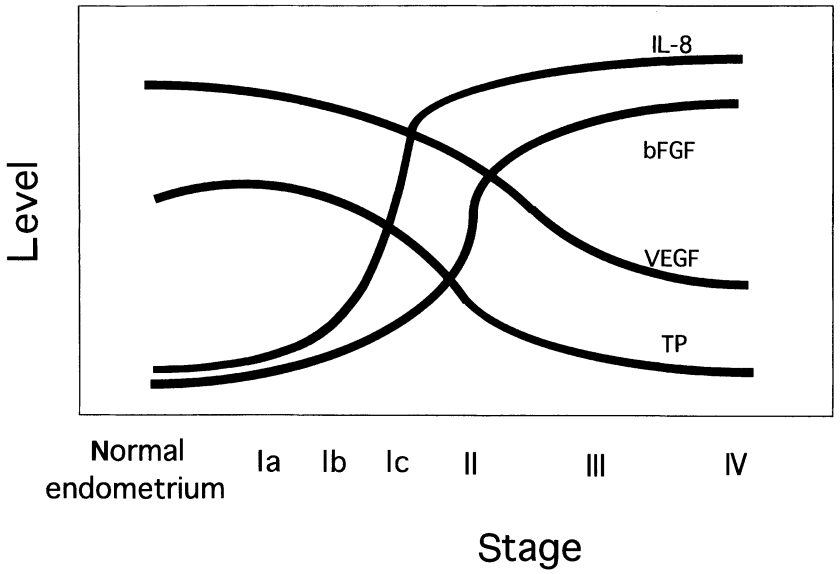

FIG. 3. Angiogenic factors in uterine endometrial cancers. *IL-8*, interluekin-8; *bFGF*, basic fibroblast growth factor; *VEGF*, vascular endothelial growth factor; TP, thymidine phosphorylase

[13], as shown in Fig. 4. The transient upregulation of VEGF is considered to be logical, because upstream of the *VEGF* gene conserves estrogen-responsive elements. Therefore, it is natural that VEGF expression should be downregulated with advancement and dediffentiation of endometrial cancers. The expression of VEGF, particularly the $VEGF_{165}$ and $VEGF_{121}$ isomers, decreases with the advancement of clinical stage and with dedifferentiation of uterine endometrial cancers [9], as shown in Fig. 3.

The localization of IL-8 is identified with that of CD-68 specific to infiltrating macrophages. IL-8 levels are correlated with the number of infiltrating macrophages. Therefore, IL-8 may be secreted from tumor-associated infiltrating macrophages. IL-8 is expressed by stromal cells of cancers and induces the migration of endothelial cells. In uterine endometrial cancers, IL-8 was rapidly up regulated from stage Ia to stage Ib and, then, was maintained at the maximum level. IL-8 expression was saturated at stage Ib. Therefore, IL-8 may work as an angiogenic switch. However, it is very difficult to suppress IL-8 within the cytokine network in order to avoid turning on the angiogenic switch [11].

ETS-1 is a transcription factor for angiogenesis in uterine cervical cancers that is linked to the expression of IL-8 and TP [14]. Localization of ETS-1 is identified with that of factor VIII-related antigen specific to endothelial cells. ETS-1 levels are correlated with the number of microvessels. Therefore, ETS-1 may also mediate angiogenic potential as a transcription factor in endometrial cancers. ETS-1 levels are correlated

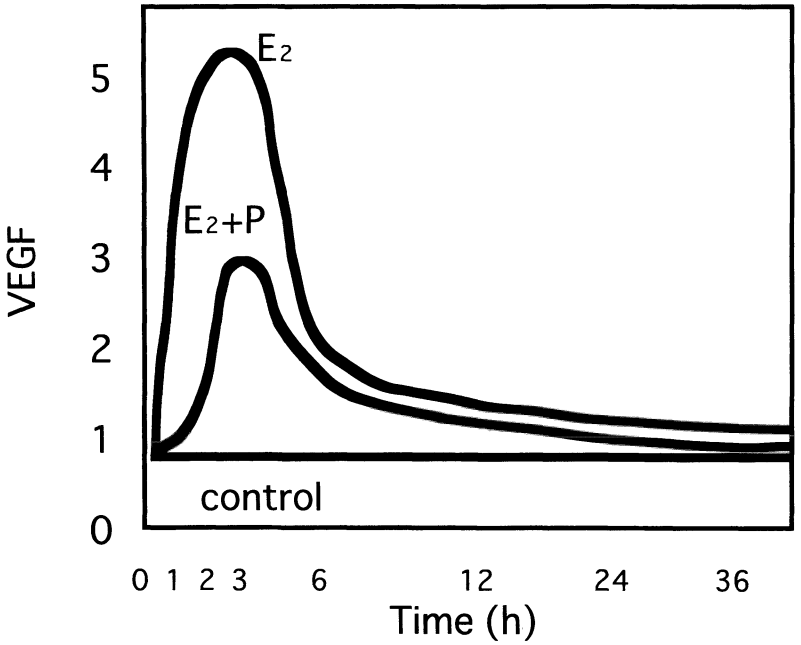

FIG. 4. Effect of sex steroids on vascular endothelial growth factor (*VEGF*) expression in well-differentiated endometrial cancer cells. *E2*, 10 nM 17β-estradiol; *P*, 10 nM progesterone. Each level is the mean of five determinations

with VEGF levels in G1. In G2 and G3, ETS-1 levels are correlated with bFGF levels. Therefore, ETS-1 may be linked to VEGF in G1 and to bFGF in G2 and G3 to mediate angiogenesis [15].

Strategy of Tumor Dormancy Therapy

Because bFGF is recognized as an excellent indicator of advancement, we considered a strategy for the suppression of angiogenic potential of endometrial cancers in vitro. Medroxyprogesterone acetate (MPA) suppressed bFGF mRNA expression and bFGF secretion into the culture media in G1 cells. In G3 cells, MPA could not suppress bFGF mRNA expression and bFGF secretion, but the antiangiogenic agent TNP 470 could [16]. Clinically, endometrial cancer tissue involves cancer cells at various stages of differentiation with diverse populations. Therefore, if we can determine the cell population, we can efficiently suppress the angiogenic potential and, subsequently, the advancement of endometrial cancers clinically.

VEGF expression in a G1 cell line has been shown to be sensitively regulated by ovarian steroids [13], as has TP expression in the uterine endometrium [12]. These results indicate that the expression of VEGF and TP may be downregulated with dedifferentiation. Therefore, if VEGF and TP in G1 and bFGF in G2 and G3 can be

suppressed, as part of the tumor-dormancy therapy, the prognosis of patients should be markedly improved without the severe side-effects seen with chemotherapy. Because chemotherapy is often not very specific to cancer cells, it produces severe effects even on normal cells, especially bone marrow cells. In contrast, tumor-dormancy therapy is specific to the rapidly growing vascular endothelial cells in tumors, without having any effect on slow-growing vascular endothelial cells and other normal cells. However, if an angiogenic factor is suppressed by tumor-dormancy therapy for a long period, another angiogenic factor may be induced by an alternatively linked angiogenic pathway, which is recognized as tolerance.

G1 tumors comprise over 70% of all endometrial cancers. During hormone therapy, G1 often become hormone-resistant tumors. In G1, inhibition of VEGF may be transiently effective in inhibiting angiogenic potential. During long-term therapy inhibiting VEGF, the treatment may become ineffective because of a loss of estrogen dependency, which is related to VEGF dependency. If so, inhibition of VEGF along with ETS-1 should be more effective in inhibiting angiogenic potential. In G2 and G3 endometrial cancers, inhibition of bFGF alone may be less effective than inhibition of bFGF with ETS-1. In the future, inhibition of not only an angiogenic factor, but also the simultaneous inhibition of a transcription factor for angiogenesis should provide a more appropriate and effective inhibition of angiogenic potential to suppress the advancement of tumors and improve patient prognosis in uterine endometrial cancers.

Conclusion

Endometrial cancers conserve hormone dependency, which leads to complicated angiogenic potential and tumor advancement. Even if dedifferentiation and angiogenic switching occur due to advancement and long-term hormone therapy, the inhibition of ETS-1 along with main angiogenic factors may be an effective strategy to suppress uterine endometrial cancers as a novel tumor-dormancy therapy. In general, tumor-dormancy therapy against transcription factors linked to the corresponding angiogenic factors may be an excellent novel strategy against various tumors.

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Part 6
Carcinogenesis

Inhibitory Effects of Phytoestrogens and Related Herbal Extracts on Mouse Endometrial Carcinogenesis: A Review

KENJI NIWA, ZENGLIN LIAN, KEIKO TAGAMI, JINGCHUN GAO, and TERUHIKO TAMAYA

Summary. The effects of isoflavones (genistein and daidzein) and related Kampo medicines (*Juzen-taiho-to* and *Shimotsu-to*) on endometrial carcinogenesis in mice were investigated separately in two experiments. In the short-term experiment (2 weeks), a single subcutaneous (s.c.) administration of genistein [(1 mg/30 g body weight (BW))] significantly decreased the levels of 17β-estradiol (E_2; 5 p.p.m. in diet)-induced expression of c-*jun*, interleukin (IL)-1α and tumor necrosis factor (TNF)-α mRNAs in the uteri of ovariectomized mice ($P < 0.005$, $P < 0.05$, and $P < 0.01$, respectively). Daidzein significantly inhibited E_2-induced expression of c-*fos* and IL-1α ($P < 0.01$ and $P < 0.01$, respectively). In the long-term experiment (30 weeks), the incidences of endometrial adenocarcinoma and atypical endometrial hyperplasia were significantly lower in the E_2 group treated with either genistein or daidzein than in the corresponding control group ($P < 0.01$ and $P < 0.05$, respectively). In the short-term experiment, *Juzen-taiho-to* or *Shimotsu-to* (Kampo formula) treatment (2 weeks) significantly decreased the levels of E_2-stimulated expression of c-*fos* mRNA ($P < 0.05$). In the long-term experiment, *Juzen-taiho-to* and *Shimotsu-to* treatment significantly decreased the incidences of endometrial adenocarcinoma ($P < 0.05$) and other preneoplastic lesions under estrogenic stimulation. It is suggested that both genistein and daidzein, as well as *Juzen-taiho-to*, have an inhibitory effect on estrogen-related endometrial carcinogenesis in mice, indicated by the suppression of estrogen-related c-*fos* and c-*jun*, as well as the suppression of cytokine IL-1α and TNF-α-expression through a cytokine- and estrogen receptor-mediated pathway. *Shimotsu-to* is considered to be a key compounet of *Juzen-taiho-to* for the prevention of endometrial carcinoma.

Key words. Isoflavones, *Juzen-taiho-to*, prevention, endometrial carcinogenesis, cytokines

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Introduction

Epidemiological studies have suggested that the consumption of diets containing soybeans and soybean-based products reduces the risk of endometrial cancers [1] and other hormone-dependent cancers [2,3]. Soybeans and soybean-related foods are a good source of phytochemicals, including phytoestrogens such as genistein and daidzein. It is well known that isoflavones have a variety of biological activities, including anticancer effects.

Genistein is an inhibitor of protein tyrosine kinase, which plays an important role in cell proliferation or transformation [4,5]. Genistein is known to inhibit the growth of the breast cancer cell line MCF-7 [6,7] and a colon tumor cell line [8]. A related compound, daidzein, has been shown to inhibit the growth of human prostate cancer cells [9–11] and to induce apoptosis in human prostatic cancer cell lines [12].

Several studies have demonstrated the inhibitory effects of genistein and daidzein on carcinogenesis in the skin, breast, prostate, and seminal vesicle [13–15]. Although the effects of isoflavones on endometrial carcinogenesis have not been reported, we have recently reported the preventive effects of the abovementioned isoflavones on endometrial carcinogenesis in mice [16].

Juzen-taiho-to is one of the phytochemical-containing tonic formulas in Kampo medicine. It is a medicine made up of herbal complexes, mainly consisting of *Shimotsu-to* and *Shikunshi-to* Kampo formulas (Table 1). *Juzen-taiho-to* reduces metastatic potential [17] and enhances both cytokine induction [18] and antitumor activity [19,20]. We have reported on the inhibitory effects of *Juzen-taiho-to* on endometrial carcinogenesis [21]. The inhibitory effect of *Juzen-taiho-to* in short-term experiments is believed to be mediated by *Shimotsu-to* [22].

The expression of estrogen-response genes c-*fos*/*jun* is considered to relate to cellular proliferation and differentiation [23–26]. Herbal Glycyrrhizae radix extract suppresses estrogen-induced c-*fos*/*jun* expression and endometrial carcinogenesis in mouse uterus [27]. The isoflavones genistein and daidzein inhibit expression of c-*fos* mRNA in some cell lines [28,29]. The internal cytokines interleukin (IL)-1α and

TABLE 1. The herbal ingredients of *Juzen-taiho-to*

Ingredients	Botanical origins	Ratio
Shimotsu-to		
Angelicae radix	*Panax ginseng*	3.0
Cnidii rhizoma	*Cnidium officinale*	3.0
Paeoniae radix	*Paeonia lactiflora*	3.0
Rehmanniae radix	*Rehmannia glutinosa*	3.0
Shikunshi-to		
Ginseng radix	*Panax ginseng*	3.0
Atractyloidis rhizoma	*Atractylodes japonica*	3.0
Hoelen	Sclerotium of *Poria cocos* Wolf	3.0
Glycyrrhizae radix	*Glycyrrhiza uralesis*	1.5
Others		
Astragali radix	*Astragalus membranceus*	3.0
Cinnamoni cortex	*Cinnamomum cassia*	3.0

Adapted from [17]–[21]

tumor necrosis factor (TNF)-α appear to be important factors for tumor promotion and progression in skin and colonic carcinogenesis [30,31]. In general, estrogens exert a proliferative effect on endometrial carcinoma, but isoflavones inhibit cell proliferation [32].

The present study was conducted to determine whether genistein and daidzein, as well as *Juzen-taiho-to* and *Shimotsu-to*, exert suppressive effects on mouse endometrial carcinogenesis induced by *N*-methyl-*N*-nitrosourea (MNU) and 17β-estradiol (E_2). It is important for the understanding of the action of estrogen to determine the effects of these compounds on the mRNA expression of the estrogen-related genes c-*fos*/*jun* and the cytokines IL-1α and TNF-α.

Methods

Animals and Chemicals

Female ICR mice were purchased from Japan SLC (Shizuoka, Japan). Oriental MF (Oriental Yeast, Tokyo, Japan) was used as the basal diet. Diet and filtrated tap water were available ad libitum throughout the experiment. MNU, E_2, or genistein (purity over 95%; Fig. 1) and daidzein (purity over 97%; Fig. 1) were purchased from Sigma Chemical (St. Louis, MO, USA) and Fujicco (Kobe, Japan), respectively. The content of E_2 and the isoflavones gensitein and daidzein in the control MF diet was confirmed to be under 0.01 p.p.m. *Juzen-taiho-to*, *Shimotsu-to*, and *Shikunshi-to* compound herbal extracts were purchased from Tsumura (Tokyo, Japan). The ingredients of *Juzen-taiho-to* are given in Table 1.

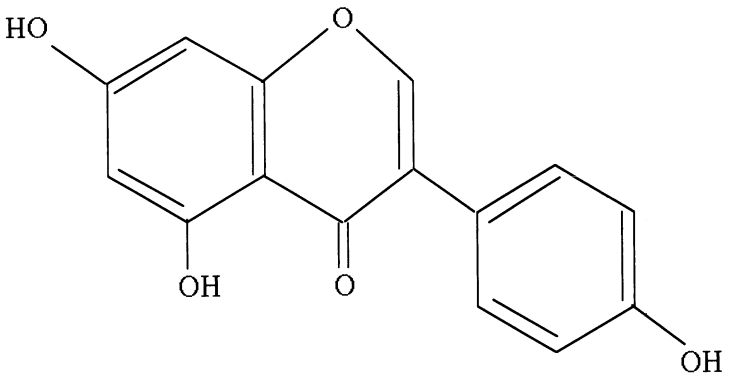
Experimental Protocol for the Short-Term Assay for Isoflavones

Female ICR mice, 12 weeks of age, were ovariectomized at laparotomy under general anesthesia with diethylether. Two weeks later, the ovariectomized mice were divided into four experimental groups (five mice in each group; Fig. 2). Groups 1, 2, and 3 were given the diet containing E_2 (5 p.p.m.). Genistein and daidzein were administered subcutaneously to avoid any interaction with the oral administration of E_2. Mice in groups 1 and 2 further received a single subcutaneous (s.c.) injection of genistein or daidzein, respectively, at a dose of 1 mg/30 g body weight (BW), 24 h prior to resection of uteri on the 13th day. The isoflavones were dissolved in ethanol and mixed with sesame oil. Group 4 served as a non-treatment control group. Two weeks later, the uteri were resected and cut in half longitudinally. One half was frozen quickly in liquid nitrogen for the experiments described below, while the other half was subjected to pathological examination.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from frozen tissues by a guanidium thiocyanate–phenol–chloroform extraction method [33]. Total RNA (3 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RTase; 200 units; Gibco BRL, Gaitherburg, MD, USA) in 20 μM Tris-HCl (pH 8.4), 50 μM KCl, 2.5 μM $MgCl_2$, 0.1 μg/mL bovine serum albumin, 10 μM dithiothreitol, and 0.5 mM deoxynucleotides to generate cDNAs, using random hexamers (50 ng; Gibco BRL) at 37°C for 60 min.

Genistein



Daidzein

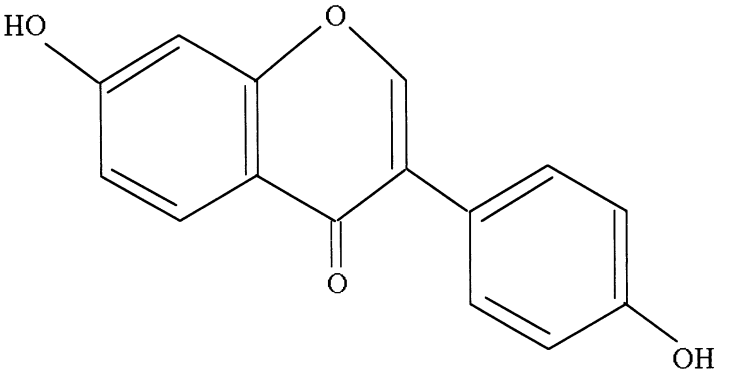


FIG. 1. The structural formula of genistein and daidzein. (Adapted from [16])

The RT reaction was heated at 94°C for 5 min to inactivate the MMLV-RTase. For c-*fos* (320 bp) and TNF-α (369 bp) mRNA expression, 30 cycles of PCR consisted of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1.5 min at 72°C for extension. For c-*jun* (257 bp) and IL-1α (401 bp) mRNA expression, 25 cycles of PCR were performed for 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for extension. The PCR reaction was performed in reverse-transcribed cDNAs and 0.1 mM specific primers described below, using an IWAKI thermal sequencer TSR-300 (IWAKI Glass, Tokyo, Japan) with Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) in 10 μM KCl, 20 μM Tris-HCl (pH 8.8), 10 μM $(NH_4)_2SO_4$, 2 μM $MgSO_4$, 0.1% Triton X-100, and 0.15 μM deoxynucleotides phos-

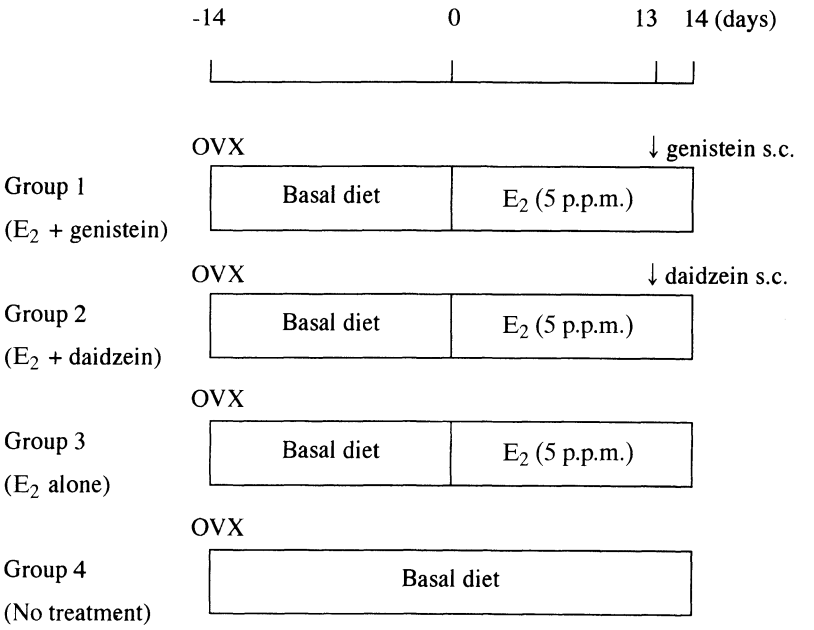


FIG. 2. Short-term experimental design for isoflavones. *OVX*, bi-lateral oophorectomy; ↓, genistein or daidzein was injected subcutaneously (*s.c.*) at a dose of 1 mg/30 g body weight; E_2, 17β-estradiol. (Adapted from [16])

phates. Twenty cycles of PCR for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; a house-keeping gene; 252 bp) mRNA as an internal standard were performed similarly. The following oligodeoxynucleotides were synthesized as specific primers in the PCR according to published information (cDNA for c-*fos* [34], c-*jun* [35], IL-1α [36], TNF-α [37], and GAPDH [38]) as follows: c-*fos*, sense 5′-GCTTCTATAAAGGCGCCAGCTGA-3′ and antisense 5′-GACAGGAGAGCCCATGCT GGAG-3′; c-*jun*, sense 5′-GGAGTGGGAAGGACGTGGCGC-3′ and antisense 5′-TCCCA GCCCTCCCTGCTTTGTG-3′; IL-1α, sense 5′-GATGGCCAAAGTTCCTGACTTG-3′ and antisense 5′-GCCTGACGAGCTTCATCAGTTT-3′; TNF-α, sense 5′-AGGCAGGTT CTGTCCCTTTCA-3′ and antisense 5′-TCCACTTGGTGGTTTGCTACG-3′; and *GAPDH*, sense 5′-CAAGGTCATCCCAGAGCTGAA-3′ and antisense *GAPDH*, 5′-GCAATGCCAGCCCCGGCATCG-3′.

Semi-Quantitative Analysis of c-fos, c-jun, IL-1α, and TNF-α mRNA Expression by Southern Blot PCR Products

PCR products were applied to a 1.5% agarose gel for electrophoresis performed at 50–100 V. The PCR products were capillary transferred to Immobilon transfer membrane (Millipore, Bedford, MA, USA) for 16 h. The membrane was dried at 80°C for 30 min and UV-irradiated to tightly fix the PCR products. The PCR products on the membrane were prehybridized in 1 M NaCl, 50 mM Tris-HCl (pH 7.6), and 1% sodium

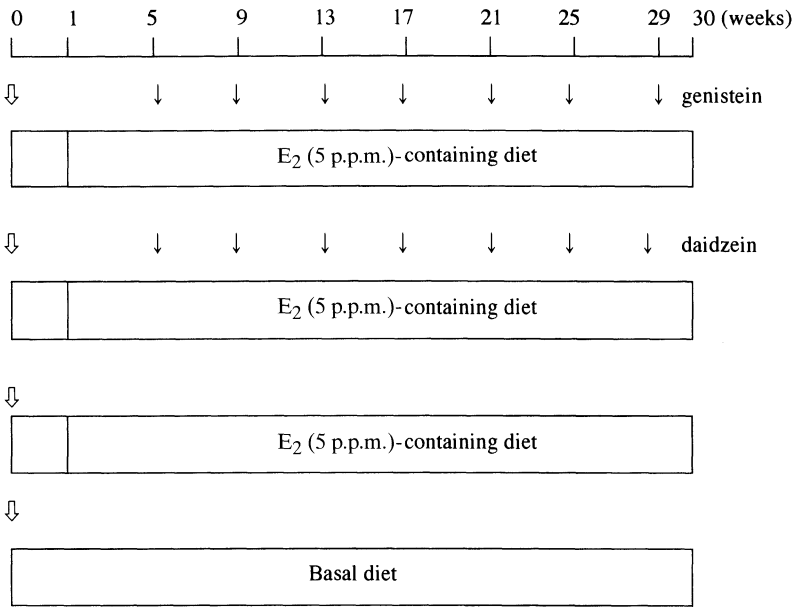


FIG. 3. Long-term experimental design for isoflavones. ⇩, *N*-methyl-*N*-nitrosourea (MNU) solution (total volume 0.1 mL), administered at a dose of 1 mg/100 g body weight, was injected into the left uterine tube and normal saline was injected into the right uterine tube; ↓, genistein or daidzein was injected subcutaneously at a dose of 1 mg/30 g body weight. (Adapted from [16])

dodecyl sulfate (SDS) at 42°C for 1 h and hybridized in the same solution with biotinylated oligodeoxynucleotides probes synthesized from the sequences between the specific individual primers of c-*fos*, c-*jun*, IL-1α, and TNF-α at 65°C overnight. Specific bands hybridized with biotinylated probes were detected with Plex Luminescent Kits (Millipore) on the membrane after it had been exposed to X-ray film at room temperature for 10 min. The quantification of Southern blot was performed with BioImage (Millipore). The intensity of specific bands was standardized against that of *GAPDH* mRNA.

Experimental Protocol for the Long-Term Effects of Isoflavones

The experimental protocol invetigating the long-term effects of isoflavones is shown in Fig. 3. A total of 110 female ICR mice, 12 weeks of age, underwent laparotomy under general anesthesia with diethylether. MNU solution (total volume 0.1 mL), at a dose of 1 mg/100 g BW, was injected into the left uterine tube and normal saline was injected into the right uterine tube. One week after MNU injection, animals were divided into six groups. Isoflavones were administered by s.c. injection. Group 1 (25 mice) was given E_2 (5 p.p.m. in the diet) and injected with genistein (1 mg/30 g BW, s.c., every 4 weeks, seven times), while group 2 (25 mice) was given the E_2 diet and injected with daidzein (1 mg/30 g BW, s.c., every 4 weeks, seven times). The doses of genistein or

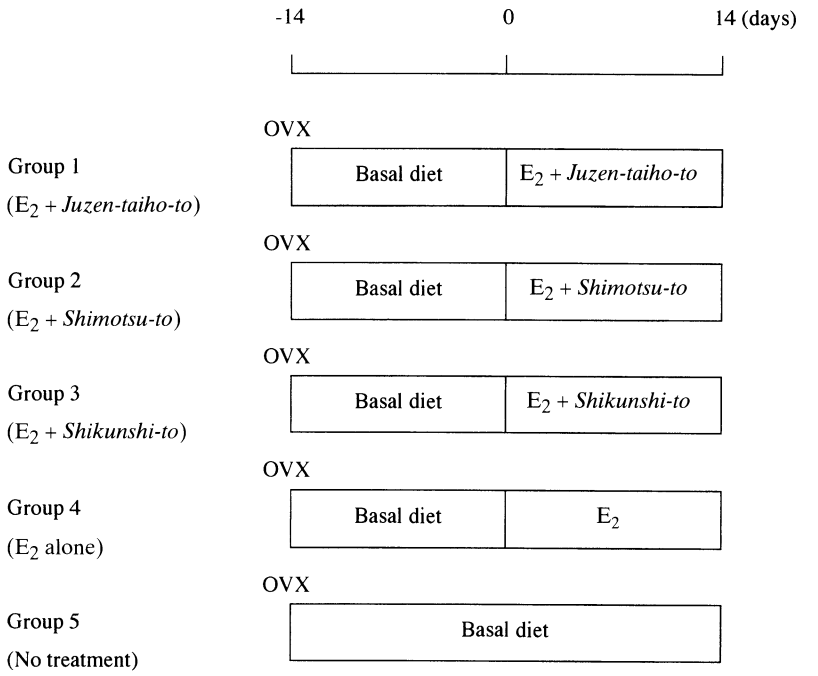


FIG. 4. Short-term experimental design for the investigation of Kampo medicines. *OVX*, bi-lateral oophorectomy; E_2, 17β-estradiol. *Juzen-taiho-to* (0.2%), *Shimotsu-to* (0.07%), and *Shikunshi-to* (0.08%) were administered as indicated. (Adapted from [22])

daidzein used in the long-term experiments were determined on the basis of results from the short-term experiment. Group 3 (30 mice) was given the E_2 (5 p.p.m.)-containing diet with s.c. injections of vehicle. Group 4 (30 mice) was given s.c. injections of the vehicle alone as a control. Thirty weeks after MNU exposure, all animals were killed and autopsied. All major organs, particularly the reproductive organs, were inspected grossly. The uterus, ovaries, and vagina, as well as any other lesions suspected of being neoplastic and hyperplastic, were cut in half. The tissues were subjected to histopathological examination. Tissues were sectioned at 3 μm and stained with hematoxylin and eosin.

Experimental Protocol Investigating the Short-Term Effects of Kampo Medicines

The experimental protocol for the investigation of the short-term effects of Kampo medicines is shown in Fig. 4. Female ovariectomized ICR mice were prepared as described for the short-term experiments invetigating the effects of the isoflavones. Ovariectomized mice were divided into five experimental groups (five mice in each group). Group 1 was given the diet containing E_2 (5 p.p.m.) and *Juzen-taiho-to* (0.2%). The 0.2% dose of *Juzen-taiho-to* in the diet is consistent with the clinical dose of

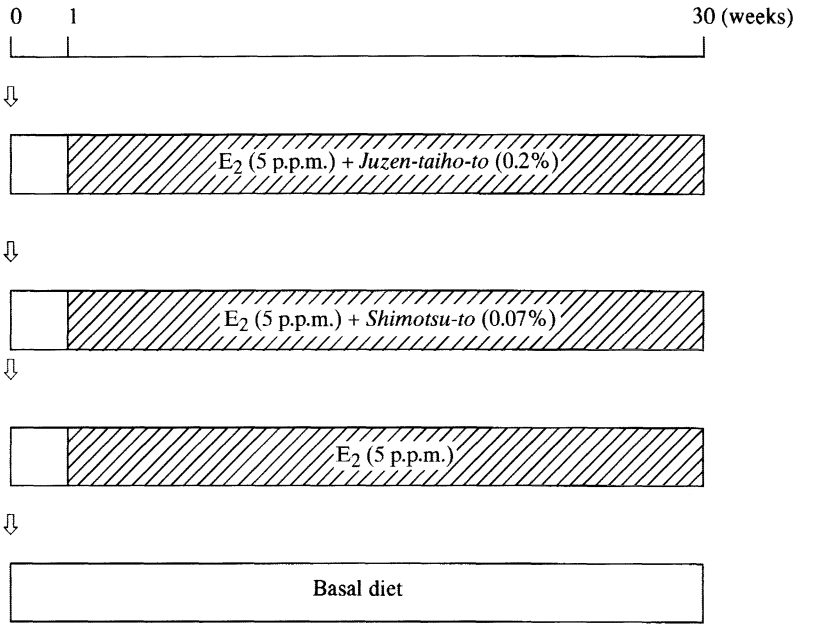


FIG. 5. Long-term experimental design for the investigation of *Juzen-taiho-to* and *Shimotsu-to*. ⇩, *N*-methyl-*N*-nitrosourea (MNU) solution (total volume 0.1 mL), at a dose of 1 mg/100 g body weight, was injected into the left uterine tube and normal saline was injected into the right uterine tube; E_2, 17β-estradiol

Juzen-taiho-to (7.5 g/50 kg, daily) used in humans. Group 2 was fed the diet containing E_2 (5 p.p.m.) and 0.07% *Shimotsu-to*; group 3 was fed with diet containing E_2 (5 p.p.m.) and 0.08% *Shikunshi-to*; group 4 was fed the diet containing E_2 alone; and group 5 was a nontreatment control. Two weeks later, resected uteri were cut in half longitudinally. One half was frozen quickly in liquid nitrogen for experiments as detailed for the invetigation of the effects of isoflavones and the other half was subjected to pathological examination.

Experimental Protocol Investigating the Long-Term Effects of Juzen-taiho-to *and* Shimotsu-to

The experimental protocol used to investigate the long-term effects of *Juzen-taiho-to* and *Shimotsu-to* is shown in Fig. 5. A total of 98 female ICR mice, 12 weeks of age, underwent laparotomy under general anesthesia with diethylether. The MNU solution was injected as described in the isoflavone experiments. One week after the injection of MNU, animals were divided into the following four experimental groups. Group 1 (18 mice) was fed a diet containing 0.2% *Juzen-taiho-to* and 5 p.p.m. E_2; group 2 (20 mice) was fed a diet containing 0.07% *Shimotsu-to* and 5 p.p.m. E_2; group 3 (30 mice) was fed a diet containing 5 p.p.m. E_2; and group 4 (15 mice) was fed the basal diet alone. Thirty weeks after MNU injection, all animals were killed and autopsied.

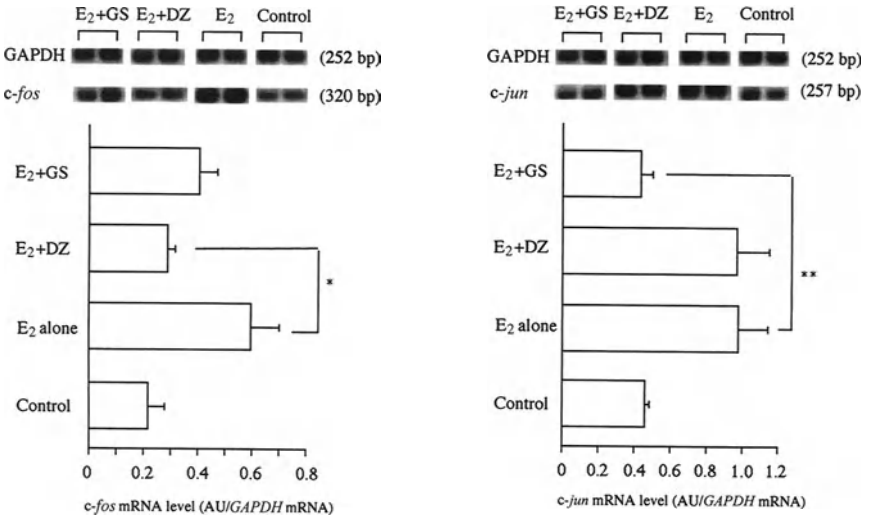


FIG. 6. Expression of c-*fos* and c-*jun* mRNAs in the uterus of ovariectomized mice, treated continuously for 2 weeks with 17β-estradiol (E_2) or E_2 plus genistein (*GS*) or daidzein (*DZ*) in the short-term experiments. $^*P < 0.01$, $^{**}P < 0.005$. *AU*, arbitrary units. (Adapted from [22])

Pathological examinations were performed as described for the isoflavone experiments.

Histology of Uterine Lesions

According to World Health Organization criteria [39], uterine endometrial lesions in the present study were divided into four categories: (1) endometrial hyperplasia, simple; (2) endometrial hyperplasia, complex; (3) atypical endometrial hyperplasia; and (4) adenocarcinoma.

Statistical Analysis

Statistical analysis was performed using the k^2-test or Student's *t*-test.

Results

The expression levels of either c-*fos* and c-*jun* or IL-1α and TNF-α mRNA determined in the short-term assay for isoflavones are shown in Figs 6,7, respectively. Genistein significantly decreased the E_2-induced expression of c-*jun* ($P < 0.005$) and cytokines IL-1α ($P < 0.05$) and TNF-α ($P < 0.05$) mRNA, whereas daidzein inhibited the E_2-induced expression of c-*fos* ($P < 0.01$) and IL-1α ($P < 0.01$) mRNA. Genistein and daidzein tended to decrease estrogen-induced c-*fos*, c-*jun* and TNF-α mRNA levels.

The incidence of neoplastic and preneopastic endometrial lesions in mice treated with MNU and isoflavones in the long-term experiments is summarized in Table 2.

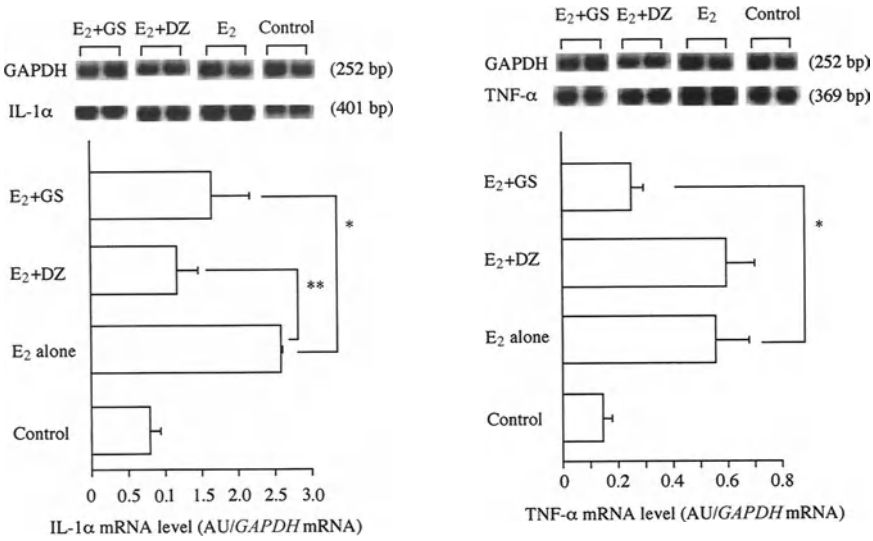


FIG. 7. Expression of interleukin (*IL*)-1α and tumor necrosis factor (*TNF*)-α mRNA in the uterus of ovariectomized mice, treated continuously for 2 weeks with 17β-estradiol (E_2) or E_2 plus genistein (*GS*) or daidzein (*DZ*) in the short-term experiment. $^*P < 0.01$, $^{**}P < 0.05$. *AU*, arbitrary units. (Adapted from [22])

TABLE 2. Incidence of neoplastic and preneoplastic endometrial leions on the treated (left) side of the uterus of mice treated with isoflavones

Group	Treatment	No. mice	ADC	AtH	EH, complex	EH, simple
1	MNU/NS + E_2 + genistein	19	0 (0%)*	5 (26%)**	18 (95%)	16 (84%)
2	MNU/NS + E_2 + daidzein	23	1 (4%)*	4 (17%)**	22 (96%)	16 (70%)
3	MNU/NS + E_2	24	8 (33%)	16 (67%)	23 (96%)	22 (92%)
4	MNU/NS alone	26	3 (12%)	8 (31%)	19 (73%)	6 (23%)

Data show the number of lesions, with percentages given in parentheses
$^*P < 0.01$, $^{**}P < 0.05$ compared with group 3
ADC, adenocarcinoma; *AtH*, atypical endometrial hyperplasia; *EH*, complex and *EH*, simple, endometrial hyperplasia, complex and simple, respectively; *MNU*, *N*-methyl-*N*-nitrosourea; *NS*, normal saline; E_2, 17β-estradiol. (Adapted from [16])

The incidence of adenocarcinoma and atypical hyperplasia was significantly lower on the MNU-treated side of the uterine corpus in groups 1 and 2 (treated with E_2 plus genistein or daidzein) compared with group 3 ($P < 0.01$ and $P < 0.05$, respectively). Meanwhile, the incidence of endometrial hyperplasia, complex or simple, in groups 1, 2, and 3 showed almost no statistical difference.

The expression levels of c-*fos* and c-*jun* or IL-1α and TNF-α mRNA in the short-term experiments investigating Kampo medicines are shown in Figs 8,9, respectively. *Juzen-taiho-to* and *Shimotsu-to* treatment significantly decreased the c-*fos* and IL-1α

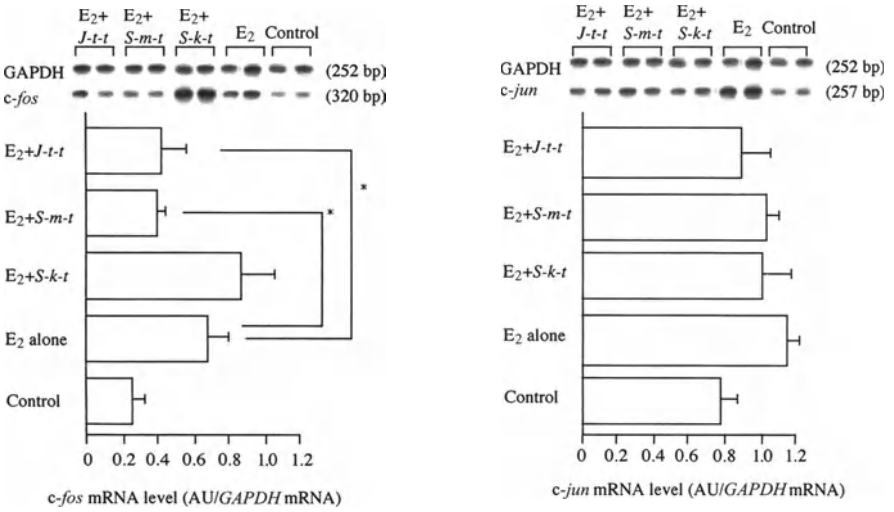


FIG. 8. Expression of c-*fos* and c-*jun* mRNA in the uterus of ovariectomized mice, treated continuously for 2 weeks with 17β-estradiol (E_2) or E_2 plus *Juzen-taiho-to* (*J-t-t*), *Shimotsu-to* (*S-m-t*), or *Shikunshi-to* (*S-k-t*) in the short-term experiment. $^*P < 0.01$. *AU*, arbitrary units. (Adapted from [22])

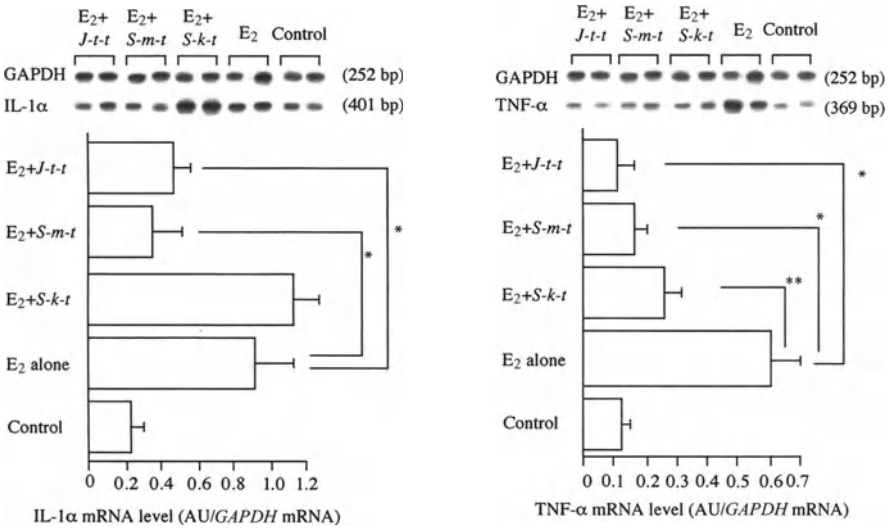


FIG. 9. Expression of interleukin (*IL*)-1α and tumor necrosis factor-α (*TNF*)-α mRNA in the uterus of ovariectomized mice, treated continuously for 2 weeks with 17β-estradiol (E_2) or E_2 plus *Juzen-taiho-to* (*J-t-t*), *Shimotsu-to* (*S-m-t*), or *Shikunshi-to* (*S-k-t*) in the short-term experiment. $^*P < 0.01$, $^{**}P < 0.05$. *AU*, arbitrary units. (Adapted from [22])

TABLE 3. Incidence of neoplastic and preneoplastic endometrial leions on the treated (left) side of the uterus of mice treated with Kampo medicines

Group	Treatment	No. of mice	ADC	AtH	EH, complex	EH, simple
1	MNU/NS + E_2 + *Juzen-taiho-to*	14	1 (7%)*	2 (14%)**	9 (64%)*	7 (50%)**
2	MNU/NS + E_2 + *Shimotsu-to*	19	1 (5%)*	6 (32%)*	12 (63%)*	17 (89%)
3	MNU/NS + E_2	24	8 (33%)	16 (67%)	23 (96%)	22 (92%)
4	MNU/NS alone	26	3 (12%)	8 (31%)	19 (73%)	6 (23%)

Data are the number of lesions, with percentages given in parentheses
*$P < 0.05$, **$P < 0.01$ compared with group 3
ADC,adenocarcinoma; *AtH*, atypical endometrial hyperplasia; *EH*, complex and *EH*, simple, endometrial hyperplasia, complex and simple, respectively; *MNU*, *N*-methyl-*N*-nitrosourea; *NS*, normal saline; E_2, 17β-estradiol

levels induced by the E_2 diet ($P < 0.01$). TNF-α mRNA expression levels induced by the E_2-diet were significantly decreased following treatment with *Juzen-taiho-to* ($P < 0.01$), *Shimotsu-to* ($P < 0.01$), and *Shikunshi-to* ($P < 0.05$).

The incidence of neoplastic and preneoplastic endometrial lesions of the left uterine corpora in the long-term experiments investigating Kampo medicines is summarized in Table 3. The incidence of adenocarcinoma was decreased significantly by *Juzen-taiho-to* ($P < 0.05$) or *Shimotsu-to* treatment ($P < 0.05$). The incidence of atypical hyperplasia was also decreased siginificantly by *Juzen-taiho-to* ($P < 0.01$) or *Shimotsu-to* treatment ($P < 0.05$). The incidence of endometrial hyperplasia, complex was decreased significantly by *Juzen-taiho-to* ($P < 0.05$) or *Shimotsu-to* treatment ($P < 0.05$). The incidence of endometrial hyperplasia, simple was significantly decreased by *Juzen-taiho-to* treatment ($P < 0.05$).

Discussion

Among the phytoestrogens, genistein and daidzein are known to have chemopreventive potential in the carcinogenesis of the mammary gland and prostate [13–15]; however, the effects of isoflavones on endometrial carcinogenesis have not been clearly established.

In the present study, genistein and daidzein suppressed the estrogen-induced expression of the cytokines IL-1α and TNF-α, as well as c-*fos* and c-*jun* in the uterine corpora of ovariectomized mice, consistent with their other antiestrogenic effects [16,25,27]. TNF-α and IL-1α act as growth factors in skin or colon carcinogenesis [30,31] and TNF-α stimulates tumor promotion and the progression of initiated or preneoplastic cells [40–43]. These observations suggest that inhibition of TNF-α expression may be a target for cancer chemoprevention [43].

As a complementary and alternative medicine, Kampo medicine, a herbal complex medicine, has been shown to be effective in the clinical field. Kampo medicine consists of complex herbal ingredients, demonstrating combined additive and adjunctive effects. Kampo medicine has been used as adjunct therapy for cancer [44].

In a previous experiment, *Juzen-taiho-to* exerted a suppressive effect on E_2-related endometrial carcinogenesis in mice, possibly through the suppression of estrogen-induced c-*fos*/*jun* expression [21]. In the short- and long-term experiments in the present study, *Shimotsu-to* exerted a similar inhibitory effect as *Juzen-taiho-to*. However, a similar effect was not found following *Shikunshi-to* treatment. Therefore, *Shimotsu-to* appears to be the component of *Juzen-taiho-to* that is active against endometrial carcinognesis.

Juzen-taiho-to possesses antimetastatic effects in animal models [17]. A possible mechanism for this effect is activation of macrophage and/or T cells in the host immune system [45], yet direct effects on cancer cells are not clearly understood.

The present study demonstrated an inhibitory effect of genistein and daidzein, as well as *Juzen-taiho-to* and *Shimotsu-to*, on endometrial carcinogenesis that was related to the suppression of IL-1α and TNF-α, as well as c-*fos* and c-*jun*, expression. Genistein and daidzein have phenolic rings and may exert their anticarcinogenic effect through estrogen receptor mechanisms related to their antiestrogenic action.

In conclusion, *Juzen-taiho-to* and *Shimotsu-to* exert antitumorigenic effects, possibly through the suppression of estrogen-related downstream events. In addition, the isoflavones genistein and daidzein, as well as *Juzen-taiho-to*, may be effective in the prevention of human endometrial cancers.

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Part 7
Hormones and Hormone Receptors

Role of Glycoprotein Hormones in Endometrial Cancer

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Summary. In vitro investigations into the reported anticancer activity of gonadotropin-releasing hormone (GnRH) in endometrial cancer have provided new information on the role of the glycoprotein hormones in this disease. GnRH analogs had no discernable effects on cell growth or on GnRH receptor activation in HEC-1A and Ishikawa cells. Rather, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), glycoprotein hormones whose levels are greatly suppressed by GnRH analogs in vivo, significantly enhanced cell numbers by up to 70% in 6 days of culture. Despite these results, the cells do not express appreciable levels of respective receptor mRNA; consequently, it seems likely that the hormones may be acting on another target. Comparisons are drawn between the actions of LH and FSH in these cell lines and the similar activity of a related glycoprotein hormone, namely human chorionic gonadotropin free β-subunit (hCGβ), in bladder carcinoma cells. This molecule is thought to be able to block transforming growth factor (TGF)-β-mediated apoptosis by acting as an antagonist at TGF-β receptors. Further evidence for this hypothesis is provided by the discovery that not only LH and FSH, but also hCGβ can increase HEC-1A and Ishikawa cell numbers in vitro. The results are discussed in the light of the knowledge that TGF-β is structurally related to the glycoprotein hormones in that they are all members of the cystine knot growth factor superfamily, thus providing a possible mechanism for the observed results.

Key words. Glycoprotein hormones, gonadotropin-releasing hormone, luteinizing hormone, follicle-stimulating hormone, transforming growth factor-β

Introduction

The growth of many cancers has long been known to be hormone dependent; for example, breast and prostate cancer growth can be stimulated by estrogens and testosterone, respectively, and, consequently, mainstay therapies for these diseases involve

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the blocking of drug action at the receptor level or by reducing steroid hormone production. This latter strategy is often used in premenopausal breast cancer and in prostate cancer, whereby gonadotropin-releasing hormone analogs (GnRHa) are able to substantially reduce gonadal sex hormone synthesis by suppressing the activity of the hypothalamic–pituitary–gonadal (HPG) axis; this effect is mediated by gonadotropin-releasing hormone (GnRH) receptor downregulation in pituitary gonadotrophs. In contrast, in the case of endometrial cancer, which is predominantly a postmenopausal disease believed (in type 1 disease) to be induced by estrogens, cancer cell growth is similarly stimulated by estrogens [1], but these are predominantly derived from extragonadal sources [2]. The HPG axis is effectively abolished postmenopausally and, therefore, one would not expect GnRHa to have an anticancer effect in these patients. However, a number of lines of evidence obtained in the recent past have suggested otherwise.

First, a number of groups have reported the presence of specific extrapituitary GnRH binding sites, together with a growth-inhibitory action of the hormone, in a range of cancer tissues, such as breast [3,4], prostate [5], ovary [6,7], and endometrium [8,9] (see also the review by Gründker et al. [10]). It follows that, if these tissues express GnRH receptors, then a possible mechanism exists for physiological control of mitosis by a GnRH autocrine loop (Fig. 1). Support for this theory is provided by reports that GnRH itself is expressed in these tissues (e.g., in the endometrium [11,12]).

Second, use of the GnRHa goserelin in a clinical setting to treat women with recurring endometrial cancer has been shown to have beneficial effects, with a long-term survival of 44%, and 28% of patients showing clinical improvement [13,14].

Expression of GnRH and its Receptor in HEC-1A and Ishikawa Cells

Studies by our group were performed to investigate further the nature of the putative GnRH loop in endometrial cancer [15] using primarily the Ishikawa [16] and HEC-1A [17] cell lines, as used previously by others for this purpose [8].

While GnRH mRNA was detected using reverse transcription-polymerase chain reaction (RT-PCR), the amount of actual peptide recorded in cell culture supernatants was very low or absent (Table 1). Only one of 10 malignant endometrial tissues secreted GnRH and neither Ishikawa nor HEC-1A cell lines secreted quantifiable levels.

GnRH receptor mRNA was not detectable using RT-PCR without subsequent Southern blotting; probed blots revealed that some expression of message was observable in HEC-1A cells, but not Ishikawa cells (Fig. 2). In agonist binding studies using ^{125}I-labelled goserelin, we found that high-affinity binding sites that were readily demonstrable in αT3-1 pituitary cells [18] were not detectable in the endometrial cancer cell lines [15]. Cell signaling studies showed that GnRH could not elicit intracellular inositol phosphate accumulation or cytosolic calcium transients, both of which are characteristic of GnRH receptor signaling [19].

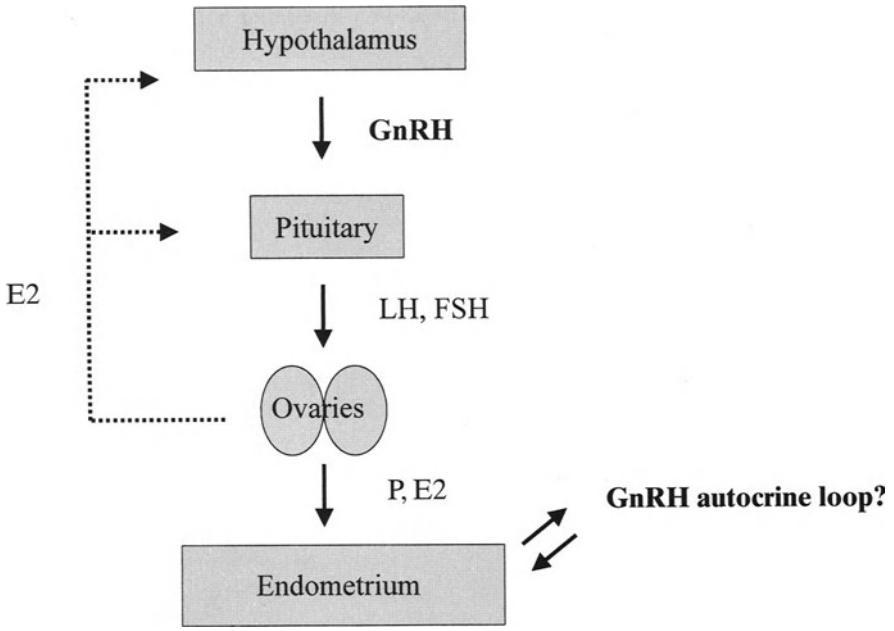

FIG. 1. Gonadotropin-releasing hormone (*GnRH*) and the endometrium. Premenopausally, GnRH is an integral part of the hypothalamic–pituitary–gonadal axis, inducing secretion of luteinizing hormone (*LH*) and follicle-stimulating hormone (*FSH*) from the pituitary gonadotroph cells (circulating levels of GnRH are considered to be negligible). Subsequent release of the sex hormones progesterone (*P*) and estrogens, particularly estrodiol (*E2*), from the ovaries can influence the endometrium (either directly or indirectly) and these can also inhibit release of GnRH by negative feeback. Postmenopausally, the axis ceases to function due to ovarian failure, but a local role for GnRH has been suggested (Emons et al 93) in which the hormone is able to suppress cell growth via an autocrine/paracrine mechanism

Growth Response of HEC-1A and Ishikawa Cells to GnRH Agonists

Measurements of cell growth using a sensitive fluorimetric DNA assay [20] were performed in carefully controlled conditions, which included the use of fetal calf serum that had been charcoal filtered to remove estrogens. Neither goserelin, leuprolide, [δ-Trp^6]GnRH, nor GnRH itself had any detectable inhibitory effect on cell growth over 6 days of treatment (Fig. 3).

Growth Response of HEC-1A and Ishikawa Cells to Gonadotropin Hormones

In the absence of any perceivable effect of the analogs on endometrial cancer cell growth rate, it was hypothesized that GnRH must be exerting the anticancer effect (reported by Jeyarajah et al. [14]) in an indirect manner, rather than via the proposed

TABLE 1. In vitro secretion of gonadotropin-releasing hormone peptide by tissues and cell lines

Tissue	Number tested[a]	[GnRH] (nM)
Primary cultures		
Trophoblast	3/3	6–66
Endometrial epithelia		
Benign premenopausal	1/10	9
Bening postmenopausal	1/4	0.6
Malignant postmenopausal	1/10	0.9
Endometrial stroma:		
Benign premenopausal	0/2	<0.001
Malignant postmenopausal	0/1	<0.001
Cell lines		
Endometrial cancer		
HEC-1A		<0.001
Ishikawa		<0.001
Ishikawa (early passage)		<0.001
COLO 684		<0.001
Breast cancer		
MCF7		<0.001
Choriocarcinoma		
JEG3		0.005
Pituitary		
αT3-1 gonadotrophs		<0.001
GH3 somatotrophs		<0.001

[a] Number of gonadotropin-releasing hormone (GnRH)-secreting primary cultures over total cultures tested

The culture supernatant was collected and stored after 4 days of culture, and the presence of immunoreactive peptide was examined by radioimmunoassay with a detection limit of 0.001 nM

GnRH endometrial autocrine loop. GnRHa are known to have a profound effect on the circulating levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), with concentrations falling to 5%–10% of pretreatment values [2,14]; this led to the hypothesis that endometrial cancers may be sensitive to gonadotropins directly (the source would, most likely, be circulating hormone, but could also conceivably be produced at a local level). The hypothesis was strengthened by the knowledge that, in postmenopausal women, LH and FSH concentrations are several-fold higher than premenopausally as a result of loss of negative feedback control by estrogens; further, one study has suggested that endometrial cancer is associated with increased plasma [LH] in postmenopausal women [21].

The gonadotropins LH and FSH are members of the glycoprotein hormone family, which also includes human chorionic gonadotropin (hCG) and thyroid-stimulating hormone (TSH). These hormones consist of two noncovalently associated subunits, α and α, with the α-subunit being common to all four glycoprotein hormones. It is therefore the β-subunit that confers hormonal specificity. Sequence homology does

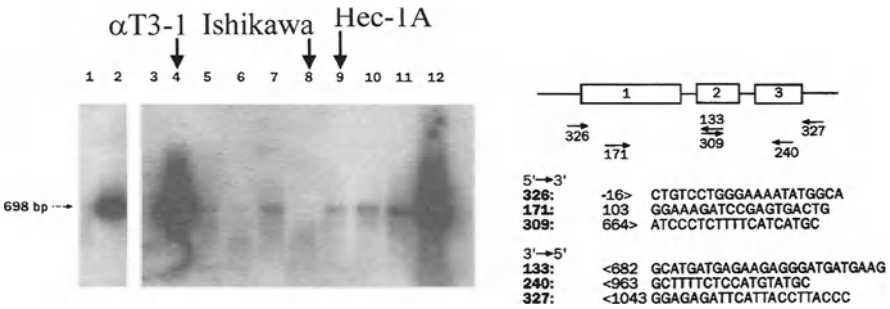

FIG. 2. Polymerase chain reaction (PCR) amplification of gonadotropin-releasing hormone (GnRH) receptor from first-strand cDNA of tissues and cell lines, primed with oligonucleotide primers 326 and 133. The autoradiogram reveals the 698-bp product following hybridization with ^{32}P-labelled oligonucleotide 171. *Lanes 1* and *3* are derived from PCR reaction in the absence of DNA and RNA, respectively, whereas *lane 2* shows amplification from control rat GnRH receptor cDNA. Also presented are the PCR products from αT3-1 mouse pituitary cells (*lane 4*), LNCaP human prostate cancer cells (*lane 5*), JEG3 human choriocarcinoma cells (*lane 6*), human placenta (*lane 7*), Ishikawa cells (*lane 8*), HEC-1A cells (*lane 9*), MCF-7 human breast cancer cells (*lane 10*), human ovarian cancer (*lane 11*), and monkey pituitary cells (*lane 12*). On the right is a schematic diagram of the human GnRH receptor gene and the position of the oligonuleotides used for PCR amplification and Southern blot analysis

exist between all β-subunits, but especially between LHβ and hCGβ (LH 81%, FSH 36%, and TSH 46% compared with hCGβ). Indeed, it is believed the *hCG*β genes arose from the *LH*β gene by duplication, mutation, and amplification [22], and their common ancestry is reflected in the fact that LH and hCG share a common receptor. Whereas LH and FSH are members of the glycoprotein hormone family, the glycoprotein hormones, in turn, are members of the cystine knot growth factor family [23]. Members of this family include hCG, nerve growth factor (NGF), platelet-derived growth factor (PDGF), and transforming growth factor (TGF)-β; they all have structural similarity and possess a central cystine knot motif.

Growth experiments using LH and FSH were performed on the endometrial cancer cell lines, using the same methodology as was used for the GnRH growth experiments (see above). On this occasion, the experiments yielded positive results [24,25] (Fig. 4). HEC-1A growth was stimulated by pituitary derived (pd) LH and pdFSH by 75% and 77%, respectively, relative to controls. HEC-1A growth also increased in a concentration-dependent manner when exposed to recombinant human (rh) LH (100–300 U/L) and rhFSH (300–1000 U/L), with maximum increases of 62% and 50%, respectively. In Ishikawa cells, pdLH and rhLH increased growth by 50% and 67%, respectively; however, no growth effect was observed with rhFSH in these cells and pdFSH only induced an 8% increase in cell number.

rhLH and pdLH increased total DNA in Ishikawa and HEC-1A cell cultures by 67% and 75%, respectively, whereas rhFSH and pdFSH increased HEC-1A DNA by 152% and 178%, respectively ($P < 0.05$ compared with controls). In contrast, rhFSH did not induce a response in Ishikawa cells and an increase in DNA of less than 10% was achieved with pdFSH.

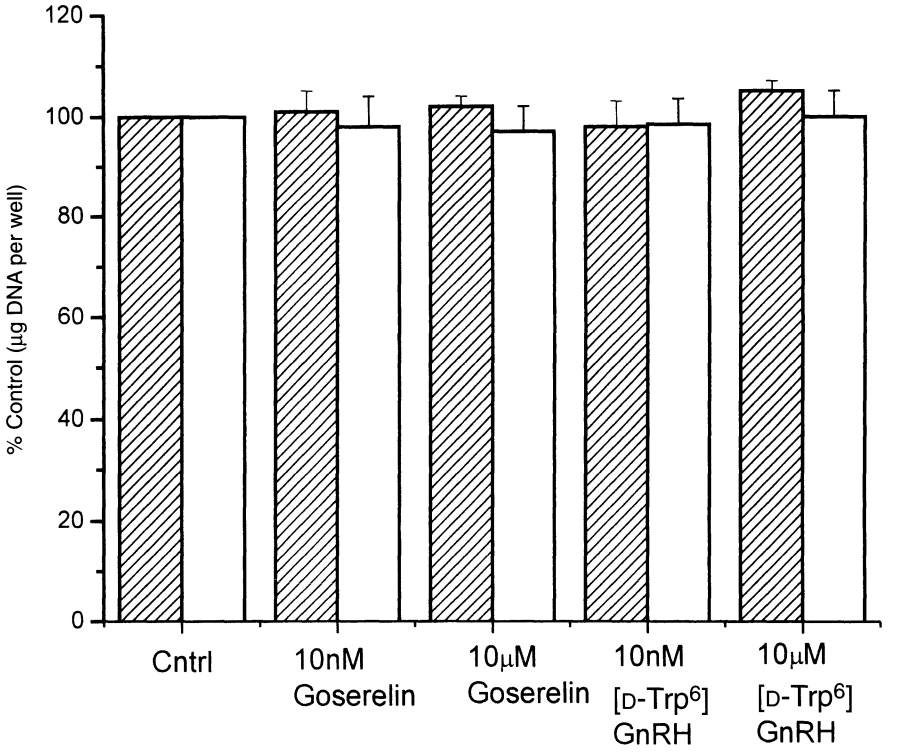

FIG. 3. Effect of the gonadotropin-releasing hormone (*GnRH*) analogs goserelin and [δ-Trp^6]GnRH (10 nM and 10 µM) on the growth of endometrial cancer cell lines HEC-1A (*hatched bars*) and Ishikawa cells (*open bars*) in the presence of phenol red-free medium supplemented with 10% charcoal-treated fetal calf serum. Growth was assessed by measuring the DNA content after 6 days. Results are expressed as a percentage of nontreated controls. Each determination was performed in duplicate, with each column representing the mean ± SEM of three independent experiments using different passages of cell lines

LH and FSH: Receptor and Hormone Expression and Signal Transduction in HEC-1A and Ishikawa Cells

RT-PCR experiments were used to investigate receptor expression. The LH/hCG receptor was expressed to a variable degree by Ishikawa cells, but was not found in HEC-1A cells. The hFSH receptor was not expressed by either cell line.

Neither LH nor FSH were able to mediate an elevation in intracellular cAMP, in contrast with positive control 293 cells transfected with glycoprotein hormone receptor (Fig. 5).

RT-PCR was also used to determine expression of the glycoprotein hormone message. mRNA for the common α-subunit and for the *hCG*β/*LH*β gene cluster were readily detectable (Fig. 6), whereas, in contrast, no FSHβ or TSHβ message was seen. Immunoassay of the homones was also performed (Table 2) and revealed that,

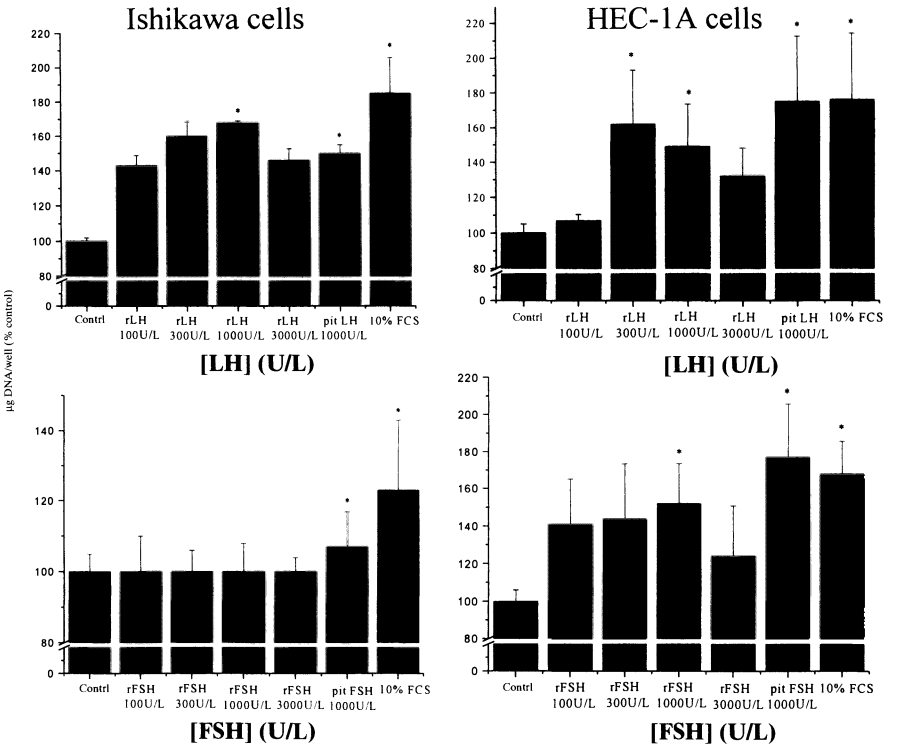

FIG. 4. Effect of varying concentrations of recombinant human (*r*) or pituitary derived (*pit*) luteinizing hormone (*LH*) or follicle-stimulating hormone (*FSH*) on the growth of Ishikawa and HEC-1A cells in phenol red-free media over 6 days of treatment. All results are expressed as a percentage of nontreated controls. Each determination was performed in fifteen replicates (data are the mean ± SEM; $^*P < 0.05$, Student's *t*-test)

although the cells may be transcribing subunit genes, hCGβ, LH, and FSH were not detectable in cell culture supernatants. Assay for the α-subunit showed that HEC-1A cells release extremely high levels, whereas any Ishikawa cell production of the subunit was below detection limits.

Glycoprotein Hormones as Putative Antiapoptotic Mediators

Recent work in this laboratory [26,27] has shown that the hCG β-subunit, which commonly secreted by (among numerous other cancers) bladder cancers, can increase bladder cancer cell numbers in vitro. Bladder cancers associated with high levels of hCGβ production have a relatively poor prognosis and this discovery provides a mechanism to explain the phenomenon in terms of autocrine/paracrine activity in

TABLE 2. Glycoprotein hormone expression in endometrial cancer cell lines

Cell line	hCGβ (pmol/mL)	hCGα (pmol/mL)	LH (pmol/mL)	FSH (pmol/mL)	TSH (mU/mL)
Endometrial carcinoma					
HEC-1A	<10.0	5744.9	<1.0	<0.8	<0.3
Ishikawa	10.5	<10.0	<1.0	<0.8	<0.3
Choriocarcinoma					
BeWo	716.5	176.6	<1.0	<0.8	<0.3
JAr	102.6	<10.0	<1.0	<0.8	<0.3
Bladder carcinoma					
T24	<10.0	<10.0	<1.0	<0.8	<0.3
RT112	50.3	<10.0	<1.0	<0.8	<0.3
5637	18.2	<10.0	<1.0	<0.8	<0.3
SCaBER	271.3	<10.0	<1.0	<0.8	<0.3
J82	93.7	<10.0	<1.0	<0.8	<0.3
Prostate carcinoma					
DU145	<10.0	<10.0	<1.0	<0.8	<0.3
PC3	<10.0	<10.0	<1.0	<0.8	<0.3
LNCaP	<10.0	<10.0	<1.0	<0.8	<0.3

Media were collected at confluence and stored at −20°C until assayed. Thyroid-stimulating hormone (*TSH*), luteinizing hormone (*LH*), and follicle-stimulating hormone (*FSH*) were measured using an immunoradiometric assay; concentrations of free α- and β-subunits of human chorionic gonadotropin (*hCG*α and *hCG*β, respectively) were quantified using immunofluorimetric assays

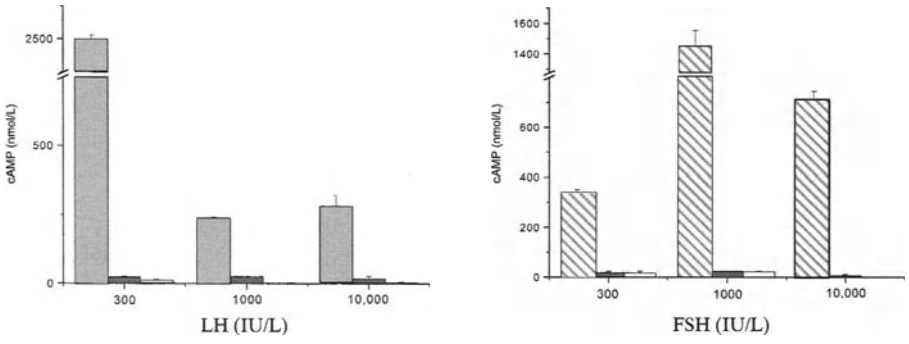

FIG. 5. cAMP production by luteinizing hormone (*LH*) receptor-positive cells 293L (*gray columns*), follicle-stimulating hormone (*FSH*) receptor-positive 293 cells (*hatched columns*), HEC-1A cells (*black columns*), and Ishikawa cells (*open columns*) in response to LH (*left*) or FSH (*right*). Data are the mean ± SEM

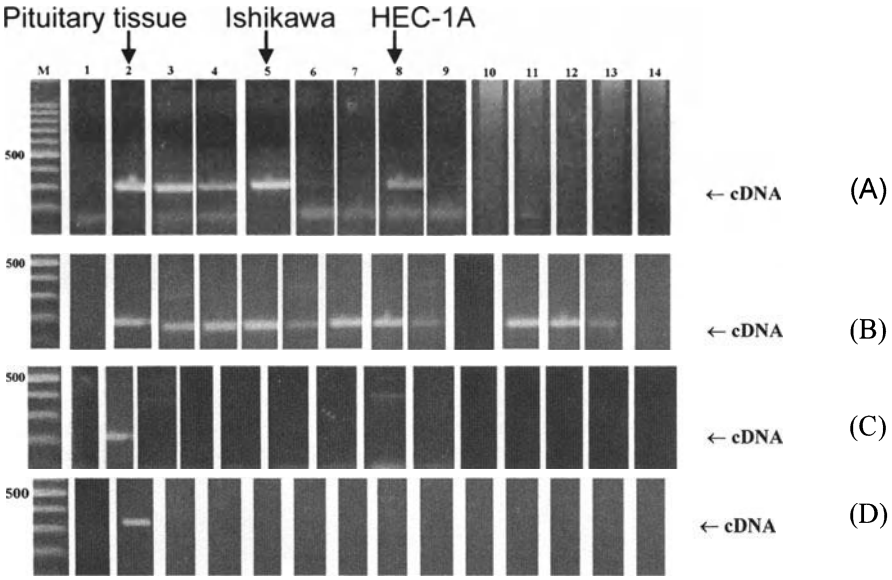

FIG. 6. Reverse transcription–polymerase chain reaction (RT-PCR) of different cell lines for glycoprotein hormone gene expression. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining under ultraviolet light. **A** Common α-subunit of the glycoprotein hormones; **B** β-Chorionic gonadotropin/β-luteinizing hormone (βCG/βLH) gene cluster; **C** βFSH gene; **D** β-Thyroid stimulating hormone gene. Relevant tissues: *lane 1*, no DNA (control); *lane 2*, pituitary tissue; *lane 5*, Ishikawa cells; *lane 8*, HEC-1A cells

bladder epithelium. However, the subunit does not act as a true growth factor and seems to have no effect on mitosis in these cells; rather, it exerts its effect on cell number by suppressing apoptosis. In addition, hCGβ is able to block the proapoptotic action of TGF-β1, a member of the cystine knot growth factor family and well-documented inducer of apoptosis in epithelia [28,29]; indeed, this may be the primary action of hCGβ in this regard. Thus, in bladder cancer, it appears that there is antagonism between two cystine knot growth factor family members, hCGβ and TGF-β1, which presumably results from the structural similarity of the two molecules. The recently described homodimeric form of hCGβ [30,31] would be a good candidate for the receptor-blocking activity, because it is known that dimeric species are necessary to activate the TGF-β1 receptor.

With regard to our endometrial cancer data (described above), there is a tantalizing similarity to the bladder cancer observations. We have demonstrated a sizeable positive effect of LH and FSH on HEC-1A and Ishikawa cell growth; are these molecules doing what hCGβ appears to do in bladder cancer, blocking local TGF-β1 activity?

In light of this and of the results obtained in HEC-1A and Ishikawa cells with the hCGβ-related hormones LH and FSH (see above), experiments were performed to examine the effect of hCGβ _on endometrial cancer cell growth. In both cell lines, significant increases in cell number were observed (Fig. 7); moreover, the maximum

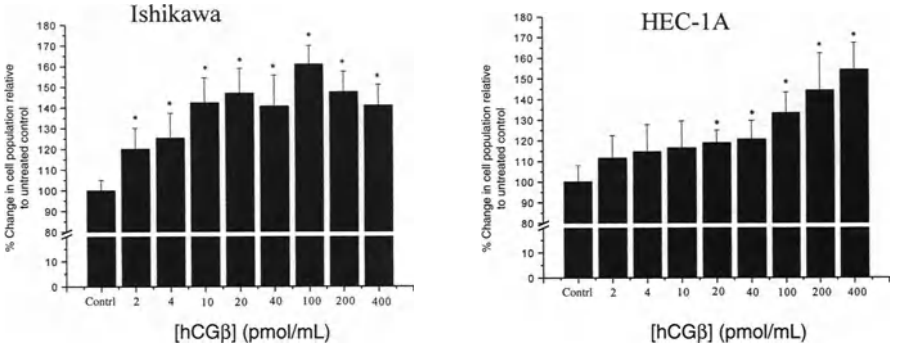

FIG. 7. Growth response of Ishikawa and HEC-1A cells following incubation with recombinant human chorionic gonadotropin free_ β-subunit (*hCG*β). Results are expressed as a percentage of nontreated controls. Data are the mean ± SEM; $^*P < 0.05$, Student's *t*-test

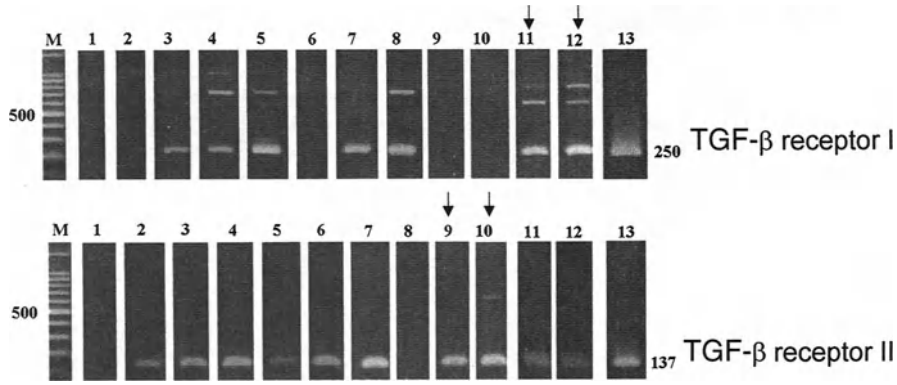

FIG. 8. Reverse transcription–polymerase chain reaction (RT-PCR) analysis of the gene expression of transforming growth factor (TGF)-β receptor types I and II. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining under ultraviolet light. Arrows indicate endometrial cancer cell lines. TGF-β receptor type I: *lane 1*, no DNA (control); *lane 11*, HEC-1A cells; *lane 12*, Ishikawa cells. TGF-β receptor type II: *lane 1*, no DNA (control); *lane 9*, HEC-1A cells; *lane 10*, Ishikawa cells

response in Ishikawa cells (this cannot be determined from the HEC-1A data) was similar to that previously seen with LH (Fig. 4). To investigate the overall hypothesis further, an analysis of TGF-β receptor gene expression was performed.

RT-PCR of the endometrial cancer cell lines showed unequivocal transcription of the TGF-β receptor I and II genes (Fig. 8). If the cells translate this into glycoprotein, then this would provide further evidence that endometrial cancer cells, like bladder cancer cells, may be able to interfere with TGF-β receptor activity to increase cell number. A recent study [32] demonstrated loss of TGF-β-mediated Smad2 signaling

in endometrial carcinoma tissue; the authors speculate that this may be due a number of factors, such as TGF-β receptor downregulation, receptor gene mutations, or decreased [TGF-β]. Blocking of TGF-β receptor activity by an endogenous antagonist would also explain this effect on downstream signaling.

In conclusion, there is considerable circumstantial evidence indicating that a potential mechanism exists whereby gonadotropin/cystine knot growth factor homologs hCGβ, LH, and FSH can act as TGF-β antagonists at the TGF-β receptor in endometrial cancer. Interplay between cystine knot family members with TGF-β receptor function provides a new insight into the endocrinology of endometrial cancer and a possible future target for therapies.

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Contribution of Estrogen Receptor-α and Progesterone Receptor-B to Oncogenic K-Ras-Mediated NIH3T3 Cell Transformation

Kiyoko Kato and Norio Wake

Summary. We investigated the biological significance of estrogen receptors (ER) in NIH3T3 cell transformation by the [^{12}Val] K-Ras mutant. This mutant enhanced the steady state level of ER. Cells expressing mutant K-Ras (K12V cells) were tumorigenic. To determine the role of ER accumulation in Ras-transformed cells, we developed cells (KwtER cells) that overexpressed both wild-type (wt) K-Ras and ER, and found that these cells were also tumorigenic. In the presence of 10% serum in the medium, the activation of ER appeared only in transformed KwtER and K12V cells. There was a significant reduction in the expression of progesterone receptor (PR)-B in Ras-mediated NIH3T3 cell transformation. Coexpression of the PR with mutant K-Ras led to suppression of tumorigenicity and inhibition of the activation of ER. Functional inactivation of ERα by a dominant negative mutant of ERα (DNER) in the presence of the activated K-Ras 4B mutant arrested the cell cycle at G_0/G_1, subsequently provoking replicative cell senescence, and finally abrogating tumorigenic potential. p53-dependent upregulation of p21 was implicated in the induction of this cell senescence. The oncogenic K-Ras 4B mutant significantly increased MDM2 (mouse double minute 2) proteins coprecipitated with p53 and suppressed p53 transcriptional activity. In turn, DNER blocked these effects of the oncogenic K-Ras 4B mutant. Finally, we demonstrated that c-Jun expression overcame the suppression and resultant enhancement of p21 protein levels in response to DNER. The data imply that the ERα/AP-1 (activator protein-1) pathway activated by the oncogenic K-Ras 4B mutant contributes to NIH3T3 cell transformation by modulating p53 transcriptional activity through MDM2.

Key words. Estrogen receptors, p53, progesterone receptors, Ras, transformation

Introduction

Ras proteins are GTP-binding proteins that control signal transduction pathways that regulate cell growth, differentiation, apoptosis, and senescence. Ras proteins function as biological signal switches that connect the cell surface with the nucleus. Mutations

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in the *K-ras* gene are detected in 20% of human endometrial cancers and it is believed that the signal pathway through the Ras protein is critical for the development of endometrial cancer [1].

Estrogen (E2) is a potent mitogen. The E2 signal is transmitted to the nucleus by binding with the estrogen receptor (ER). The dimerized E2–ER complex binds tightly to estrogen-responsive DNA sequences (ERE), acting as transcription factors that can regulate gene expression, a phenomenon that depends on the presence of E2. E2 has an important role in both the etiology and treatment of hormone-dependent, breast, and endometrial cancers. Cancers that have developed in nonovariectomized women often regress following E2 removal or following treatment with the antiestrogen tamoxifen [2]. Moreover, it has been suggested that a mutation, a deletion, or alternative splicing (resulting in deletions of ER exons 3, 4, 5, or 7) are observed frequently in breast cancer [3]. The event causes deregulation of signals transmitted by E2 or ER and, in turn, is linked to the development of hormone-independent cancer [3]. A recent study demonstrated the functional activation of ER through ER-activation function 1 (AF1) by mitogen-activated protein kinase (MAPK) [4]. Therefore, the E2/ER binding effect is somehow modified by the signal transduction cascade, although it remains unclear whether ER function is affected by deregulated signals from the activated Ras mutant. Therefore, we investigated the contribution of ERα to Ras-mediated cell transformation.

Enhancement of ER Activity in K-Ras–Mediated Transformed Cells

To investigate the modulation of ER activity by Ras, we established NIH3T3 cell lines that expressed wild-type K-ras (Kwt cells), activated [^{12}Val] K-ras (K12V cells) and wild-type human ER (ER cells) by cotransfection. Mock cells were established by cotransfection of an empty pZIP-NeoSV(X)1 retrovirus vector and a pSG5 eukaryotic expression vector. The Kwt and K12V cells carried an empty pSG5 vector and pZIP-NeoSV(X)1 retrovirus vector containing cDNA sequences encoding either wild-type K-Ras 4B or [^{12}Val] K-ras 4B. In contrast, ER cells carried an empty pZIP vector and a pSG5 vector containing cDNA sequences encoding the human ER.

We determined the steady state levels of ER proteins in these cells by Western blots using an ER monoclonal antibody. Low levels of ER were expressed in parent NIH3T3 cells, mock cells, and in Kwt cells. In comparison, an approximate 2.5-fold increase in ER levels was observed in K12V cells. This protein level of the ER was almost equal to that of ER cells expressing exogenous human ER (data not shown). The K12V cells expressing activated K-Ras exhibited an altered cellular morphology and formed colonies in soft agar and palpable tumors in nude mice. Furthermore, in order to evaluate the biological significance of overexpressed ER for Ras transformation, we established a cell line coexpressing both wild-type K-Ras and ER by cotransfecting the vectors containing each cDNA (KwtER cells). Cells expressing wild-type K-Ras or ER individually were not tumorigenic. In contrast, KwtER cells, which expressed both wild-type K-Ras and ER, were tumorigenic, as were K12V cells (Table 1).

In order to investigate changes in transcriptional activity in response to ERα in these reconstituted cells, we transiently transfected them with pCAT-enhancer vector, which contained an SV40 enhancer element and vitellogenin A2 ERE. The transfec-

TABLE 1. Summary of tumorigenicity

Cell line	Morphology	Soft agar colony formation[a]	Tumor growth in nude mice[b]
Mock	N	0.0	0/4
Kwt	N	0.0	0/4
K12V	T	1.0	4/4
ER	N	0.0	0/4
KwtER			
C1	N	0.0	ND
C2	T	0.9	4/4
C3	T	0.6	ND
C4	N	0.0	0/4
C5	T	0.5	4/4
C6	T	0.4	ND
K12VPR1			
C1	T	0.4	4/4
C2	T	0.2	4/4
C3	R	0.1	4/4

[a] Colonies were counted 3 weeks after cell seeding
Each value was normalized to the number of colonies observed in K12V cells. Experiments were repeated three or four times
[b] Data show the number of animals positive for tumor growth/number of animals inoculated. Palpable tumor (<1 cm in diameter) formation required 1 week in K12V cells, 1–2 weeks in KwtER cells and 3–5 weeks in K12VPR cells. By 3 weeks after cell inoculation, K12V cells had produced massive tumors (almost 3 cm in diameter). In contrast, K12VPR cells formed much smaller tumors (almost 1 cm in diameter)
N, normal; *T*, transformation; *R*, revertant; *ND*, not determined

tion efficiency of each cell line was normalized by a β-galactosidase assay. In the presence of 10% calf serum, enhancement of ER activity was observed only in transformed K12V cells and KwtER cells (Fig. 1).

Induction of Cell Senescence by a Dominant Negative Mutant of ERα in the Presence of Activated K-Ras 4B in NIH3T3 Cells

To clarify the biological significance of ER function for Ras transformation, we investigated the effect of activated [^{12}Val] K-Ras 4B on NIH3T3 cell transformation in the absence or presence of a dominant negative ER that inhibited its AF2 function. The dominant negative ER mutant (DNER) is an ER mutant containing a frame shift substitution at 554 codon Ser (S554fs ER), which is generated by random chemical mutagenesis [5]. A previous report demonstrated that S554fs ER suppressed the activity of wild-type ER by 80% when equal amounts of the plasmid encoding the ER mutant and the wild-type ER were used [5].

We established four types of reconstituted NIH3T3 cells that expressed the DNER and/or constitutively activated [^{12}Val] K-Ras 4B proteins: (*1*) mock cell cotransfection of an empty pZIP-Neo(X)1 retrovirus vector and an empty pSG5 eukaryotic expres-

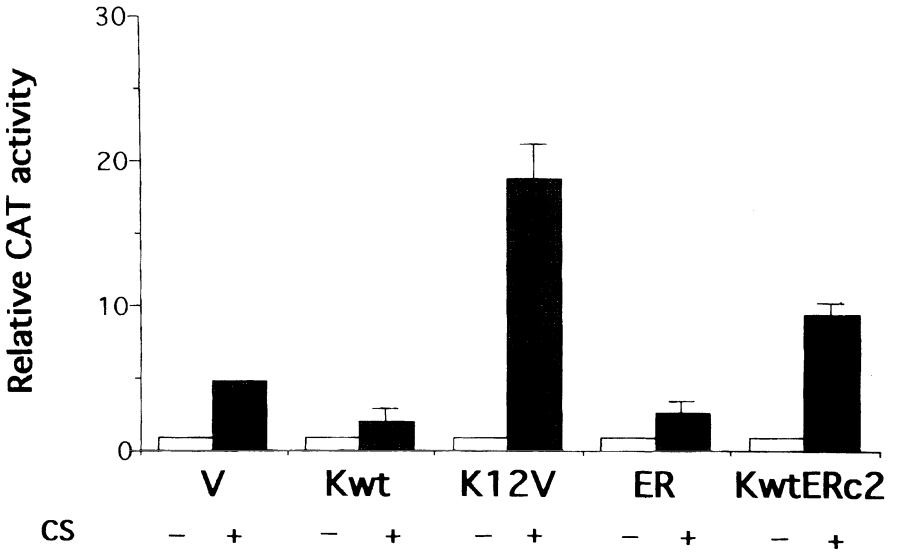

FIG. 1. Activation of estrogen-response element (ERE)-dependent chloramphenicol acyltransferase (*CAT*) activity by normal calf serum. CAT activity was investigated in both the presence (+) and absence (–) of 10% normal calf serum (*CS*). Data show the mean ± SEM of four to six experiments. Transactivation in the presence of calf serum is shown as a fold-induction compared with that in the absence of calf serum. In the presence of calf serum that contained various growth factors and steroid hormones, stimulation of the CAT activity was shown in both transformed K12V and KwtER cells. *V*, mock cell; *ER*, estrogen receptor

sion vector; (*2*) DNER cell cotransfection of an empty pZIP vector and a pSG5 vector containing ER cDNA with a frame shift substitution (S554fs); (*3*) K12V cell cotransfection of a pZIP vector containing [^{12}Val] K-Ras 4B cDNA and an empty pSG5 vector; and (*4*) K12V DNER cell cotransfection of a pZIP containing [^{12}Val] K-Ras 4B and a pSG5 vector containing ER cDNA with an S554fs mutation.

To investigate the changes in transcriptional activity induced by ERα in these reconstituted cells, we transiently transfected them with a pCAT-enhancer vector, which contained an SV40 enhancer element and vitellogenin A2 ERE. The transfection efficiency of each cell line was normalized by a β-galactosidase assay. Under conditions of charcoal treatment and low serum (1%), E2 stimulated pCAT activity eightfold in mock cells and 50-fold in K12V cells. In contrast, DNER expression completely abrogated the activation of ER transcriptional activity in response to E2: 1.2-fold in DNER cells and 1.0-fold in K12V DNER cells (data not shown).

Whereas mock cells displayed spindle-shaped morphology, K12V cells grew as rounded, refractile cells and had the potential to form tumors in nude mice. In response to DNER expression in K12V cells, we observed profound alterations in both cell proliferation and morphology, whereas none of these alterations was detectable by DNER expression in mock cells (Fig. 2a). In order to characterize these effects, cells were seeded at a density of 2.5×10^4 cells/well and cell numbers were obtained as a

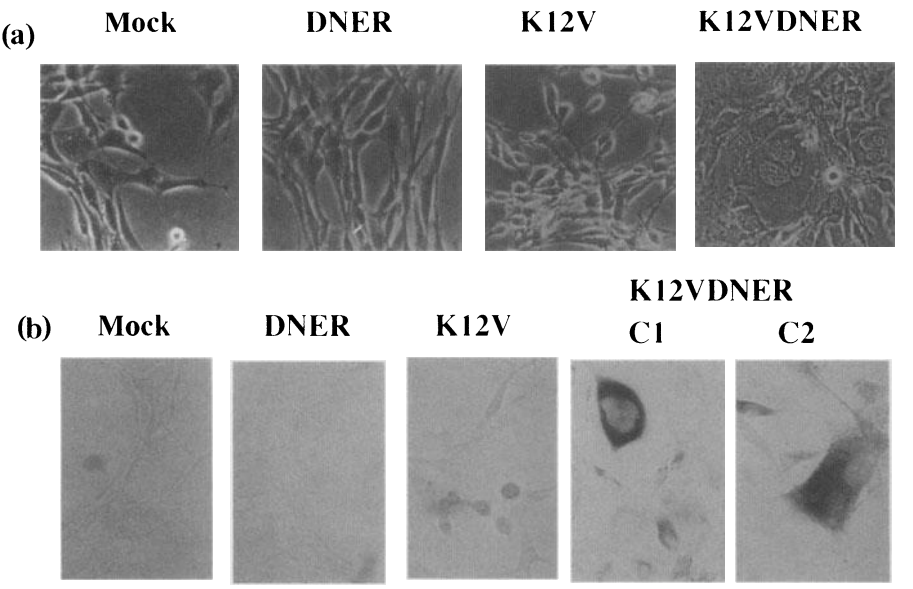

FIG. 2. Induction of senescence by coexpression of [^{12}Val] K-Ras and dominant negative estrogen receptor (*DNER*). **a** The morphology of each reconstituted cell line (original magnification ×200). K12VDNER cells acquired a flat and enlarged morphology. **b** The senescence-associated β-galactosidase (SA-β-gal) activity at pH 6.0 was investigated in each cell line. SA-β-gal activity is shown in K12VDNER cells. Very strong SA-β-gal activities in enlarged K12VDNER cells with multi-nuclei are shown. *C1*, *C2*, clones 1 and 2, respectively

function of time. K12V cell proliferation was markedly inhibited by DNER expression, but there were no alterations in mock cell proliferation observed. In addition, DNER expression inhibited the colony forming ability of K12V cells in soft agar (data not shown). There were also dramatic alterations in cell morphology. Expression of DNER caused cells to exhibit an increased size and flattened morphology, as well as enlarged nuclei in all three individual clones from K12V DNER cells. A significant increase in the proportion of enlarged cells with multinuclei was detectable in these cells (a six fold increase) compared with mock, DNER and K12V cells (Fig. 2a).

Therefore, we analyzed K12V cells for changes in cell cycle progression in the absence or presence of DNER by fluorescence-activated cell sorting (FACS) with propidium iodide staining. Expression of DNER resulted in a marked reduction in the S phase population, which declined from 13.2% to5.0% in K12V cells. Conversely, the percentage of cells in the G_0/G_1 phase population increased from 65% to 81%, indicating a correlation of DNER expression with the arrest of K12V cells in the G_0/G_1 phase (data not shown). Of note, there was no evidence indicating the accumulation of a sub-G_1 population, as seen in apoptosis. Senescent, but not presenescent, quiescent cells express a senescence-associated β-galactosidase (SA-β-gal) activity that can be detected by incubating cells at pH 6.0. Because the coexpression of DNER and [^{12}Val] K-Ras proteins is a striking feature characteristic of senescent cells, we exam-

ined whether these cells expressed this senescence-specific marker (Fig. 2b). SA-β-gal-positive mock, DNER and K12V cells were undetectable. In contrast, almost 30%–50% of K12V DNER cells were SA-β-gal positive. Very strong SA-β-gal activities were shown in enlarged K12V DNER cells with multinuclei. These results indicate that DNER expression is able to induce replicative cell senescence in NIH3T3 cells transformed by activated K-Ras 4B, but not in untransformed mock cells.

Induction of the p21 CDK (cyclin dependent kinase) Inhibitor Through a p53-Dependent Pathway in Senescent K12V DNER Cells

During the replicative senescence of normal diploid human fibroblasts, the expression of both p21 and p16 CDK inhibitors increases dramatically. The increase is sequential; p21 expression increases when the majority of cells have lost their growth potential, whereas p16 expression increases in the terminal stages of senescence when all cells have lost their growth potential [6]. The NIH3T3 cells sustain a homozygous deletion at the INK4a/ARF locus, which encodes p16INK4a and p19ARF with different reading frames. In agreement with the homozygous deletion, the expression of p16 and p19 was not detected in reconstituted NIH3T3 cells, whereas A431 cells and MEF (mouse embryonic fibroblast) expressed detectable levels of these proteins (Fig. 3a). As expected, DNER expression increased p21 expression levels 3.5–4.2-fold in K12V cells, but not in mock cells (Fig. 3a).

It is well known that p21 is under the transcriptional control of p53, whereas p53-independent modulation of p21 has also been established [7]. To determine whether p21 induction in senescent K12V DNER cells was p53 dependent, we investigated alterations in p53 transcriptional activity with a luciferase assay using a luciferase vector containing p53-responsible consensus sequences in the promoter. Expression of activated [^{12}Val] K-Ras 4B in NIH3T3 cells resulted in a significant reduction of p53 transcriptional activity (42% of that in mock cells; Fig. 3b). In turn, DNER expression in the presence of activated [^{12}Val] K-Ras 4B corresponded to the marked enhancement (3.4–3.7-fold) of p53 transcriptional activity in individual K12V DNER clones C1 and C2.

Contribution of ERα to the p53-Independent Upregulation of MDM2

MDM2 acts as a major regulator of p53 by targeting its destruction. It has been shown recently that the *mdm2* gene is upregulated by the Ras-driven Raf/mitogen-activated protein kinase kinase (MEK)/MAPK pathway in a p53-independent manner [8]. The Ras-driven pathway also increases the steady state level of ERα, functioning as a transcription factor in NIH3T3 cells [9]. According to this line of reasoning, ERα is predicted to contribute to the p53-independent upregulation of MDM2 downstream of Ras. We investigated whether the levels of the MDM2 protein involved in the p53 binding complex were altered in response to activated [^{12}Val] K-Ras 4B and/or DNER. First, we performed semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) to evaluate the level of *mdm2* mRNA in each cell line. We obtained the PCR exponential phase and determined the optimal number of PCR cycles (25 cycles).

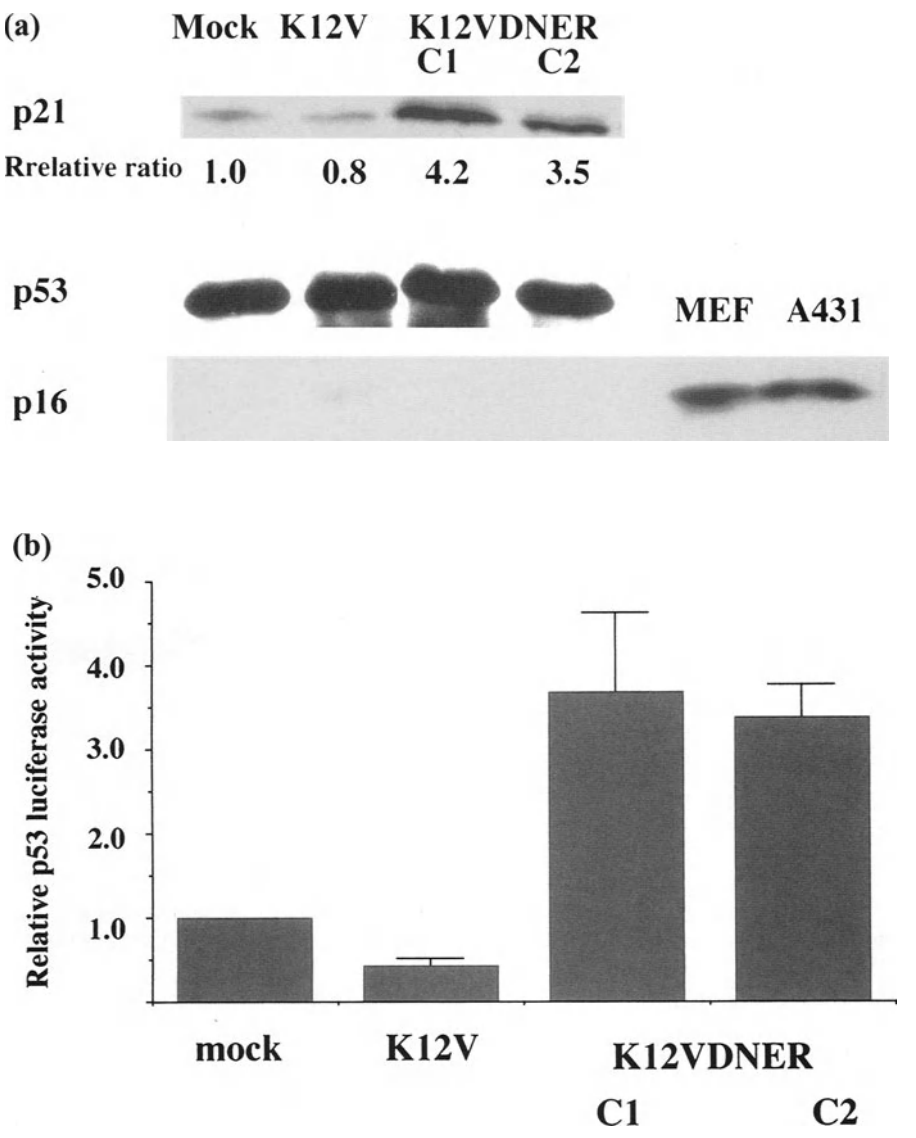

FIG. 3. Cell senescence induction in K12V cells in response to dominant negative estrogen receptor (*DNER*) via p53-dependent p21 upregulation. **a** Western blots were performed using each antibody (p21, p53, and p16). The marked induction of p21 proteins was noticeable in K12VDNER cells. No significant difference in p53 expression was observed in any cell line. Expression of p16 proteins was not detected in the reconstituted NIH3T3 cell lines. MEF cells and A431 cells were used as positive controls for the detection of p16 protein. The relative ratio of the p21 expression level to that in mock cells is shown. *C1*, *C2*, clones 1 and 2, respectively. **b** Transient transfections of each cell line with a p53 luciferase reporter gene were used to monitor p53 activity in the steady state. Seventy-two hours after transfection, cells were lysed for analysis. p53 activity was decreased in K-Ras-transformed cells (42%) and increased in senescent K12VDNER cells (3.7-fold and 3.4-fold in C1 and C2, respectively) compared with mock cells

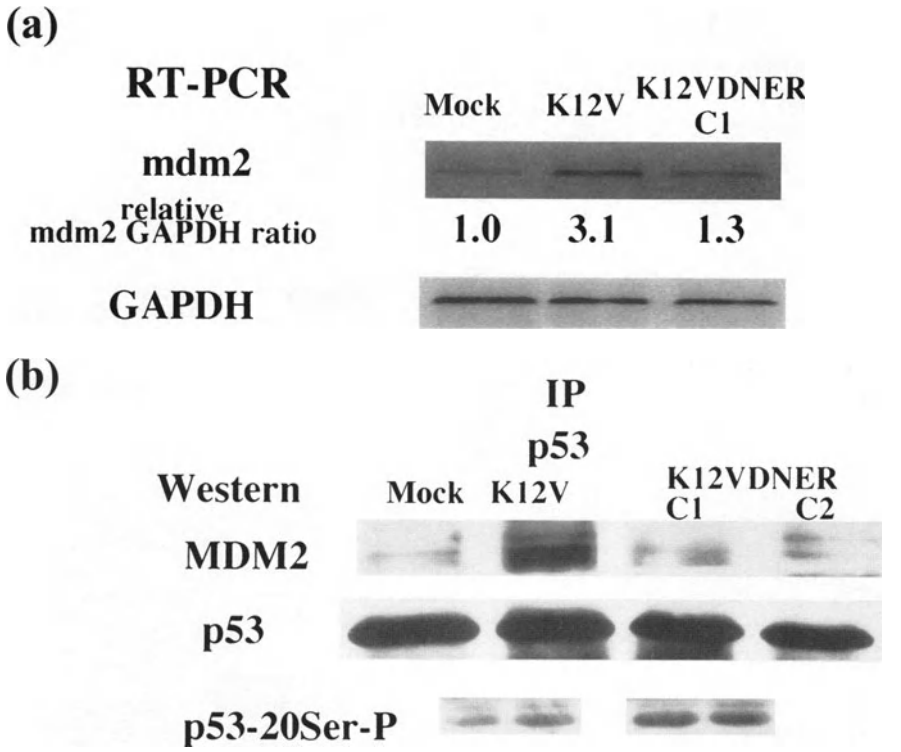

FIG. 4. Implication of estrogen receptor (*ER*) α in p53-independent upregulation of MDM2 (mouse double minute 2). The levels of *mdm2* mRNA were analyzed by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) using a 5′ primer in exon 2 and a 3′ primer in exon 3. The PCR exponential phase was 20–27 cycles and the optimal number of PCR cycles was determined to be 25. The mRNA level of *mdm2* in each cell line was evaluated by RT-PCR for 25 cycles. The mRNA level of *mdm2* in K12V cells (*lane 2*) was enhanced compared with that in mock cells (*lane 1*) and the level in K12VDNER cells (*lane 3*) was almost equal to that in mock cells. The relative ratio of the *mdm2*/GAPDH expression level in each cell line to that in mock cells is shown. The association of MDM2 with p53 was analyzed by immunoprecipitation with an anti-p53 antibody, followed by Western blots with anti-MDM2 antibody. The levels of p53 and phosphorylated p53 proteins were determined by Western blots using anti-p53 and antiphospho-specific p53 antibody, respectively. The association of both proteins was markedly enhanced in K12V cells (approximately six fold) compared with mock and K12VDNER cells. No significant difference in phosphorylation at the 20 codon Ser of p53 was dectable between K12V and K12VDNER cells

As reported previously [8], activated [^{12}Val] K-Ras 4B induced the expression of *mdm2* mRNA in NIH3T3 cells (3.1-fold; Fig. 4a). In turn, DNER expression in K12V cells blocked this stimulatory effect (1.3-fold; Fig. 4a, lane 3). By using immunoprecipitation with an anti-p53 antibody followed by Western blots with an anti-MDM2 antibody, we detected a significant increase in MDM2 protein in p53 precipitates in the presence of activated [^{12}Val] K-Ras 4B, which was blocked by DNER expression in an NIH3T3 cell system (Fig. 4b). The phosphorylation levels of p53 protein at Ser20

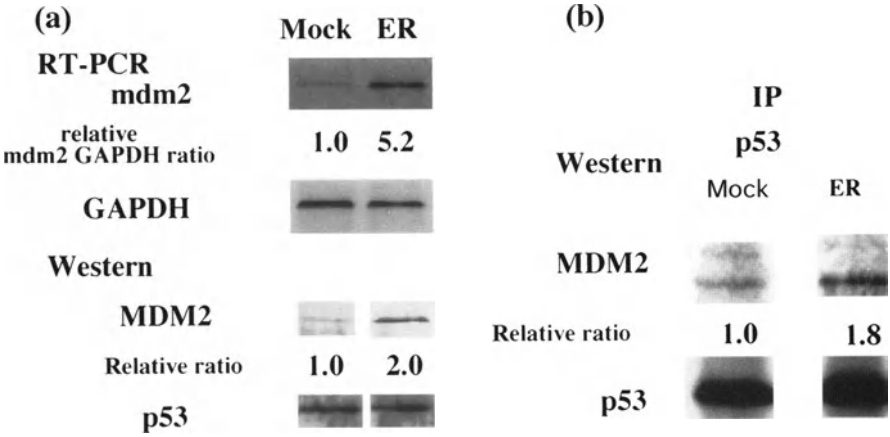

FIG. 5. Enhancement of the levels of MDM2 expression by overexpression of estrogen receptor (ER) α in NIH3T3 cells. **a**, The levels of *mdm2* mRNA and protein were investigated in ER-overexpressing cells (*ER cells*). Reverse transcription–polymerase chain reaction (*RT-PCR*) for 25 cycles and Western blots were performed as described in Fig. 4. The levels of mRNA and protein expression were increased in ER cells compared with mock cells (5.2-fold and 2.0-fold respectively). **b**, The interaction between MDM2 and p53 was also enhanced (1.8-fold). The association between the MDM2 protein and the p53 protein was examined as described in Fig. 4

(Fig. 4c) or Ser15 (data not shown) seemed to be analogous between K12V cells and K12VDNER cells. These results imply that the signal mediated by activated K-Ras 4B accelerated MDM2–p53 interaction through the transcriptional upregulation of the *mdm2* gene, whereas DNER blocked this effect of activated K-Ras 4B in NIH3T3 cells that sustained a homozygous deletion at the INK4a/ARF locus.

To address whether the ERα was a downstream effector of the K-Ras 4B-promoted MDM2–p53 interaction, we established ER cells overexpressing wild-type ERα by transfecting NIH3T3 cells with a pSG5 vector containing wild-type ER cDNA. These ER cells did not exhibit any transformed phenotypes or tumorigenic potential. Both RT-PCR and Western blots showed that ERα overexpression resulted in a increase in the level of *mdm2* mRNA (5.2-fold; Fig. 5a) and protein (2.0-fold; Fig. 5a) in the presence of 10% serum compared with the level observed in mock cells. The enhancement of MDM2–p53 interaction (1.8-fold) was also demonstrated in ER cells cultured with 10% serum compared with mock cells (Fig. 5b). The ERα-mediated enhancement of MDM2 involved in the p53 immunoprecipitate resulted in the suppression of transcriptional activity by p53 (20%) in ER cells (data not shown).

The expression of MDM2 is modulated by the Ras–MAPK pathway through activation of Ets and AP-1 sites in the MDM2 p2 promoter and, then, overexpression of constitutively activated forms of c-Ets1-2 and c-Jun, which are known as downstream targets of Ras, leads to a marked induction of *mdm2* p2 promoter activity [7]. The ERα also enhances the transcription of genes that contain AP-1 sites, the cognate binding sites for the Jun/Fos complex [10]. Previous reports [10] have shown that transactivation functions of the ERα through AP-1 sites required the LBD (ligand

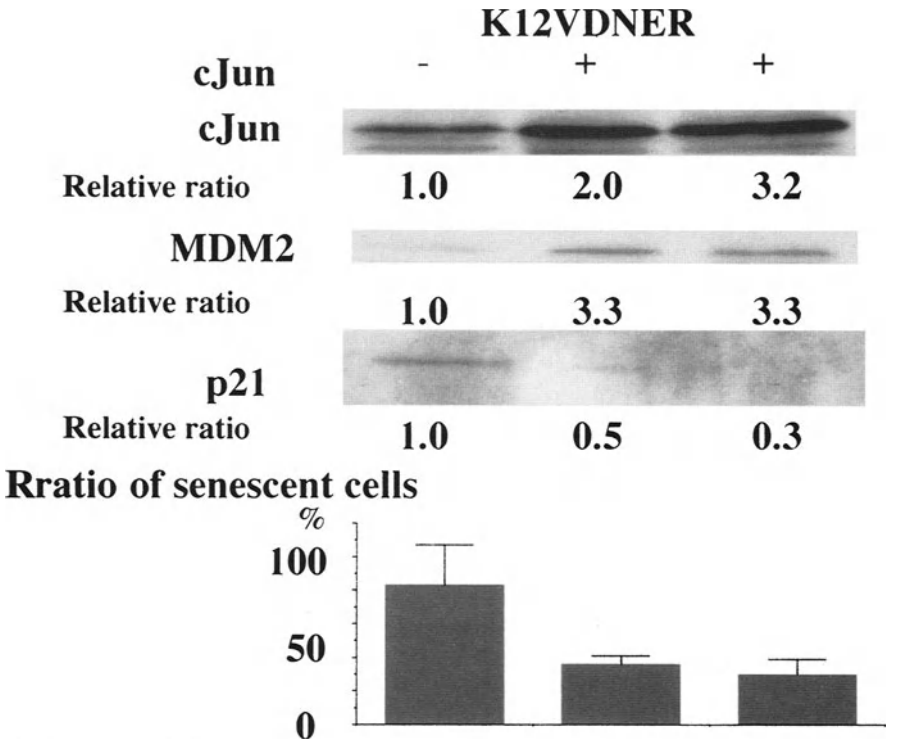

FIG. 6. Escape from cell senescence by overexpression of c-Jun in K12VDNER cells. pcDEBΔc-jun cDNA was transiently transfected into K12VDNER cells. Consistent with the increase in c-Jun levels, MDM2 protein levels were elevated and p21 protein levels were decreased, resulting in a decrease in the percentage of senescent cells. The relative ratio of c-Jun, MDM2, and p21 protein expression levels to that in the absence of c-Jun is shown

binding domain) and AF-2 (activation function-2) of ERα. The AF-2 function was eliminated in DNER (S554fs) that was used in the present study. To investigate further whether the suppression of AP-1 activity by DNER in K12V cells resulted in a decrease in MDM2 levels, we transiently transfected pcDEBΔc-jun cDNA into K12VDNER cells [11]. Transient transfection of pcDEBΔc-jun cDNA into K12V DNER cells resulted in an increase in the c-Jun protein level (2.0–3.2-fold; Fig. 6). Consistent with the increase in c-Jun, MDM2 protein levels were elevated, followed by a subsequent decrease in p21 protein levels despite the presence of DNER. Consequently, c-Jun expression resulted in the escape of NIH3T3 cells from senescence. These results demonstrate that ERα functions downstream of Ras and that the MDM2 modulation by ERα is regulated by AP-1 activity, indicating a signaling route that involves Ras/ERα/AP-1/MDM2/p53 in the regulation of NIH3T3 cell senescence.

Inhibitory Action of Progesterone Receptor-B in NIH3T3 Cell Transformation Mediated by Activated K-Ras Mutant

In Ras-mediated NIH3T3 cell transformation, we also found significantly lower levels of progesterone receptor (PR)-B expression in tumorigenic KwtER and K12V cells compared with nontumorigenic mock, Kwt, and ER cells. The PR-A protein was undetectable in NIH3T3 cells (Fig. 7a).

We established NIH3T3 cell clones that expressed both the activated [^{12}Val] K-Ras mutant and PR-B by cotransfection with the corresponding expression vectors (K12VPR cells). Translation of PR-B was demonstrated in all three clones from K12VPR cells (K12VPR C1, C2 and C3). The steady state level of the PR was high in K12VPR C3, but lower levels were detected in K12VPR C1 and C2 (Fig. 7b). The expression of PR in K12VPR C3 resulted in cells reverting to a morphology of large

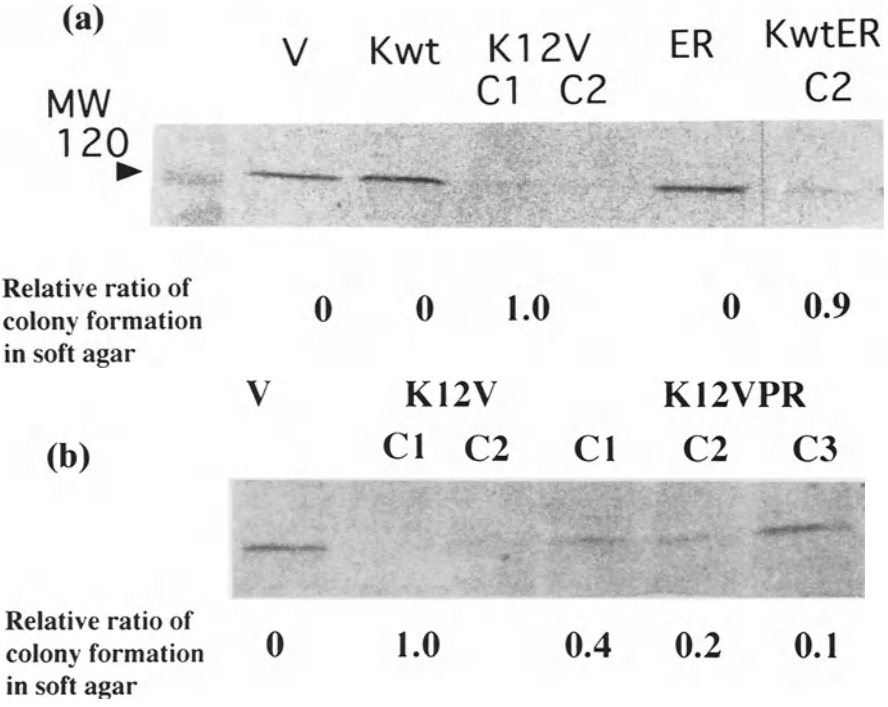

FIG. 7. Alterations in the progesterone (PR)-B expression levels between nontumorigenic and tumorigenic cells. **a** We established NIH3T3 cell lines that expressed empty vector (mock cells; *V*), wild type K-Ras (*Kwt*), activated [^{12}Val] K-Ras (*K12V*), wild-type estrogen receptor-α (*ER*), both wild-type K-Ras and ERα (*KwtER*), and both activated [^{12}Val] K-Ras and PR-B (*K12VPR*). We have reported previously that KwtER cells are tumorigenic, as are K12V cells [9]. A marked reduction of PR-B protein levels was observed in both tumorigenic K12V cells and KwtER cells. **b** Coexpression of PR-B with activated [^{12}Val] K-Ras resulted in the suppression of tumorigenicity. NIH3T3 cells expressed PR-B protein but not PR-A protein. *MW*, molecular weight

and flat cell types, which was similar to that of the parent NIH3T3 and mock V cells (data not shown). This contrasts with the cell morphology of K12VPR C1 and C2, which was similar to that of transformed K12V cells. Thus, changes in cell morphology probably depend on the level of expression of the PR.

Inhibitory actions of the PR were also demonstrated for the colony forming efficiency and tumor-forming ability of K12V cells (Table 1). The rate of soft agar colony formation was suppressed in K12VPR C3 to one-tenth of that in K12V cells. The latency period for palpable tumor formation was markedly prolonged when K12VPR C3 cells were inoculated. In addition, the size of the tumors that developed after 3 weeks of K12VPR C3 cell inoculation was significantly reduced. In contrast, the suppression of tumor size was less marked in K12VPR C1 and C2. These findings are in accord with the assumption that a high level of PR expression has the potential to suppress cell transformation mediated by mutant K-Ras.

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Part 8
Genes and Gene Expression

Regulation of Gene Expression in Endometrial Cancer Cells: Role of Extracellular Matrix in Mitochondrial Gene Expression

ELISABETH STRUNCK, KIRSTEN FRANK, and GÜNTER VOLLMER

Summary. The biological role of mitochondria becomes increasingly important in the understanding of the regulation of physiological and pathophysiological conditions. Nevertheless, comparatively little is known about the possible factors regulating mitochondrial activity and, in particular, mitochondrial gene expression. Recently, we analyzed the molecular basis of the interaction between endometrial adenocarcinoma and reconstituted basement membrane. Applying a differential display reverse transcription–polymerase chain reaction, we identified several gene fragments with apparently differential expression patterns of corresponding mRNA. Here we report on the identification and characterization of a fragment representing the mitochondrial encoded protein NADH-dehydrogenase 6 (ND6), a protein of the electron transport chain. Northern blot hybridization confirmed the downregulation of ND6 in endometrial adenocarcinoma cells in response to a reconstituted basement membrane. We further confirmed that contact with basement membrane components represses the gene transcription of both independently controlled overlapping transcription units of the mitochondrial genome. Treatment of cells with cycloheximide did not block the mitochondrial transcription rate. Nevertheless, nuclear extracts were capable of shifting the promoter elements of mitochondrial low and heavy strand promoters, providing evidence in favor of a nuclear/mitochondrial signaling pathway. Furthermore, our study clearly shows that extracellular factors are involved in the regulation of intracellular signaling, as well as signal transfer between cellular organelles. The understanding of the molecular basis of this mechanism, its regulation, and its metabolic consequences may provide an important insight into the involvement of mitochondrial activity in the process of carcinogenesis.

Key words. Basement membrane, mitochondrium, gene regulation, NADH-dehydrogenase subunit 6, differentiation

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Introduction

Endometrial adenocarcinoma is the most frequently diagnosed malignancy of the female genital tract in Western countries [1,2]. To understand the basic mechanisms involved in endometrial carcinogenesis and metastasis, we investigated the potential involvement of cell/matrix interactions in this processes. We were particularly interested in the detection of matrix-regulated genes. Therefore, we studied the effect of components of a reconstituted basement membrane on gene expression. Applying a differential display of mRNA [3] and other methods to detect differential gene expression, we found that the expression of several, so far unknown, genes was regulated by contact of the endometrial adenocarcinoma cell line HEC 1B(L) with a reconstituted basement membrane [4,5].

Extending our studies, we successfully characterized one of the gene products. We could demonstrate that L-3-phosphoserine phosphates and some members of this gene family are regulated in response to reconstituted basement membrane [6]. In addition, we observed that products generated by differential display and, therefore, potentially representing genes with a differential gene expression shared sequence homologies with mitochondrially-encoded genes. Differential gene expression screens used by others in numerous different experimental setups produced almost always the result that mitochondrial gene expression is very sensitive to changes in the cellular/extracellular milieu. For example, the expression of NADH-dehydrogenase subunits has been associated with the transition from a normal to a diseased or senescent state of a cell [7–9], the effect of stress or ionizing radiation on a cell [10,11], or has been shown to result as a consequence of external stimuli, such as hormones or vitamins [12–15].

Because comparatively little is known about the regulation of mitochondrial gene expression, we decided to characterize mitochondrial gene expression in our experimental model. We studied the time dependency of mitochondrial gene expression in response to contact of endometrial adenocarcinoma cells with reconstituted basement membrane. We further investigated whether both strands of the mitochondrial genome are equally regulated and finally, we examined whether nuclear-derived factors and/or nuclear gene expression are involved in the regulation of mitochondrial genome transcription.

Mitochondrial transcription occurs by transcription of each individual strand. The start sites are two individual promoters without overlap. After production of primary transcripts, enzymatic activities are believed to cleave these long RNA products into tRNAs, rRNAs, and mRNAs (for a review see, Jeong-Yu and Clayton [16]). The regulation of mitochondrial (mt) DNA expression involves interactions of the mtDNA with proteins and factors that are nuclear gene products. For human mitochondrial transcription, the involvement of a major transcriptional accessory protein (TFA) is almost certainly required; however, whether additional factors are involved, as described for the yeast system [17], remains to be elucidated. Further levels of regulation of mitochondrial gene expression are most likely represented by the processing of the primary transcript and by the translation of the mature mitochondrial mRNA into protein. The latter aspect is particularly interesting because translational defects represent a common phenotype in human disease [18].

Methods

Cell Lines

The endometrial adenocarcinoma cell line HEC 1B(L) is a subline of the HEC 1B cell line (American Type Culture Collection, Rockville, MD, USA). The endometrial adenocarcinoma cell line Ishikawa was kindly provided by Dr. M. Nishida (Kasumigaura National Hospital, Ibaraki, Japan).

Cell Culture

Endometrial adenocarcinoma cells were cultured as described previously [4,19,20]. Briefly, cultures were either maintained in the presence of Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing 10% fetal calf serum (FCS), 10% dextran-coated charcoal stripped FCS (DCC-medium) or defined supplements (serum-free, defined medium; SFDM) or on Matrigel (SERVA, Heidelberg, Germany; or Becton-Dickinson, Heidelberg, Germany) in the presence of SFDM.

Isolation of RNA, Differential Display Reverse Transcription–Polymerase Chain Reaction, Northern Blotting, and Polymerase Chain Reaction

Total cellular RNA was isolated by the standard guanidinium isothiocyanate/CsCl gradient centrifugation method. Cells of 50%–80% confluent cultures on plastic were harvested by trypsin–EDTA treatment and resuspended in homogenization buffer. Cells growing on or in Matrigel were lysed directly.

For differential display reverse transcription–polymerase chain reactions (DDrtPCR), total cellular RNA (0.5 μg) prepared from individual experimental conditions was reverse transcribed, using 2.5 μM oligo-dT primers. Polymerase chain reactions (PCR) were performed in 1.5 mM $MgCl_2$, 2 μM of each dNTP and included 1.25 μM of the oligo dT primer AF (5′-TTTTTTTTTTTGG-3′) in combination with 1 μM random decamer primer DD4 (5′-GGGTAACGCC-3′), as well as 1 U Taq-DNA Polymerase (Roche, Mannheim, Germany). The PCR products were labeled with 148 kBq ^{33}P-dATP in each reaction. Samples were subjected to 40 cycles of PCR amplification with a chosen annealing temperature of 42°C. An aliquot of each amplification reaction was separated on a 6% polyacrylamide gel. Differentially expressed gene fragments were cut out, purified, reamplified, and cloned using the TA-cloning kit (InVitrogen, Breda, The Netherlands) and submitted for customized sequencing. For Northern blotting, 10 μg cellular RNA of each experimental condition was subjected to electrophoresis in 0.9% agarose gels containing 2.2 M formaldehyde. RNA was vacuum blotted onto Hybond$^+$ nylon membranes (Amersham-Pharmacia, Braunschweig, Germany) and cross-linked by ultraviolet (UV) irradiation. A [^{32}P]-labeled cDNA probe was generated from cDNA fragments generated by DDrtPCR. Following hybridization, membranes were first washed for 2 min at room temperature using 3× standard saline citrate (SSC) containing 0.1% sodium dodecyl sulfate (SDS) and then washed for 20 min at 55°C using 0.5× SSC containing 0.1% SDS. After autoradiography and stripping, the filter was rehybridized with an 18S rRNA probe

(kindly provided by Dr. N. Schütze, University of Würzburg, Würzburg, Germany), which was used as the loading control.

PCR of Genomic and Mitochondrial DNA

Total cellular DNA was isolated using a commercial kit according to the manufacturer's instructions (High pure PCR template preparation kit; Rodre, Mannheim, Germany). As a representative gene for nuclear genes, we amplified β-actin; as a representative mitochondrial-encoded gene, we used the *16SrRNA* gene. The following primer pairs were used. 16SrRNA forward 5′-TATCTCCAAACCCACTCCACCT-3′, reverse 5′-CTTGGACATACATAGACGGGTG-3′; actin forward 5′-ATCATGTTTGA GACCTTCAA-3′, reverse 5′-CATCTCTTGCTCGAAGTCCA-3′. Amplification was performed using the primers described above and the following PCR protocol. For amplification of actin and 16SrRNA, 25 and 30 cycles were performed, respectively, using the following conditions: 92°C for 1 min, 55°C for 1 min, and 72°C for 30 s.

Gel Mobility Shift Assay

Gel mobility shift assays were performed using standard conditions. Because the high strand promoter (hsp) and the low strand promoter (lsp) are represented by short nucleotide sequences, the shifted promoter elements were created by customized oligonucleotide synthesis (MWG-Biotech, Ebersberg, Germany). The selected sequences were as follows: hsp1 5′-GGCAACCCCATACCCCGAACCAACCAAACCCCAAAGA-CACC-3′; hsp2 5′-CCGGGGTGTCTTTGGGGTTTGGTTGGTTCGGGGTATGGGGTTGC-3′; lsp1 5′-GGCCGCTGTGTTAGTTGGGGGGTGACTGTTAAAAGTGCAC-3′; lsp2 5′-CCGGGTGCACTTTTAACAGTCACCCCCCAACTAACACAGC-3′.

Results and Discussion

Our studies were focused on the regulation of gene expression following contact of the endometrial adenocarcinoma cell lines HEC-1B and Ishikawa with basement membrane, which, as already shown, promotes differentiation of these cells [19,20]. To detect basement membrane-regulated genes, we applied DDrtPCR. Using this method, we detected several cDNAs representing differentially expressed genes [4]. In addition to the genes and gene products described in the previous report [4], we found the cDNA MESR62. According to database searches, this sequence represents the NADH-dehydrogenase subunit 6 (ND6; Fig. 1). This gene is encoded in the mitochondrial genome and was found to be downregulated in response to contact of endometrial adenocarcinoma cells with reconstituted basement membrane (Fig. 1a). This downregulation is a time-dependent process, occurs between 24 and 72 h of growth of endometrial adenocarcinoma cells on Matrigel (reconstituted basement membrane), and does not reflect downregulation of mitochondrial numbers or the amount of mitochondrial DNA. In each individual experiment, we checked for the presence of the *16SrRNA* gene and the β-*actin* gene within a total preparation of cellular DNA using PCR (Fig. 1b). Because ND6 is encoded on the mitochondrial light strand, we investigated whether gene expression on the heavy strand was equally affected. With the aid of a probe specific for the *16SrRNA* gene, a mitochondrial heavy strand encoded gene,

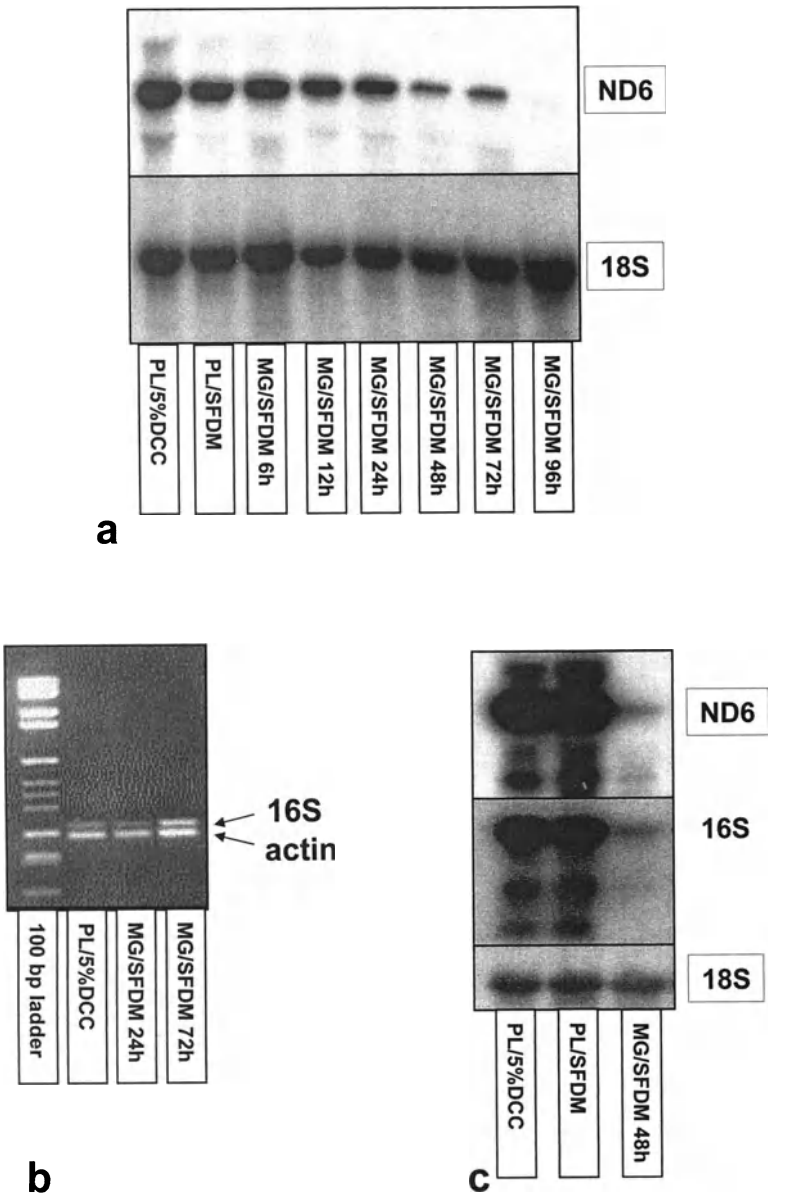


FIG. 1a–c. Regulation of mitochondrial gene expression in response to reconstituted basement membrane. In a time-dependent downregulation of NADH-dehydrogenase subunit 6 (*ND6*) in response to reconstituted basement membrane Matrigel (*MG*) is shown (**a**). Because this gene is encoded on the mitochondrial light strand, regulation of gene expression for genes encoded on the mitochondrial heavy strand, e.g. *16S rRNA* (*16S*) (**b**) was verified by comparing gene expression pattern for genes representative for light strand encoded genes (*ND6*) and heavy strand encoded genes (*16S*) directly (**c**). PL/5%DCC, cell culture plastic and 5% charcoal-stripped fetal calf serum; PL/SFDM, cell culture plastic and serum-free defined medium; MG/SFDM, Matrigel and serum-free defined medium

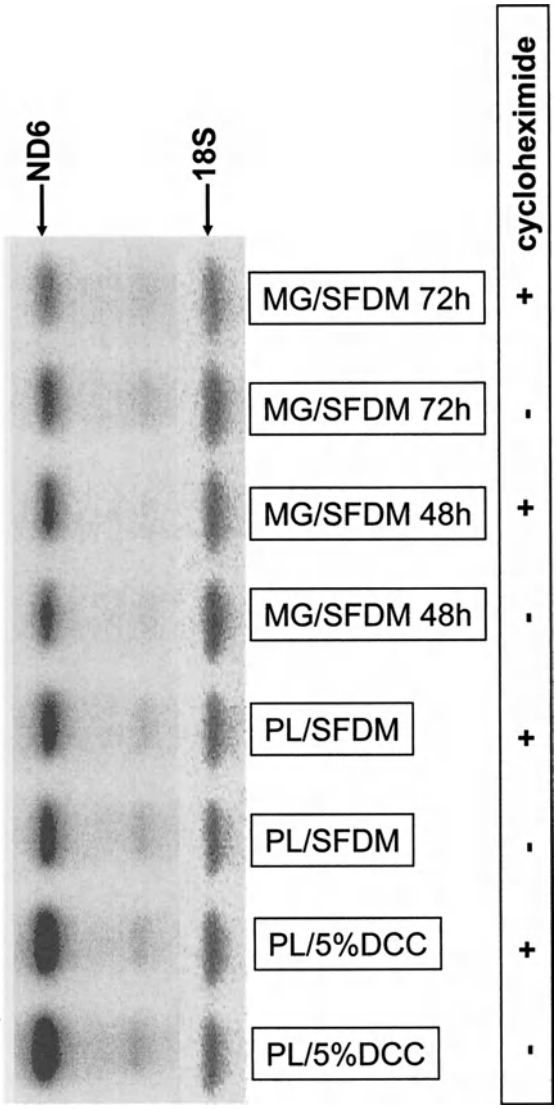


FIG. 2. Cycloheximide treatment of cell. This experiment was performed to determine whether downregulation of *ND6* is dependent on protein synthesis. +/–, with/without cycloheximide treatment

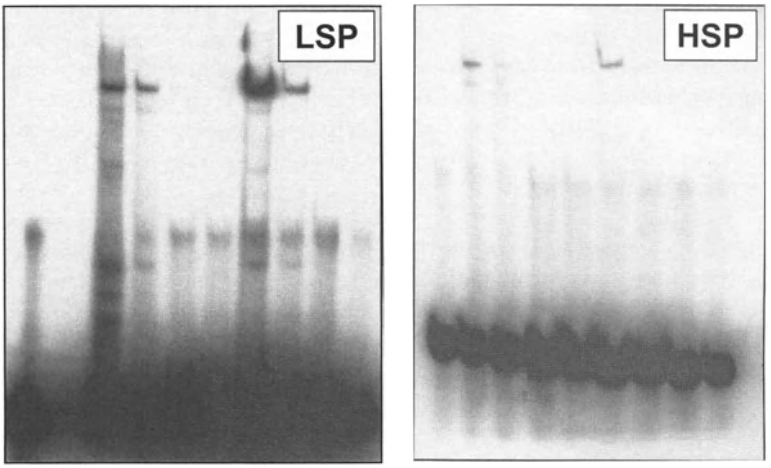
we demonstrated that transcription of heavy strand-originating mRNA is also downregulated in response to prolonged contact (48–72 h) of endometrial adenocarcinoma cells with basement membrane (Fig. 1c). To test whether this downregulation is dependent on protein synthesis, we investigated whether the downregulation could be blocked by cycloheximide. This was clearly not the case, because cycloheximide treatment did not affect gene expression of ND6 (Fig. 2).

Transcription of the mitochondrial genome occurs from two relatively small promoters, the heavy strand promoter (HSP) and the light strand promoter (LSP) of the mitochondrion. To test whether cellular proteins are capable of binding these promoters, we performed gel mobility shift assays with oligonucleotides representing the respective promoter. Following end-labeling with ^{32}P-ATP, promoter DNA was incubated with nuclear and cytosolic extracts and mitochondrial extraction did not yield a sufficient quantity of proteins. A significant alteration in the gel mobility of the oligonucleotides tested was seen following incubation with nuclear extracts (Fig. 3), but not with cytosolic extracts (data not shown). The altered mobility shift was detectable for both LSP and HSP. Competition with an excess of amount of unlabeled DNA probe revealed that the binding of nuclear proteins to the DNA probe was specific (Fig. 3). Interestingly, shifted promoter sequences were only detectable with nuclear extracts from cells grown in conventional cell cultures on plastic in the presence of serum, but not with extracts of cells grown on Matrigel. These findings are indicative of nuclear proteins that are capable of keeping mitochondrial gene expression at a high level. Apparently, mitochondrial gene expression is regulated, at least in part, by nuclear factors.

In the yeast *Saccharomyces cerevisiae*, specific mitochondrial transcription requires only two components: a core mitochondrial RNA polymerase [21] and a single specificity/transcription factor [22]. In the human situation, recombinant mitochondrial transcription factor A protein and partially purified core polymerase were sufficient for the activation of transcription from LSP and HSP in vitro [23]; however, attempts to reconstitute human mtDNA transcription entirely using these recombinant or purified proteins were unsuccessful [24]. This indicates clearly that other factors are missing. Recently, the identification of two ubiquitously expressed mitochondrial transcription factors, namely TFBM1 and TFBM2, has been reported; both transcription factors are capable of activating transcription from human mtDNA [25], but their cellular localization has not been reported as yet. Whether these factors directly regulate mitochondrial gene transcription in our system remains unanswered; however, their ubiquitous expression would fit into our observation that regulation of mitochondrial gene transcription is independent of protein synthesis.

As a working hypothesis, we assume that reconstituted basement membrane induces a "differentiation signal" and consecutively stimulates mitochondrial transcription (summarized in Fig. 4). We assume that either an integrin-mediated signal transduction pathway, conformational changes, or both are triggered, which are known to modify nuclear transcription and/or activation [26–28]. Mediated by this activated nuclear factor(s), the activation of mitochondrial transcription factors TFBM1, TFMB2, or both is stimulated. These mitochondrial transcription factors are now able to constitute and initiate the mitochondrial transcription machinery consisting of core polymerase and the human mitochondrial transcription factor A [29]. Whether there is a complete mitochondrial/nuclear feedback loop remains to be elucidated, particularly because there is only little information regarding mitochondrial factors that regulate mitochondrial to nuclear signaling [30].

In conclusion, our studies demonstrate that the extracellular surrounding triggers cellular functions in endometrial adenocarcinoma cells, including regulation of mitochondrial gene transcription. From numerous mutations reported for the mitochondrial genome, we know that there is a strong association between compromised



Lane	1	2	3	4	5	6	7	8	9		1	2	3	4	5	6	7	8	9
Cells		H	H	H	H	I	I	I	I			H	H	H	H	I	I	I	I
PL/FCS		+	+			+	+					+	+			+	+		
MG/SFDM				+	+			+	+					+	+			+	+
Competitor			+		+		+		+				+		+		+		+

FIG. 3. Mobility shift assay of mitochondrial heavy strand promoter (*HSP*) and light strand promoter (*LSP*) by nuclear extracts. Mobility shift assays with HSP and LSP were performed with nuclear extracts from HEC 1B (*H*) and Ishikawa (*I*) cells. Prior to extraction, cells were cultured under the conditions indicated. To verify specificity of the reaction, parallel incubations with an excess amount of the non-labelled promoter DNA (*competitor*) were performed

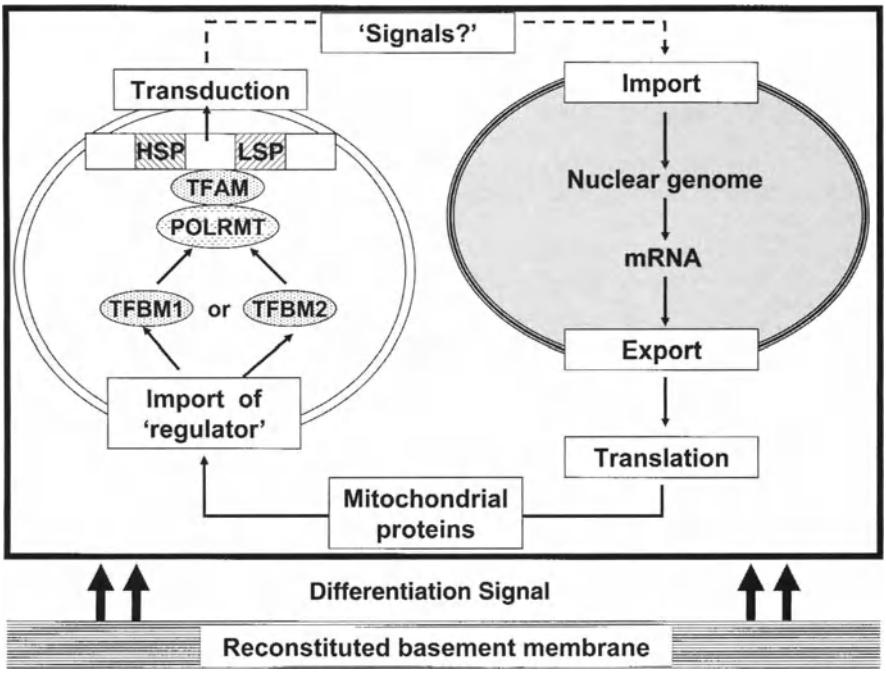


FIG. 4. Proposed hypothetical model of nuclear/mitochondrial interaction. *HSP*, *LSP*, heavy and light strand promoter, respectively; *TFAM*, mitochondrial transcription factor A; *TFBM1*, *TFBM2*, mitochondrial transcription factors *B1* and *B2*, respectively; *POLRMT*, core RNA polymerase

integrity of the mitochondrial genome, the resulting pathophysiological mitochondrial function, and the pathogenesis of several diseases, among them cancer. Identification of factors that activate mitochondrial gene transcription machinery may be helpful for studies aimed at the development of treatments for these types of human diseases.

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Role of *hMLH1* Gene Hypermethylation in Endometrial Carcinogenesis*

Satoru Kyo, Taro Kanaya, and Masaki Inoue

Summary. Inactivation of the *hMLH*1 gene by promoter hypermethylation is one of the major mechanisms that induces the microsatellite instability phenotype in endometrial cancers. The aim of the present study was to examine the methylation profile of a large number of CpG sites spread over the *hMLH*1 promoter in endometrial cancers, as well as in normal endometria, and to investigate the correlation with protein expression and MSI phenotype. We found that the *hMLH*1 promoter is frequently methylated in endometrial cancers. Surprisingly, the histologically normal endometrium of patients with endometrial cancers also exhibited frequent hypermethylation of *hMLH*1 promoter and with a similar profile of methylation to that in cancer lesions. These findings suggest that hypermethylation of the *hMLH*1 gene is the early step for endometrial carcinogenesis, supporting the concept that epigenetic changes in DNA mismatch repair genes are the initial events that trigger the genetic alterations involved in endometrial carcinogenesis. In addition, irregular methylation of the *hMLH*1 promoter may be a useful molecular marker with which to screen or diagnose precancerous and early endometrial malignancies.

Key words. hMLH1, methylation, promoter, endometrial cancer, microsatellite instability

Introduction

Endometrial carcinogenesis is a multistep process involving the abnormal function of cellular genes, including K-*ras*, *p*53, or *PTEN*, which are usually induced by genetic mutations [1]. Another factor involving endometrial carcinogenesis is the genetic instability of microsatellite sequences, namely microsatellite instability (MSI), which are common in tumors associated with hereditary nonpolyposis colorectal cancer syndrome (HNPCC) [2–6]. The microsatellite instability phenotype is associated with

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the dysfunction of DNA mismatch repair genes (MMR), including *hMLH*1 and *hMSH*2, and HNPCC is generally associated with germ-line mutations in either of *hMLH*1 and *hMSH*2, with mutations of other mismatch repair genes being rare [7–10]. However, in a significant subset of MSI-positive sporadic tumors, including endometrial cancers, no mutations of mismatch repair genes were identified, indicating that nonmutational mechanisms are responsible for the defect [11,12].

Alternative modes of gene inactivation during the development of cancer include an epigenetic process marked by promoter region hypermethylation associated with transcriptional loss, as demonstrated for several tumor suppressor genes [13]. The *hMLH*1 promoter is frequently hypermethylated in colorectal and gastric tumors, as well as in endometrial cancers associated with the MSI phenotype [14–18]. Most of these studies examined the methylation status of the CpG sites within the limited areas of the *hMLH*1 promoter, so the regions of methylated CpGs critical for gene silencing are not well understood. The present study investigates the methylation profile of a large number of CpG sites spread over the *hMLH*1 promoter in endometrial cancers and examines whether specific methylated sites or regions of hypermethylated CpGs are critical to regulate protein expression or the MSI phenotype [19].

Emerging evidence shows that the *hMLH*1 promoters in precursors of some tumor types are frequently hypermethylated, suggesting that these changes represent precursor lesions preceding the development of cancers [20,21]. However, little information is available about the methylation status of the *hMLH*1 promoter in the normal endometrium. The present study also examines the methylation status of the *hMLH*1 promoter in normal areas of endometrium adjacent to endometrial cancers and compares the profiles with those in cancers [19].

Methods

Tissue Samples

All tumors and corresponding normal tissues were obtained from patients who underwent surgery to treat primary endometrial cancer at the Department of Obstetrics and Gynecology, Kanazawa University Hospital. Normal endometrial samples were obtained from patients who underwent hysterectomy to treat other types of diseases, such as uterine myoma, adenomyosis, and ovarian tumors. All samples were collected after receiving written, informed consent from the patients. Half the tissue samples were examined histologically and the remaining portions of samples were frozen at −80°C until DNA extraction.

Methylation-Specific Polymerase Chain Reaction

Genomic DNAs extracted from the samples using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) were modified with sodium bisulfite using a CpGenome DNA Modification Kit (Intergen, Purchase, NY, USA), then methylation-specific PCR (MSP) proceeded as described previously [22]. Briefly, genomic DNAs modified with sodium bisulfite served as templates for MSP using the primer sets specific for methylated and unmethylated versions of CpG islands in the *hMLH*1 promoter. Primer sequences of hMLH1 for the unmethylated reaction were 5′-TTTTGATGTAGATGTTT

TATTAGGGTTGT-3′ (sense) and 5′-ACCACCTCATCATAACTACCCACA-3′ (antisense), and for the methylated reaction they were 5′-ACGTAGACGTTT TATTAGGGTCGC-3′ (sense) and 5′-GACGAAACTCTAATTTTCCGACCCG-3′ (antisense). The PCR mixture contained 1 × PCR buffer II (Perkin-Elmer, Foster City, CA, USA), 2.5 mM $MgCl_2$, 200 μM each dNTP, 0.5 μM each PCR primer, 0.5 units AmpliTaq Gold (Perkin-Elmer), and approximately 40 ng modified DNA in a final volume of 25 μL. Amplification proceeded at 95°C for 10 min, followed by 40 cycles at 94°C for 45 s, 62°C for 30 s, and 72°C for 60 s, with a final 5-min extension at 72°C.

The cell lines SW48 and SW480 (American Type Culture Collection, Rockville, MD, USA) served as positive and negative controls for hypermethylation of *hMLH*1, respectively [14]. Universal methylated DNA (Intergen New York, NY, USA) also served as a positive control for the methylated status of *hMLH*1. The PCR products were resolved by electrophoresis on a 3% agarose gel and visualized by ethidium bromide staining and ultraviolet (UV) illumination.

Bisulfite Sequencing

Methylation status spanning the 5′ 700-bp upstream region of the *hMLH*1 promoter was also examined by bisulfite sequencing as described previously [20]. Briefly, methylated and unmethylated alleles from bisulfite-modified DNA were amplified using the primers hMLH1-P1 (5′-TTTTTAGGAGTGAAGGAGGAGGTT-3′) and hMLH-P3 (5′-ACCTTCAACCAATCACCTCAATA-3′). Nested PCR followed using the methylation-specific primer hMLH1-Pm (5′-ACGTAGACGTTTTATTAGGGTCGC-3′) and hMLH1-P3. The PCR products were purified using a Qiagen Gel Extraction kit, cloned into the TA vector pGEM-T Easy (Promega, Madison, WI, USA) and sequenced. To examine methylation status among PCR clones, we compared the methylation profiles of five independent clones selected from five random endometrial cancer samples. We identified less than a 10 % variation in the number of methylated CpGs among the clones under our assay conditions.

Immunohistochemistry

Immunohistochemical analyses of hMLH1 were performed on formalin-fixed, paraffin-embedded specimens of endometrial tissues using the ABC-elite kit (Vector Laboratories, Burlingame, CA, USA). Sections were autoclaved for 10 min in 1 × Antigen Retrieval Solution (Biogenex, San Ramon, CA, USA), then incubated for 16 h at 4°C with an hMLH1 monoclonal antibody (PharMingen, San Diego, CA, USA; clone G168–15, code 13271A) at 10 μg/mL. Staining intensity was evaluated as ++ (positive in over 75% of tumor cells), + (positive in 25%–75% of tumor cells), or – (positive in less than 25% of tumor cells).

Analysis of MSI

Samples of DNA from endometrial cancers and normal areas of the endometrium were analyzed using a panel of seven microsatellite markers for the dinucleotide repeat sequences D2S119, D2S123, D2S147, D10S197, D13S175, D18S58, and D18S69, as described previously [12]. Myometrial samples from each patient served as controls. The PCR products of the microsatellite markers were analyzed using an ABI

GeneScan (Applied Biosystems, Foster City, CA, USA). When the PCR bands had shifted compared with those of the myometrial control samples in two or more of the seven loci, the samples were classified as MSI positive [12].

Statistical Analysis

Statistical analysis was performed using Student's *t*-test and Fisher's exact test to evaluate the significance of differences. $P < 0.05$ was considered statistically significant.

Results

Frequent Hypermethylation of hMLH1 Promoter in Endometrial Cancers

A consecutive series of 59 endometrial cancers were examined initially for *hMLH*1 hypermethylation using MSP. Of 59 endometrial cancers, 21 (36%) were methylated in the *hMLH*1 promoter, whereas 38 (64%) were not. We then analyzed the methylation profile of CpG islands in the *hMLH*1 promoter spanning nucleotides –695 and –74 (first ATG of exon 1 is represented as +1) containing 48 CpGs using bisulfite sequencing (Fig. 1). The findings were classified as fully methylated (over 80% methylated CpGs), partially methylated (10%–80% methylated CpGs), and unmethylated (less than 10% methylated CpGs). Sixteen samples (27%) were fully methylated, 14 (24%) were partially methylated, and 29 (49%) were not methylated. Thus, bisulfite sequencing showed that alleles were methylated in 30 of 59 endometrial cancers (51%). The CpGs at the 5′ region appeared to be more preferentially methylated than those at the 3′ region of the promoter (Fig. 1). The 5′ region between nucleotides –694 and –670, corresponding to CpGs 42–48 in Fig. 2, was methylated in more than 90% of the methylated samples. However, a computer-assisted search did not detect any known transcription factor binding sites in this region. A comparison of the status of *hMLH*1 hypermethylation with clinicopathological features of the patients, such as age, tumor stage, and histopathological type or degree of differentiation (Table 1), revealed no statistical correlations.

Bulky Hypermethylation of hMLH1 *Promoter is Associated with loss of Protein Expression and the MSI Phenotype*

To examine the relationship between the status of promoter methylation and protein expression of hMLH1, we analyzed hMLH1 immunohistochemically in samples from 32 patients with endometrial cancer. Expression of hMLH1 was observed in normal endometrial glands, as well as in the stromal tissues of all samples examined (Fig. 2c). In contrast, hMLH1 expression was decreased or absent in some cancer samples. When staining was classified as intense (over 75% positive in tumor cells), intermediate (25%–75% positive in tumor cells), or weak/absent (less than 25% positive in tumor cells), eight, 15, and nine, respectively, were stained in endometrial cancers (Fig. 2a–c). We then examined the correlation between protein expression and promoter hypermethylation (Table 2). The *hMLH*1 promoter was fully methylated in eight of nine samples with weak or absent expression, whereas none of the eight samples with

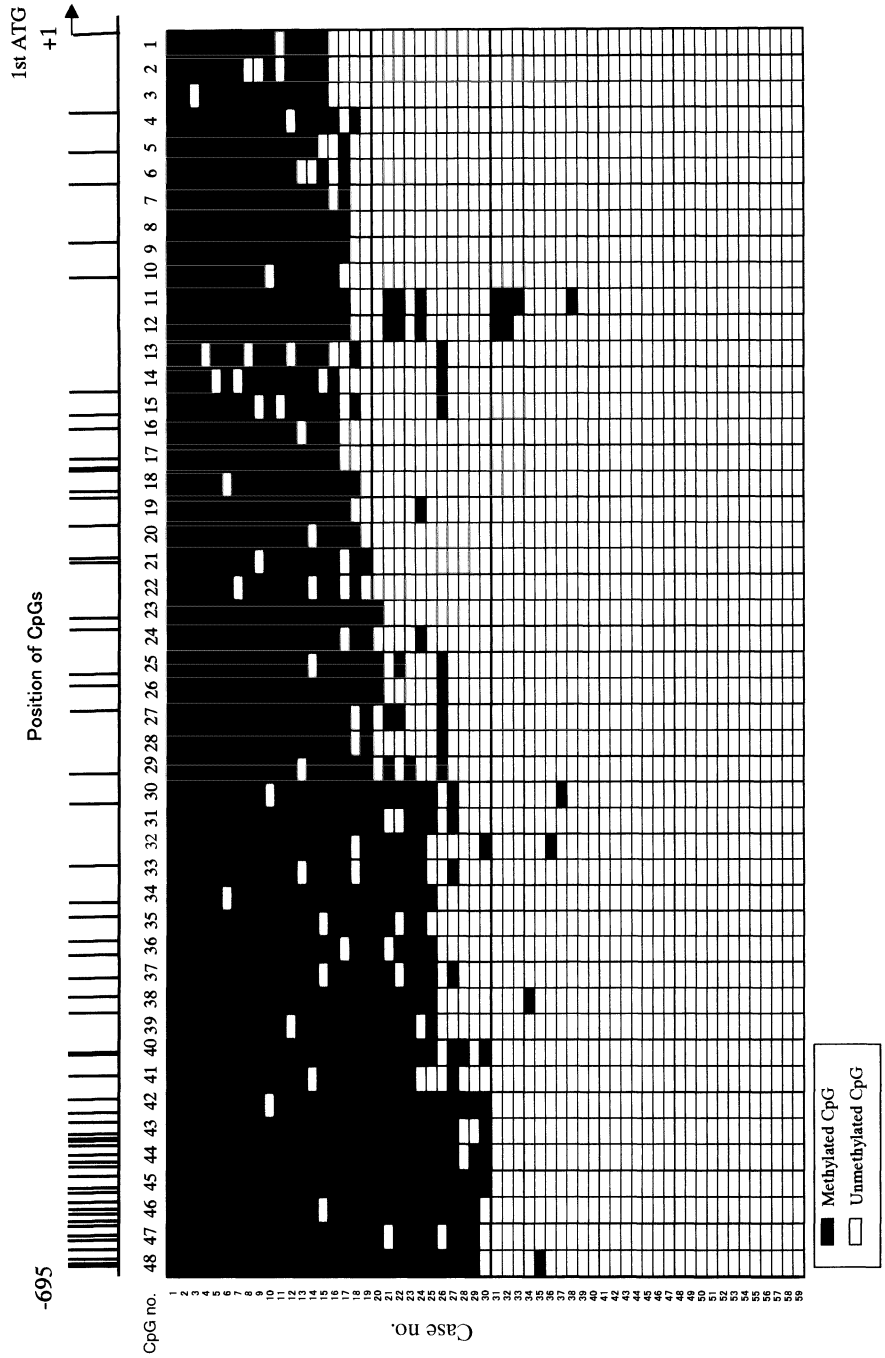

FIG. 1. Bisulfite sequencing analysis of the *hMLH1* promoter in endometrial cancers. Map of 5′ promoter region: individual CpG sites are represented by *vertical lines*. The first ATG of exon 1 is represented as +1. Each *horizontal compartment* represents one sample of endometrial cancer. Forty-eight CpGs within 700-bp upstream of the *hMLH1* promoter are numbered 1–48 (*top*)

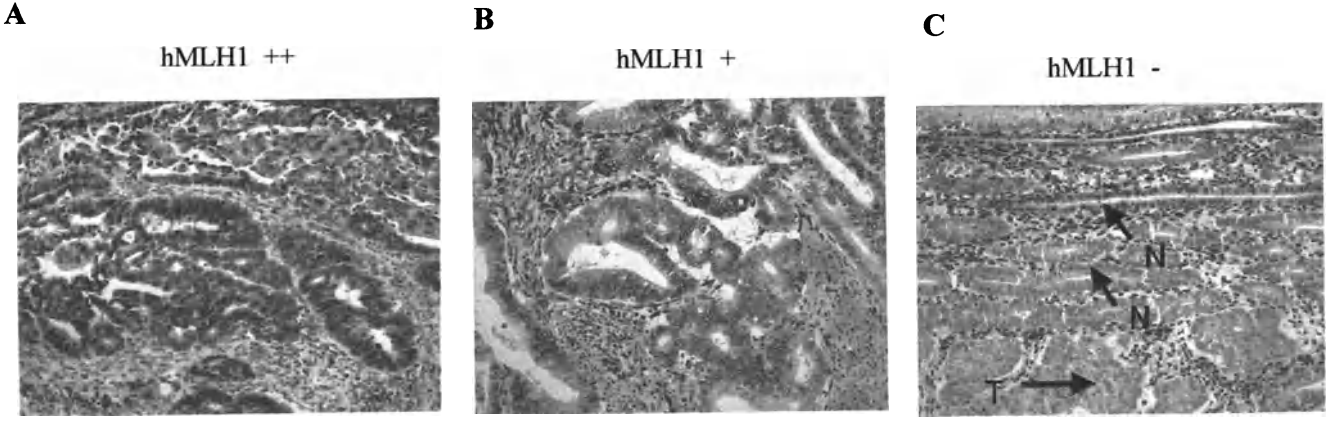

FIG. 2. Immunohistochemical analysis of hMLH1 in endometrial cancers. Representative sections are stained with anti-hMLH1 monoclonal antibody. A Intensely positive (++) tumor cells with over 75% staining. B Intermediate positive case (+) with 25%–75% positive staining. C Weak or absent expression (−−) with less than 25% positive staining. In C, tumor cells (*T*) are not stained, whereas stromal cells are stained intensely. Corresponding normal endometrial glands (*N*) with or without hMLH1 expression are colocalized in this area

TABLE 1. Clinicopathological characteristics of endometrial cancers

		Methylated CpGs		
		>80%	10–80%	<10%
n		16	14	29
Age* (years)		55.5 ± 8.0	57.2 ± 7.8	59.5 ± 11.9
Histology**	Endometrioid	16	14	26
	Serous	0	0	1
	Undifferentiated	0	0	2
FIGO stage**	Ia	5	5	11
	Ib	8	6	10
	Ic	2	2	5
	IIIa	0	0	1
	IIIc	1	1	1
	IVa	0	0	1
Grade**	1	13	8	17
	2	2	4	7
	3	1	2	5

No statistical significance by Student's *t*-text (*) or by Fisher's exact method (**)

TABLE 2. Correlation between methylation status of *hMLH1* and protein expression

hMLH1 expression	Status of *hMLH1* methylation		
	>80%	10%–80%	<10%
–	8	1	0
+	2	5	8
++	0	0	8

$P < 0.05$ (Fisher's exact method)

intense expression was methylated. Two of fifteen samples with intermediate expression were fully methylated, whereas five and two were partially methylated and not methylated, respectively. The correlation between expression and promoter hypermethylation of hMLH1 was significant ($P < 0.05$, Fisher's exact method). In cancer samples with weak or little hMLH1 expresion, some normal endometrial glands adjacent to cancer lesions had lost hMLH1 expression (Fig. 2c).

MSI was next investigated in the seven fully methylated, three partially methylated, and seven unmethylated samples of endometrial cancers (Fig. 3; Table 3). Myometrial tissue samples collected at surgery served as controls for MSI analyses. All seven fully methylated samples were MSI positive, whereas six of seven nonmethylated samples were MSI negative. All three partially methylated samples were MSI negative. Thus, MSI phenotype and *hMLH1* hypermethylation were significantly correlated ($P < 0.05$, Fisher's exact method).

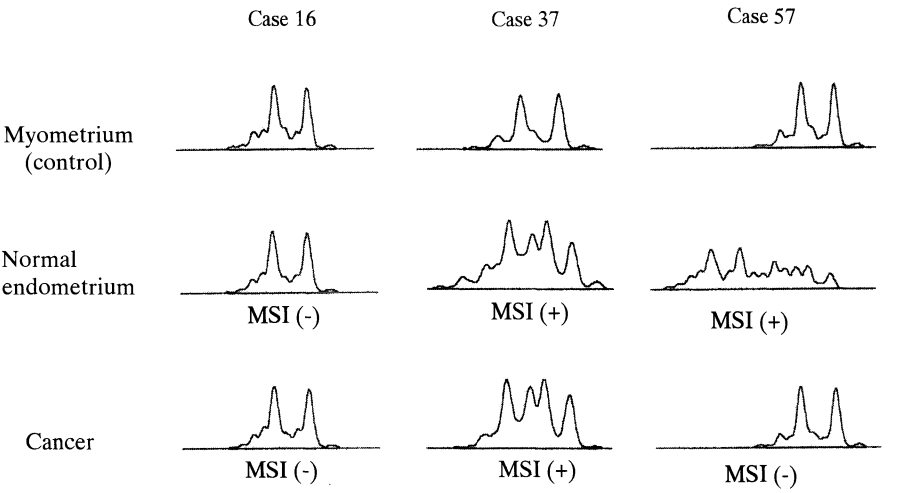

FIG. 3. Microsatellite instability (*MSI*) analysis in endometrial cancers and corresponding normal endometria. Polymerase chain reaction products of seven microsatellite markers were analyzed using an ABI GeneScan (Applied Biosystems, Foster City, CA, USA). Myometrial samples from each patient served as controls. Representative results use microsatellite marker D2S119. Patient 37 has MSI in cancer and normal endometrium. Patient 57 has MSI positivity in cancer, but not in normal endometrium. Patient 16 is MSI positive in both cancer and normal endometrium

TABLE 3. Correlation between the microsatellite instability phenotype and the methylation status of *hMLH1*

	Status of *hMLH1* methylation			
MSI	>80%	10%–80%	<10%	
Endometrial cancer ($n = 17$)				
–	0	3	6	*
+	7	0	1	*
Adjacent normal endometrium ($n = 17$)				
–	1	1	10	*
+	3	1	1	*

* $P < 0.001$ (Fisher's exact method)

TABLE 4. Status of *hMLH1* methylation in normal endometrium with or without endometrial cancers

Primary disease	Status of *hMLH1* methylation in normal endometria		
	Methylated	Unmethylated	
Endometrial cancer (Methylated CpG > 10%)	5	7	*
Other than endometrial disease	1	30	

* $P < 0.05$ (Fisher's exact method)

The hMLH1 *Promoter is Frequently Hypermethylated in Normal Endometrium of Patients with Endometrial Cancers and is Associated with MSI Phenotype*

We investigated the methylation status of the *hMLH*1 promoter by MSP in normal endometrium adjacent to methylated endometrial cancers from 12 patients. Normal endometria from 31 patients with benign uterine diseases at sites other than the endometrium or ovarian tumors were also examined. Of 12 fully or partially methylated endometrial cancer samples, the normal endometria of five (42%) were also methylated (Table 4). These five samples were further analyzed by bisulfite sequencing. Both cancerous and normal endometria from the four samples were fully methylated and the methylation profiles of the normal endometria were similar to those in cancer lesions (Fig. 4). The promoters in the other normal and cancerous endometria were partially (40% of CpGs methylated) and fully (85% of CpGs methylated) methylated. In this sample, the methylation status of the CpGs at the 5′ region was similar in normal and cancer lesions. Normal endometrial samples from 31 patients with benign uterine diseases or ovarian tumors were analyzed by MSP. The promoters of all but one (97%) were unmethylated. One methylated sample was from a 70-year-old woman with carcinoma in situ of the uterine cervix who had undergone surgery (hysterectomy). Histologically, endometrial diseases were undetectable in this patient. Taken together, these findings indicate that normal endometria adjacent to endometrial cancers are frequently methylated, with profiles similar to those of cancer lesions.

Finally, we examined whether methylation of the *hMLH*1 promoter is associated with the MSI phenotype in the normal endometrium (Fig. 3; Table 3). We investigated the presence of MSI in 17 normal endometria adjacent to endometrial cancers. Three of four normal endometria with methylated promoters were MSI positive, whereas 10 of 11 unmethylated samples were MSI negative. Thus, *hMLH*1 hypermethylation and the MSI phenotype were significantly correlated in normal endometria adjacent to endometrial cancers ($P < 0.05$, Fisher's exact method).

Discussion

The present study showed that hypermethylation of the *hMLH*1 promoter is frequent in endometrial cancers, consistent with previous observations [5,17,18]. Approximately 50% of endometrial cancers had methylated alleles, whereas only 3% of normal

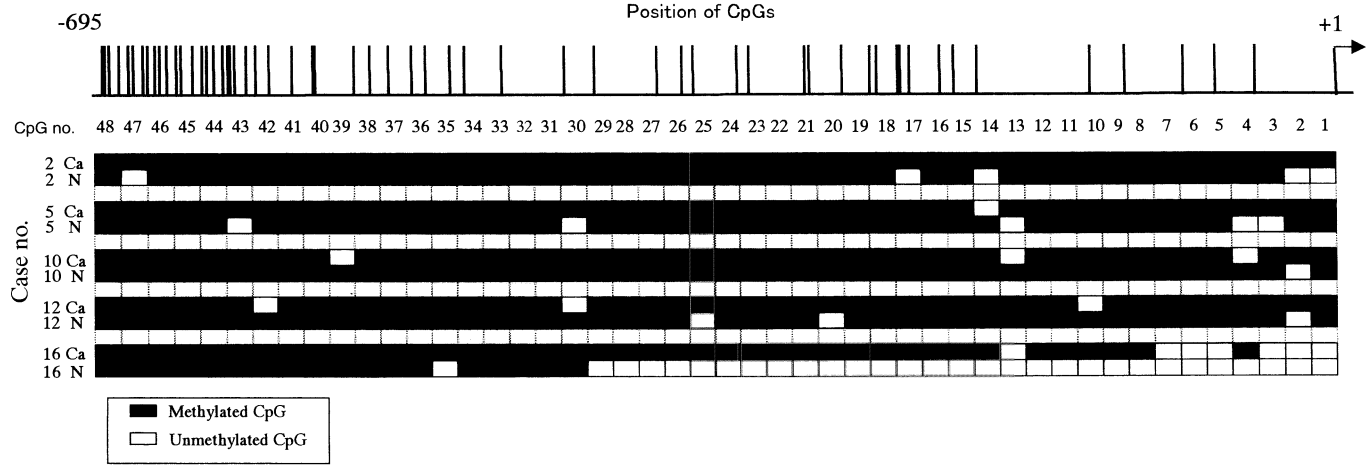

FIG. 4. Bisulfite sequencing of the *hMLH1* promoter in endometrial cancers and corresponding normal endometria. Map of the 5′ promoter region shows individual CpG sites represented by *vertical lines*. Each *horizontal compartment* represents one sample of endometrial cancer and corresponding normal endometria. Forty-eight CpGs within 700-bp upstream of the *hMLH1* promoter are numbered 1–48 (*top*). *Ca*, endometrial cancer lesions; *N*, corresponding normal endometrium

endometria from control patients were methylated, supporting the notion that hypermethylation of the *hMLH1* promoter plays a role in endometrial carcinogenesis. Bisulfite sequencing revealed variations in the methylation profiles of CpGs. Of 59 endometrial cancer samples, 16 (27%) were fully methylated, whereas 14 (24%) were partially methylated and 29 (49%) were not methylated. Immunohistochemical analyses revealed that hypermethylation of *hMLH1* was significantly associated with decreased protein expression. Deng et al. have recently correlated a small proximal region between nucleotides –272 and –202 (the first ATG is represented as +1) in the promoter with the absence of hMLH1 expression in colorectal cancer cell lines [23]. They also demonstrated that methylation at a CpG near the CCAAT motif in the *hMLH1* promoter interferes with its binding to the transcription factor CBF (CCAAT-binding factor), leading to the inhibition of gene expression in colorectal cancers [24]. The CCAAT sequence and the adjacent CpG described by Deng et al. are located at nucleotides –285 and –289 (CpG no. 12 in Fig. 2), respectively. However, we did not find that methylation of this CpG was prevalent in endometrial cancers. Thus, our data, using clinical samples, do not agree with the observations of Deng et al. using colorectal cancer cell lines. The reasons for this discrepancy remain unclear, but the methylation profiles of *hMLH1* may be tumor type specific. We found that a series of CpGs at the 5′ region between nucleotides –694 and –670 was preferentially methylated in endometrial cancers. However, a computer-assisted sequence analysis did not reveal any known specific transcription factor binding sites in this region. Thus, our data support the notion that the degree, rather than the region specificity, of methylation targeting specific transcription factor binding sites is responsible for transcriptional silencing and the decreased expression of hMLH1 protein.

We found a significant correlation between the methylation status of *hMLH1* and the MSI phenotype. This is consistent with the proposed concept that silencing of the *hMLH1* promoter by hypermethylation and the subsequent loss of protein expression causes defects in mismatch repair, leading to the MSI phenotype.

The present study found frequent hypermethylation of *hMLH1* in the normal endometrium of patients with endometrial cancers. In cancer patients with hypermethylated promoters, approximately 40% of the adjacent normal endometria also had methylated promoters. Endometrial cancers frequently involve hyperplasias in adjacent areas, which sometimes appear macroscopically normal. We therefore confirmed that the adjacent normal regions we collected did not include hyperplasia according to histological examinations by three independent pathologists. Notably, we found that some normal endometrial glands adjacent to hMLH1-negative endometrial cancers also lacked hMLH1 expression (Fig. 2c). Although we did not analyze the methylation status of *hMLH1* in individual glands by microdissection techniques, these apparently normal glands may have methylated promoters, which may be latent precancers. The methylation profiles of adjacent normal endometria were concordant with those in cancer lesions in four of five concurrent methylated samples. The remaining one sample (patient 16) exhibited 40% and 85% methylation of CpGs in normal endometrium and cancer lesions, respectively (Fig. 4). The methylated status of the CpGs at the 5′ region was almost concordant in normal and cancer lesions. These findings further support the notion that the *hMLH1* promoter becomes hypermethylated during the early stages of endometrial carcinogenesis. We also investigated the prevalence of *hMLH1* hypermethylation in normal control patients. Alleles

in all but one of 31 (3%) normal endometria in patients with benign uterine diseases or ovarian tumors were methylated. Thus, hypermethylation of the *hMLH*1 promoter is specific to the normal endometria concurrent with endometrial cancers. Recent studies have demonstrated that the *hMLH*1 promoter is methylated in endometrial hyperplasia [21]. Although we did not examine such samples, our data do not contradict this finding and extend the concept that the h*MLH*1 promoter is already hypermethylated in the normal endometrium. We further examined the MSI status in these normal endometria and identified a close correlation between hypermethylation of *hMLH*1 and the MSI phenotype, suggesting that hypermethylation of hMLH1 significantly influences MSI phenotype in the normal endometrium.

In summary, we found that the *hMLH*1 promoter is frequently methylated in the histologically normal endometrium of patients with endometrial cancers and that the methylation status is similar to that in cancer lesions. These findings support the notion that epigenetic changes in DNA mismatch repair genes are the initial events that trigger the genetic alterations involved in endometrial carcinogenesis. In addition, irregular methylation of the *hMLH*1 promoter could be a useful molecular marker with which to screen or diagnose precancerous and early endometrial malignancies.

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Molecular Genetic Profiles of Serous and Endometrioid Endometrial Cancers, Genes Downregulated in Endometrial Cancers, and the Role of Epigenetics

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Summary. Endometrial cancers, like most other malignancies, develop, in part, as the result of oncogene activation and tumor suppressor and DNA repair gene inactivations. Available molecular data suggest that endometrial cancers of endometrioid and nonendometrioid histology develop by distinct etiologies. Endometrioid lesions are typified by *PTEN*, *CTNNB*1, and *KRAS*2 mutations, DNA mismatch repair deficiency, as evidenced by microsatellite instability, and are usually near diploid. Nonendometrioid histologies are characterized by mutation of *TP*53, overexpression of *Her-2/neu*, and are usually nondiploid, suggesting widespread chromosomal instability. Although these changes are observed in many endometrial cancers, they do not occur universally, suggesting that there are unrecognized pathways involved in the development of endometrial cancer. We examined the prevalence of these prototypical defects in a panel of 87 cancers. Interestingly, more than half the cancers did not contain mutations of *PTEN*, *KRAS*2, and *TP*53, or have microsatellite instability. Based, in part, on this observation, we believe that epigenetic mechanisms likely play a major role in the development of some endometrial cancers. Multiple epigenetic mechanisms are likely involved, including loss of imprinting, chromatin remodeling, and promoter hypermethylation. Our group has recently evaluated the role of promoter methylation in endometrial carcinogenesis and has found that methylation of specific promoters varies between endometrioid and nonendometrioid histologic types. We have also performed in vitro manipulation of endometrial carcinoma cells with agents that demethylate promoters prior to microarray analysis to identify genes that are up- or downregulated. In addition, we have analyzed a panel of endometrial cancers using expression microarrays to identify downregulated genes in both endometrioid and nonendometrioid endometrial cancers. Following a cross-referencing of both array databases, genes common to both the global expression of primary endometrial cancers and in vitro experiments can be identified. These genes may be downregu-

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lated via epigenetic mechanisms and our laboratory is currently working to validate this hypothesis.

Key words. Endometrial cancer, epigenetics, hypermethylation, expression array

Molecular Genetics of Endometrial Cancers

Two subtypes of endometrial carcinoma have been described based on both clinical and histopathologic variables [1]. Type I endometrial cancers account for the majority of endometrial cancer cases and these cancers are usually well differentiated and endometrioid in histology. Type I tumors are frequently associated with a history of unopposed estrogen exposure or other hyperestrogenic risk factors, such as obesity. Patients with type I endometrial cancer typically have early stage disease and a favorable prognosis with appropriate therapy. In contrast, type II endometrial cancers are often poorly differentiated, nonendometrioid, and are not associated with hyperestrogenic factors. These tumors are more likely to be metastatic at presentation and may recur despite aggressive clinical interventions.

Molecular genetic evidence indicates that endometrial carcinoma likely develops as the result of a multistep process of oncogene activation and tumor suppressor gene inactivation. We and others have described some of these changes and demonstrated that these molecular alterations appear to be specific for type I (endometrioid) and type II (serous or clear cell) endometrial cancers. Endometrioid cancers are characterized by mutation of *PTEN*, *KRAS2*, and *CTNNB1*, defects in DNA mismatch repair (as evidenced by the microsatellite instability phenotype), and a near-diploid karyotype. Serous tumors often contain mutations of *TP53*, display chromosome 1 loss of heterozygosity, and are usually nondiploid. Several reviews have discussed the prevalence and relevance of these alterations in endometrial cancers and their associations and histologic types in much greater detail [2–5].

Epigenetics Role in Endometrial Cancer

Although these specific genetic alterations are observed in many endometrial cancers, none of these changes occurs in all cases. We have observed the existence of cancers without any evidence of genetic alterations (i.e., no detectable loss of heterozygosity or of mutation in genes frequently altered in endometrial cancers, such as *PTEN*, *KRAS2*, *CTNNB1*, or *TP53*). To examine further the molecular etiology of endometrial cancers, we assembled 87 cancers and analyzed our mutation database for these most frequent alterations. Surprisingly, 41% of cases, many of which were advanced-stage cancers, did not contain alterations at any of these loci (Table 1). Some mutations could have been missed due to less-than-perfect sensitivity of the mutation-detection methodologies used. Nonetheless, this suggests that there are several unrecognized pathways that can lead to the development of endometrial cancer. Based, in part, on this observation, we hypothesize that epigenetic mechanisms may play a role in the development of a significant fraction of endometrial cancers. These epigenetic changes may include loss of imprinting, chromatin organization, and gene promoter hypermethylation; all these mechanisms could affect gene expression.

TABLE 1. Alterations in *PTEN*, *TP53*, and microsatellite instability in early and advanced endometrial cancers

Gene	Early		Advanced	
PTEN	15/46	35%	6/41	15%
MSI	10/46	20%	8/41	20%
TP53	3/46	7%	17/41	41%
No change	27/46	59%	17/41	41%

MSI, microsatellite instability

TABLE 2. Selected genes upregulated following 5-aza-2′-deoxycytidine treatment in AN3CA endometrial cancer cells

Affymetrix identification	Gene symbol	UniGene cluster	Fold expression
202520_s_at	MLH1	Hs.57301	6.1
203404_at	ALEX2	Hs.48924	11.3
204523_at	ZNF140	Hs.154205	16.6
209243_s_at	PEG3	Hs.139033	3.9
201010_s_at	TXNIP	Hs.179526	7.7
209560_s_at	DLK1	Hs.169228	42.2
209278_s_at	TFPI2	Hs.295944	175
210546_x_at	CTAG1	Hs.167379	97
205081_at	CRIP1	Hs.17409	25.1
209035_at	MDK	Hs.82045	15.5

Recent experiments in our laboratory are designed to investigate this hypothesis. One aspect of this research involves those transcripts downregulated by various mechanisms. One important potential mechanism involves promoter hypermethylation. Various gene promoters, particularly those rich in GC content and fulfilling the classic CpG island criteria, often undergo methylation and transcription can be silenced when these promoters become saturated in methyl groups. There is already considerable evidence implicating promoter hypermethylation as a mechanism downregulating genes in many cancers, including endometrial cancers.

Approximately 20%–25% of endometrial cancers display the microsatellite instability phenotype, yet very few of these cancers have gene mutation in one of the DNA mismatch repair genes [6–8]. It is now clear that hypermethylation of the *hMLH1* gene promoter is causative of reduced *hMLH1* transcript and the defective DNA mismatch repair in those cancers occurring outside the hereditary nonpolyposis syndrome [9–12]. In addition, other loci, including the *APC* tumor suppressor gene, estrogen, and progesterone receptors, frequently contain hypermethylated promoters, although an effect on gene expression has not yet been conclusively demonstrated [13–18]. We hypothesize that promoter methylation may be a feature of endometrial cancers that contain few large-scale genomic abnormalities or gene mutations, whereas other epigenetic mechanisms may be involved in the development of cancers with gene mutations or chromosomal instability. In addition, endometrioid and serous cancers are likely to involve gene silencing at distinct loci and with different frequencies.

Recent experiments in our laboratory are investigating the role of promoter methylation in the epigenetics of endometrial cancers. We surveyed 11 genes for promoter

hypermethylation using methylation-specific polymerase chain reaction (PCR) on a panel of 77 endometrial cancers (J. I. Risinger et al., unpublished data, 2000). These promoter loci were previously shown to be commonly hypermethylated in several other cancer types. As predicted, the results of this experiment confirm a clear distinction between the two most common histologic types. Specifically, no serous cancer exhibited a methylated promoter in any of 11 genes examined. In contrast, frequent hypermethylation events were detected at several loci, including the *hMLH*1, *APC*, and *MGMT* genes, and occasionally at the other loci in endometrioid endometrial cancers. These results support our hypothesis that endometrioid cancers that contain few large-scale genomic changes are more likely to evolve by silencing genes through promoter hypermethylation rather than through deletion or rearrangements, which are common in serous cancers. However, the promoter survey approach is extremely limited and biased based on the loci examined. It will be necessary to identify and examine loci that are proven relevant to the development of these two differing histologies. There are likely loci present in both serous and endometrioid cancers silenced by this mechanism that are yet to be described.

Experimental Approaches for Identifying Epigenetically Silenced Genes in Endometrial Cancer

In vitro cell culture manipulations using DNA methylation inhibitors (i.e., 5-aza-2′-deoxycytidine (5AZAdC) or zebularine) or histone deacetylase inhibitors (i.e., tricostatin A, butyrate, suberoylanilide hydroxamic acid (SAHA)) can be used to reactivate the transcription of silenced genes. These treatments can be used to identify candidates of epigenetic silencing through either promoter hypermethylation or chromatin remodeling. Preliminary experiments in our laboratory suggest that this approach, particularly when combined with expression array technology, can identify plausible gene candidates for epigenetic silencing. For example, treatment of the AN3CA endometrial cancer cell line with 5AZAdC can restore expression of *hMLH*, *PEG*3, or *APC*, which are normally silenced by promoter hypermethylation in these cells. Similarly, treatment of these cells with tricostatin A, an extremely potent histone deacetylase inhibitor, will result in expression of the *PEG*3 gene in the absence of 5AZAdc, suggesting that chromatin remodeling of this loci is also important. When these same untreated and treated RNAs are examined using high-density oligonucleotide array technologies, these and other genes are identified by an increase in transcript levels. These upregulated transcripts represent a pool of potential genes regulated by epigenetic mechanisms. Of course, some of these transcripts may be upregulated by secondary events taking place in these cells or may represent inappropriately expressed genes that are normally silenced.

A selection of some of these transcripts is shawn in Table 2.

Expression Profiling and Epigenetics in Endometrial Cancer

In an effort to identify genetic loci that could be affected by epigenetic mechanisms in primary endometrial cancers, we used expression array technology to identify

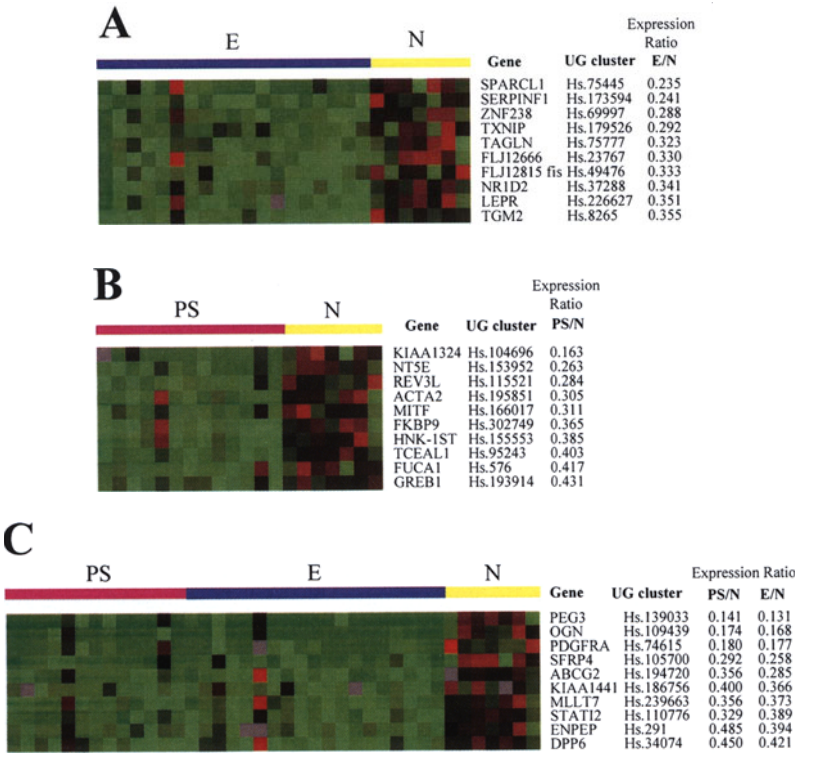

FIG. 1. Downregulated genes in endometrial cancers as determined by cDNA microarray technology. Microarray data indicating the 10 most downregulated genes based on the expression ratio to normal (*N*) endometrial samples for endometrioid endometrial (*E*) carcinomas (**A**), papillary serous (*PS*) endometrial carcinomas (**B**), and papillary and endometrioid histologies (**C**). Red indicates upregulation, whereas green indicates downregulation in reference to a universal reference RNA standard. *UG*, UniGene cluster

those genes that are downregulated in endometrioid and serous endometrial cancers. Genes downregulated in relation to normal endometria were identified following expression profiling performed in our laboratory. Specifically, we examined global gene expression changes in 19 endometrioid endometrial cancers, 13 serous endometrial cancers, and seven normal endometria using a cDNA array containing over 9000 genes (J. I. Risinger et al., unpublished data, 2002). A portion of these data listing some of the most downregulated genes is depicted in Fig. 1. Several aspects of this analysis are worth noting. This analysis is seeking those genes downregulated in the majority of cases in the individual groups. Therefore, loci known to be hypermethylated in the minority of cases, like *hMLH1*, will not appear. In addition, genes overexpressed in the normal stromal components but absent from the stromal components in the tumor samples may give the appearance of relevant loci. We are currently examining some of these loci in more detail with regard to their expression in normal endometrial epithelium and in cancer. Despite the noted limitations of array experiments, this approach supplies an abundant set of data that is likely

relevant to the disease. Cross-referencing data obtained from mechanistic cell line experiments can quickly be examined for relevance by analysis with the primary tissue data.

For example, some genes universally downregulated in our endometrial cancer array (Fig. 1) were shown to be upregulated in AN3CA cells following treatment with 5AZAdC, including *PEG3* and *TXNIP*. These data suggest that these two transcripts are likely regulated, at least in part, by hypermethylation events. In this regard, the *PEG3* gene has recently been shown to be silenced in human gliomas by a promoter hypermethylation mechanism [19]. The role of *PEG3* disregulation in endometrial carcinogenesis is unknown, but appears worthy of further examination. Several candidate loci were identified after cross-referencing with one agent (5AZAdC) and one cell line (AN3CA). Therefore, this approach provides reasonable candidates that can be examined more thoroughly using other methods. A similar approach has been used successfully to identify candidate genes silenced in colorectal cancer, however without cross-reference to primary cancer array data [20].

Future experiments in our laboratory will extend these reactivation experiments to additional cell lines and compounds, including some with more likely clinical application, including zebularine and SAHA. We believe these approaches and others are necessary to determine the molecular events that contribute to endometrial cancer development. This information will be essential in developing novel prevention and nonsurgical therapies in the future for this most common gynecologic cancer.

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Biological Implications of Survivin Gene Expression in the Development of Endometriosis and Endometrial Carcinoma

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Summary. A total of 63 pigmented or non-pigmented endometriotic, 26 endometrial carcinoma, and 12 normal eutopic endometrial tissues were examined for mRNA expression of survivin, matrix metalloproteinase (MMP)-2, MMP-9, and membrane-type 1 (MT1)-MMP. The expression levels of the survivin and MMPs genes in clinically aggressive pigmented lesions were significantly higher than those in normal eutopic endometrium, and survivin gene expression in pigmented lesions was also higher than that in non-pigmented lesions ($P < 0.05$). There was a close correlation between the expression levels of the survivin and MMPs genes in 63 endometriotic tissues examined ($P < 0.01$). Apoptotic cells detected by dUTP nick-end labeling were rare in 11 ovarian endometriotic tissues, which showed positive immunohistochemical expression for survivin and MMPs. Expression levels of the survivin and MMPs genes in endometrial carcinomas were higher than those in normal eutopic endometrium and were well correlated with the depth of myometrial invasion ($P < 0.05$). There was a close correlation between the expression levels of the survivin and MMPs genes in 26 endometrial carcinoma tissues examined ($P < 0.01$). These findings suggest that upregulation of survivin and MMPs may contribute cooperatively to survival and invasion of endometriosis and endometrial carcinomas.

Key words. Survivin, apoptosis, invasion, endometriosis, endometrial carcinoma

Introduction

Endometriosis, the presence of endometrium outside of the uterine cavity, is a common disease, causing abdominal pain, dysmenorrhea, dyspareunia, and infertility in 10%–15% of menstruating women [1]. Its etiology is unclear, but it is thought

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to be due to the implantation and maintenance of disseminated uterine endometrium, predominantly on the ovary and pelvic peritoneum [2]. Normal epithelial cells undergo apoptosis when they separate from their primary tissue. However, spontaneous apoptosis of ectopic endometrial tissue is impaired in women with endometriosis and its decreased susceptibility to apoptosis may participate in the growth, survival, and invasion of endometriotic tissue [3,4]. Clinical observations have led to the assessment that endometriosis is an invasive disease [5,6]. In endometriotic lesions, although derived from normal endometrium, decreased expression of adhesion molecules and increased expression of proteolytic enzymes seem to contribute to the establishment of endometrial glands and stroma at ectopic sites, likely as a behavior of cancer cells [5–8]. Although there have been some reports on the induction of apoptosis in endometriotic lesions [9,10], there is no consensus on the mechanism of escape from apoptosis in endometriosis and little is known on the correlation between survival activity and invasive phenotype in endometriotic cells.

Among the regulators of cell death, inhibitor of apoptosis (*IAP*) proteins have recently emerged as modulators of an evolutionarily conserved step in apoptosis, which may potentially involve the direct inhibition of terminal effector caspases 3 and 7 [11]. Recently, a novel and structurally unique member of the *IAP* gene family designated 'survivin' was identified [12]. Unlike other IAP proteins, survivin was found during embryonic and fetal development, was completely downregulated and undetectable in normal adult tissues, and became prominently reexpressed in all of the most common human cancers [12]. However, there has been no report on the biological significance of survivin in endometriosis, an aggressive tumor-like benign disease. Moreover, some previous investigators have demonstrated that survivin gene expression may reflect an important mechanism in tumor progression of the endometrial mucosa [13,14]. In the present study, we investigated survivin gene and protein expression in surgical specimens from patients with endometriosis and correlated them with apoptosis and the invasive phenotype of endometriotic tissues. We also examined survivin gene expression in endometrial carcinomas and evaluated its biological significance in tumor invasion and progression.

Methods

Patients and Tissue Samples

A total of 63 endometriotic tissues for mRNA analysis were removed with biopsy forceps under laparoscopy or laparotomy from 35 women with endometriosis and included 43 pigmented (red or black; blueberry spot, blood breb, and chocolate cyst) and 20 non-pigmented (white or yellow-brown; serous breb and surface elevation) lesions. Stages of the disease and macroscopic findings of endometriotic lesions were classified according to the revised American Fertility Society classification. Of these patients, two had stage I, one had stage II, eight had stage III, and 24 had stage IV disease. Control eutopic endometrial tissues were collected from 12 women without endometriosis by endometrial curettage. Cycle phase for each subject was assigned on the basis of histologic evaluation of eutopic endometrium or basal body temperature. Endometrial carcinoma tissues for mRNA analysis were obtained from 26

women who underwent surgical treatment. Stages of the disease were classified according to the FIGO classification. Of these patients, 16 had stage I, three had stage II, five had stage III, and two had stage IV disease. All tissue specimens were frozen immediately in liquid nitrogen and then stored in –80°C until use.

Tissue samples for terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) and immunohistochemistry were obtained from 11 women who underwent ovarian cystectomy or oophorectomy under the diagnosis of chocolate cyst of the ovary. Resected ovarian tissues were fixed in 10% formalin and embedded in paraffin wax. Eutopic endometrial tissues collected from seven women without endometriosis were also used for TUNEL and immunohistochemical studies.

RNA Isolation and cDNA Preparation

RNA was extracted from homogenized tissue samples by phenol/chloroform extraction as per the RNA STAT-60 protocol (Tel-Test, Friendswood, TX, USA) according to the manufacturer's recommendation. cDNAs were prepared using at least 2 μg total RNA and SUPERSCRIPTII reverse transcriptase (GIBCO BRL Life Technologies, Gaithersburg, MD, USA) with random hexamers as primers and were finally dissolved in diethyl pyrocarbonate-treated water and then frozen at –20°C until use.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Oligonucleotide primers for RT-PCR were designed using a published sequence of survivin, matrix metalloproteinase (MMP)-2, MMP-9, membrane-type 1 (MT1)-MMP, and β-actin genes. Detailed PCR conditions and primer sequences used for amplification are available from the authors upon request.

Amplification of cDNA was performed according to the method described previously [15,16]. The cDNA template was amplified by PCR in a final volume of 20 μL reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM dNTP, 0.5 μM each primer, and 1.25 units Taq polymerase (Perkin-Elmer, Norwalk, CT, USA). After an initial denaturation at 96°C for 3 min, various cycles of denaturation (94°C for 1 min), annealing (57–60°C for 1 min), and extension (72°C for 2 min) for the respective target genes were performed on a Perkin-Elmer GeneAmp PCR System 9700. The final extention was performed at 72°C for 10 min. The number of cycles in the RT-PCR was determined so as to obtain logarithmic amplification of each gene for semiquantitative analysis of the expression levels of the genes. Reaction products were visualized on a 1.5% agarose gel with ethidium bromide. The relative expression levels were calculated as the density of the product of each target gene divided by that of β-actin from the same cDNA. Each analysis was performed in triplicate.

DNA Nick-End Labeling

DNA breaks were detected in situ by TUNEL according to the method described previously [17,18]. Dewaxed and rehydrated paraffin sections were digested with

20 μg/mL proteinase K (Sigma, St. Louis, MO, USA) for 15 min at room temperature and then incubated with a solution containing 2% H_2O_2 in phosphate-buffered saline (PBS) to inhibit endogeneous peroxidase activity. The TdT buffer solution (100 mM potassium cacodylate, 2 mM cobalt chloride, 0.2 mM dithiothreitol, pH 7.2), containing 0.3 U/μL TdT (Oncor, Gaithersburg, MD, USA) and 0.04 nmol/μL digoxigenin-dUTP (Oncor), was added to cover the tissues, which were then incubated in a humidified atmosphere for 60 min at 37°C. The tissues were washed with buffer solution containing 300 mM sodium chloride and 30 mM sodium citrate for 30 min at 37°C to terminate the reaction, and then washed with PBS. Tissues were incubated with antidigoxigenin–peroxidase complex for 30 min at room temperature and stained with a solution of 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2 in Tris-HCl buffer (DAB solution) at pH 7.6 for 3–6 min at room temperature. Sections were counterstained with hematoxylin. Negative controls were obtained by omitting TdT from the buffer solution. Slides were evaluated independently by two investigators who had no knowledge of the clinicopathologic features of the patients. Three different fields containing 100 glandular cells per field were examined at a magnification of ×400. By evaluating the ratio of positively stained cells, apoptosis was assessed as negative (−) if no cells were stained, weakly positive (+) if <25% of cells were stained, and extremely positive (++) if >25% of cells were stained.

Immunohistochemistry

For immunohistochemical staining, rabbit antihuman survivin polyclonal antibody (SURV11-A; Alfa Diagnostic International, San Antonio, TX, USA) and mouse antihuman MMP-2, MMP-9, and MT1-MMP monoclonal antibodies (Fuji, Toyama, Japan) were used for the avidin–biotin–peroxidase complex procedure. Slides were incubated overnight at 4°C with primary antibodies at a 1:50 dilution and washed with PBS. Biotinylated goat antirabbit or horse antimouse immunogloblin (Dako, Kyoto, Japan) was then added to the sections for 30 min at room temperature. Peroxidase-conjugated avidin (Dako) was applied and peroxidase activity was detected by exposure of the sections to the DAB solution.

Sections were counterstained with hematoxylin. Normal rabbit or mouse IgG (Dako) was used as a substitute for the primary antibody for the negative controls. The number of positively stained cells was counted and evaluated using the method indicated for the TUNEL protocol.

Statistical Analysis

All statistical calculations were performed using StatView statistical software. The significance of differences between groups was calculated by applying a nonparametric test. The Spearman rank correlation coefficient was also used to analyze the relationship between two different values. $P < 0.05$ was accepted as statistically significant.

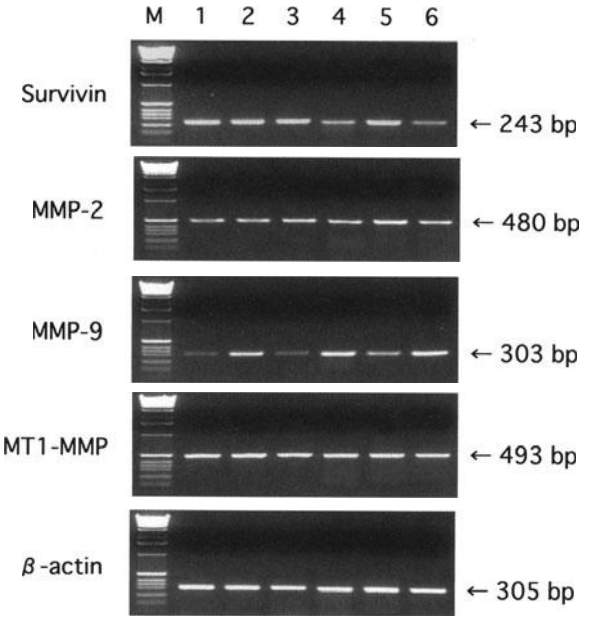

FIG. 1. Reverse transcription–polymerase chain reaction analysis of survivin, matrix metalloproteinase (*MMP*)-2, MMP-9, and membrane-type 1 (*MT*1)-MMP in normal endometrium and endometriotic tissues. *Lanes* 1–2, samples from normal eutopic endometrium; *lanes* 3–4, samples from non-pigmented lesions; *lanes* 5–6, samples from pigmented lesions. *M*, 100-bp DNA size marker

Results

Figure 1 shows an example of mRNA expression of survivin, MMP-2, MMP-9, and MT1-MMP in endometriotic tissues and normal endometrium. Their relative gene expression levels in comparison with β-actin expression ranged widely among the lesions examined; however, there was no significant difference in the expression level of survivin and MMPs between stages of the disease and cyclic phases of the patients (data not shown). As can be seen in Fig. 2, gene expression levels of survivin, MMP-2, MMP-9, and MT1-MMP in pigmented lesions were significantly higher than those in normal eutopic endometrium. Moreover, survivin gene expression in pigmented lesions was statistically higher than that in non-pigmented lesions, whereas there was no significant difference in MMP-2, MMP-9, and MT1-MMP gene expression levels between the two groups. As shown in Fig. 3, there was a close correlation between survivin and MMP-2, MMP-9, or MT1-MMP and between MMP-2 and MT1-MMP gene expression levels in 63 endometriotic tissues examined.

Seven specimens from normal endometrium and 11 from ovarian endometriosis were examined for apoptosis and expression of survivin and MMPs. Intense TUNEL signals were sometimes observed in the small nuclei of normal endometrial cells (Fig. 4a); however, signals were rarely seen in the nuclei of endometriotic cells (Fig. 5a). In contrast, the immunoreactivity of survivin and MMP-2 was negative or weakly positive in the cytoplasm of eutopic endometrial cells (Fig. 4b,c), whereas it was strongly positive in that of ectopic endometrial cells (Fig. 5b,c). MT1-MMP expression was observed mainly on the cell membrane of glandular epithelial cells in endometriotic tissues (Figs 4d,5d). The immunoreactivity of MMP-9 in eutopic and

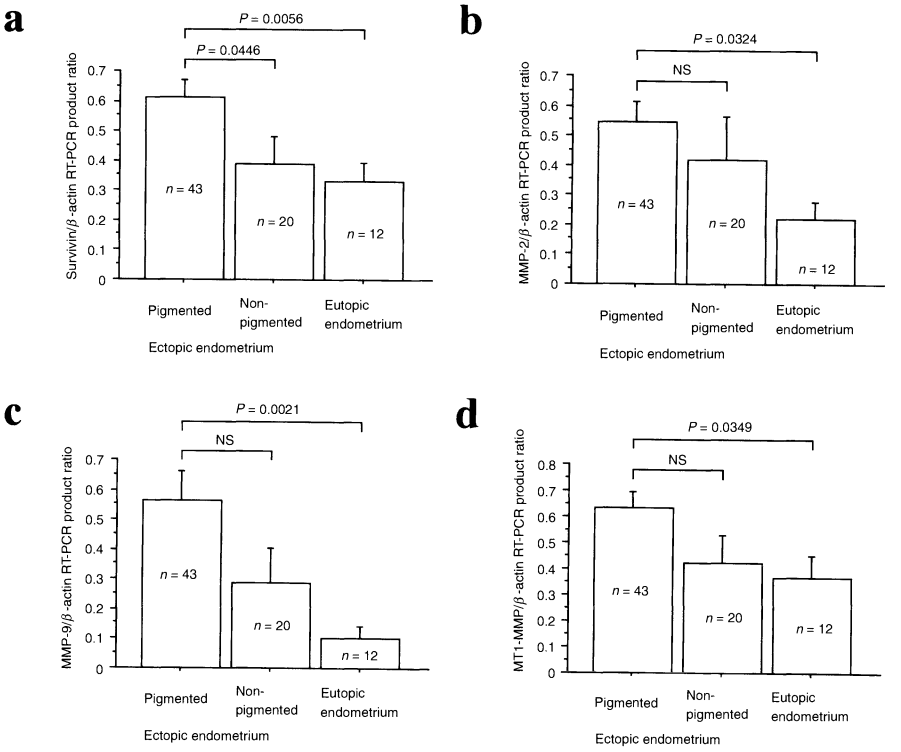

FIG. 2. Gene expression levels of survivin (**a**), matrix metalloproteinase (*MMP*)-2 (**b**), MMP-9 (**c**), and membrane-type 1 (*MT1*)-MMP (**d**) in pigmented and non-pigmented endometriotic tissues and normal eutopic endometrium. Values are expressed as relative expression levels of each target gene in comparison with β-actin expression (mean ± SEM). *NS*, not significant

ectopic endometrium was similar to that of MMP-2 (data not shown). Table 1 shows the overall results, including clinical data and histochemical staining outcomes. The apoptotic epithelial cells positive for TUNEL were detected in two of seven and only one of 11 cases in eutopic and ectopic endometrium, respectively. Positive immunostaining for survivin and MMPs was observed in all 11 specimens from ovarian endometriosis, regardless of the menstrual cycle phase. Glandular epithelial cells that overexpressed MMP-2 were also strongly positive for MT1-MMP in ectopic endometrial tissues. However, survivin, MMP-2, MMP-9, and MT1-MMP expression was weak and detected in one, two, two, and three of seven specimens from normal endometrium, respectively.

As shown in Fig. 6, survivin, MMP-2, and MT1-MMP mRNA expression levels in endometrial carcinomas were significantly higher than those in normal eutopic endometrium. Moreover, expression was well correlated with the depth of myometrial invasion (Fig. 7). There was a close correlation between survivin and MMP-2 or MT1-MMP and between MMP-2 and MT1-MMP gene expression levels in 26 endometrial carcinoma tissues examined (Fig. 8).

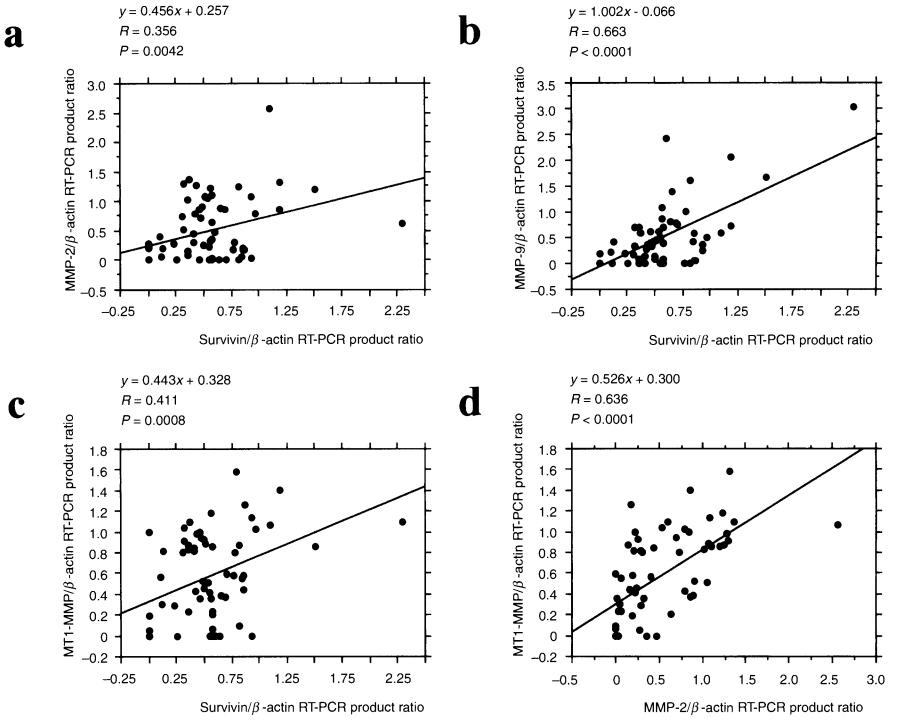

FIG. 3. Correlation between survivin and matrix metalloproteinase (*MMP*)-2 (**a**), MMP-9 (**b**), or membrane-type 1 (*MT*1)-MMP (**c**), and between MMP-2 and MT1-MMP (**d**) gene expression levels in 63 endometriotic tissues examined. Each point represents the mean of triplicates

TABLE 1. Clinical data and histochemical staining outcomes

Patient no.	Cycle	TUNEL	Survivin	MMP-2	MMP-9	MT1-MMP
Normal endometrium						
1	EP	−	−	−	−	−
2	LP	−	−	+	+	+
3	ES	+	−	−	−	+
4	ES	−	−	−	−	−
5	ES	+	−	−	−	−
6	ES	−	−	−	−	−
7	LS	−	+	+	+	+
Ovarian endometriosis						
8	EP	−	+	+	+	+
9	LP	−	++	++	++	++
10	LP	−	+	+	+	+
11	LP	−	+	+	+	+
12	ES	−	+	++	+	++
13	ES	−	+	+	+	+
14	ES	−	++	+	++	+
15	LS	+	+	+	+	+
16	LS	−	+	++	++	++
17	LS	−	++	+	+	+
18	LS	−	++	++	+	++

TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick-end labeling; *EP*, early proliferative; *LP*, late proliferative; *ES*, early secretory; *LS*, late secretory; *MMP*, matrix metalloproteinase; *MT1*, membrane-type 1

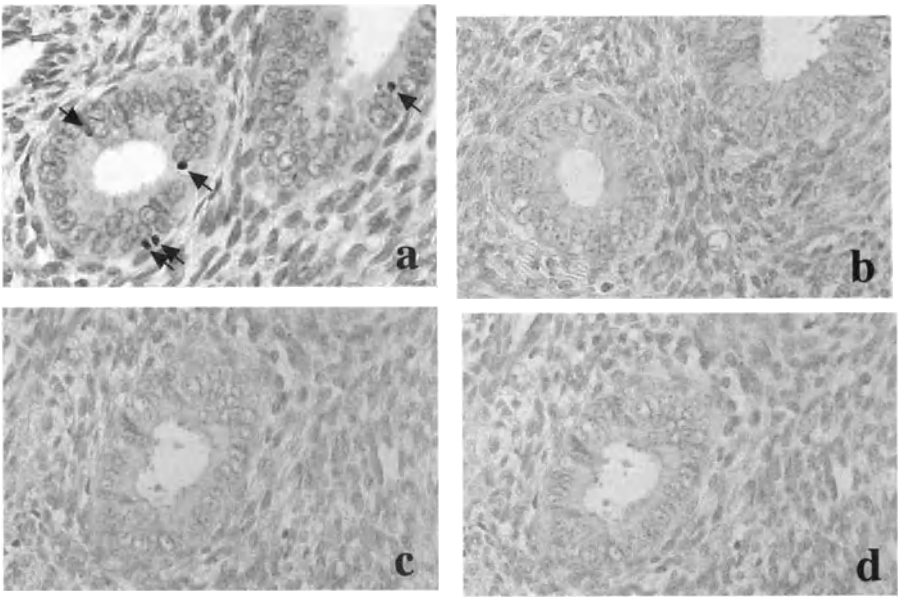

FIG. 4. Histochemical staining of normal eutopic endometrial tissue. Case 5 (Table 1) in the early secretory phase showed weakly positive staining for terminal deoxyribonucleotidyl transferase-mediated dUTP–biotin nick-end labeling (**a**; *arrows*), but negative immunostaining for survivin (**b**), matrix metalloproteinase (MMP)-2 (**c**), and membrane-type 1-MMP (**d**). Original magnification ×400

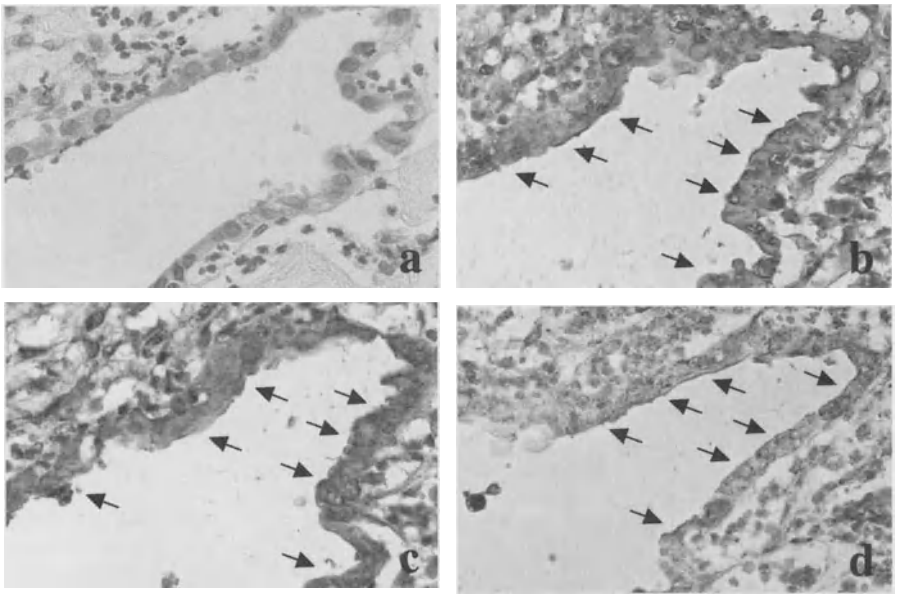

FIG. 5. Histochemical staining of ectopic endometrial tissue from ovarian endometriosis. Case 9 (Table 1) in the late proliferative phase showed negative staining for terminal deoxyribonucleotidyl transferase-mediated dUTP–biotin nick-end labeling (**a**), but strongly positive immunostaining for survivin (**b**), matrix metalloproteinase (MMP)-2 (**c**), and membrane-type 1-MMP (**d**) (*arrows*). Original magnification ×400

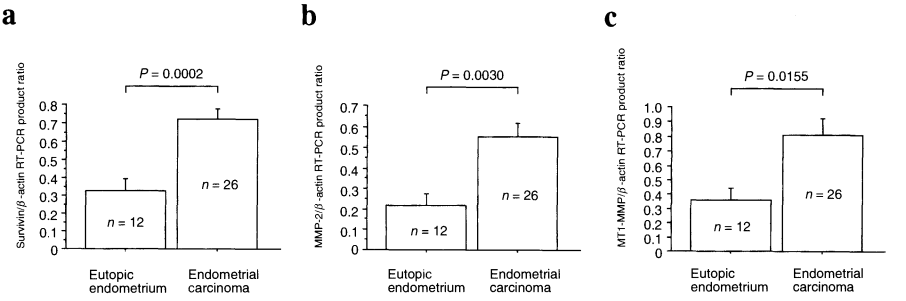

FIG. 6. Gene expression levels of survivin (**a**), matrix metalloproteinase (*MMP*)-2 (**b**), and membrane-type 1 (*MT*1)-MMP (**c**) in endometrial carcinoma tissues and normal eutopic endometrium. Values are expressed as relative expression levels of each target gene in comparison with β-actin expression (mean ± SEM)

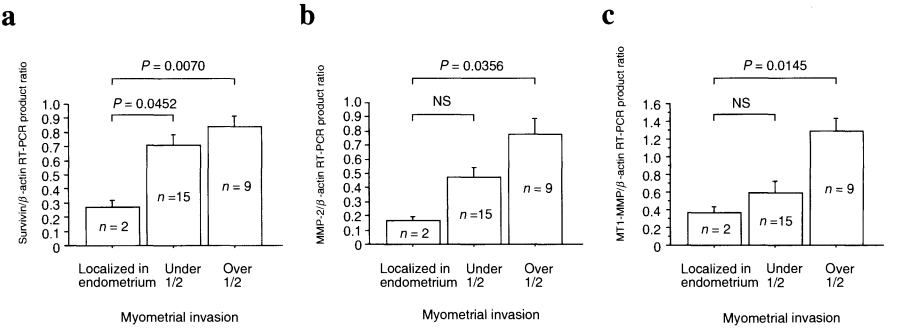

FIG. 7. Correlation between survivin (**a**), matrix metalloproteinase (*MMP*)-2 (**b**), and membrane-type 1 (*MT*1)-MMP (**c**) gene expression levels and the depth of myometrial invasion in 26 endometrial carcinoma tissues examined. Values are expressed as relative expression levels of each target gene in comparison with β-actin expression (mean ± SEM). *NS*, not significant

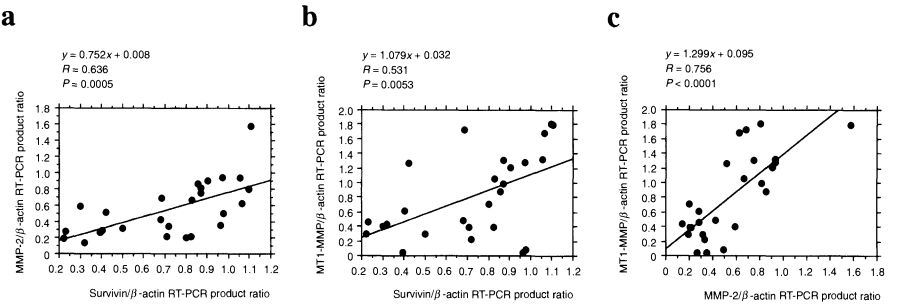

FIG. 8. Correlation between survivin and matrix metalloproteinase (*MMP*)-2 (**a**) or membrane-type 1 (*MT*1)-MMP (**b**), and between MMP-2 and MT1-MMP (**c**) gene expression levels in 26 endometrial carcinoma tissues examined. Each point represents the mean of triplicates

Discussion

Survivin is a novel inhibitor of apoptosis and is expressed during fetal development and in cancer tissues [11,12], but there have been very few reports on its biological significance in endometrial lesions [13,14,16]. Konno et al. [19] demonstrated that survivin gene and protein expression was detected in normal human endometrium and that survivin could play an important role in physiological homeostasis during the normal menstrual cycle. It could be expected that survivin is also expressed in ectopic endometriotic tissues and endometrial carcinomas.

In the present study, we investigated gene expression of survivin and MMPs in eutopic and ectopic endometrial tissues and found that survivin, MMP-2, MMP-9, and MT1-MMP mRNA expression levels in pigmented endometriotic lesions were significantly higher than those in normal eutopic endometrium. Moreover, survivin gene expression in pigmented lesions was higher than that in non-pigmented lesions. In patients with endometriosis, visual findings of peritoneal endometriotic foci are often recognized as typical red or black pigmented lesions and non-pigmented lesions seem to occur earlier during the development of the disease [20]. We reported previously that pigmented lesions, such as blueberry spot, blood breb, and chocolate cyst, were clinically more active and aggressive [21]. It is likely that upregulation of survivin and MMPs expression may contribute cooperatively to survival and invasion of endometriotic tissues. Interestingly, there was a close correlation between survivin and MMP-2, MMP-9, or MT1-MMP gene expression levels in 63 endometriotic tissues examined. Invasive endometriotic cells may escape from apoptosis by expressing higher levels of the survival gene survivin.

To confirm this hypothesis, we then performed a histochemical study on eutopic and ectopic endometrial tissues. Endometriotic lesions rarely contained apoptotic epithelial cells and only one of 11 cases showed weakly positive staining for TUNEL. Jones et al. [22] also demonstrated that apoptotic cells were rare in ovarian endometriosis and there was no significant difference in the number of apoptotic cells between eutopic and ectopic endometrium. Interestingly, immunohistochemical expression of survivin and MMPs was positive for all 11 specimens from ovarian endometriosis, and survivin expression in ectopic endometrium was markedly stronger than that in eutopic endometrium. Moreover, endometriotic cells that overexpressed MMP-2 were also strongly positive for MT1-MMP. MMP-2 has been shown to be activated by MT1-MMP, which possesses a functional transmembrane domain and acts as cell-surface receptor and activator for proMMP-2 [23,24]. Relative gene expression levels of MMP-2 were well correlated with those of MT1-MMP in endometriotic lesions, as described above. The upregulated invasiveness of endometriotic cells by MMP-2 may be closely linked to its activation by MT1-MMP. These results suggest that overexpression of both survivin and MMPs in endometriotic tissues may contribute to escape from apoptosis and development of the disease.

Takai et al. [13] reported that survivin expression was significantly associated with proliferating cell nuclear antigen-labeling index, clinical stage, histological grade, myometrial invasion, clinical outcome, and survival rate and suggested that survivin protein is a defining diagnostic marker for endometrial carcinomas that may also yield prognostic information. Lehner et al. [14] also demonstrated that survivin mRNA levels were increased in correlation with ascending grade in endometrioid ade-

nocarcinomas. Our present results revealed that survivin, MMP-2, and MT1-MMP mRNA expression levels in endometrial carcinomas were significantly higher than those in normal eutopic endometrium and were well correlated with the depth of myometrial invasion. Moreover, there was a close correlation between survivin and MMP-2 or MT1-MMP, and between MMP-2 and MT1-MMP gene expression levels in 26 endometrial carcinoma tissues examined. Survivin gene expression may be also closely associated with the invasive process of endometrial carcinomas.

In conclusion, these findings suggest that upregulation of survivin and MMPs may contribute cooperatively to the survival and invasion of endometriosis and endometrial carcinomas. However, at this stage, molecular interaction between survivin expression and invasive phenotype in these diseases remains unclear. Further studies are needed to clarify the molecular events that coregulate the expression of survivin and MMP genes in the development of endometrial lesions.

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Part 9
Endometrial Receptivity

Endometrial Cancer Cells as Models to Study Uterine Receptivity

Bruce A. Lessey, Aleksandr E. Vendrov, and Lingwen Yuan

Summary. Endometrial estrogen and progesterone receptors mediate gene expression during the menstrual cycle in all mammalian species. At the time of implantation, differential patterns of gene expression occur on both glandular and luminal (surface) epithelium. Glandular secretions appear to be essential for embryonic development and viability, whereas luminal epithelium may provide a site for attachment and subsequent invasion of the nascent embryo. We have characterized previously integrins and other extracellular matrix (ECM) molecules as differentiated markers of endometrial epithelium. Both luminal and glandular epithelium express constitutive $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins and hormonally regulated $\alpha 1\beta 1$, $\alpha 4\beta 1$, $\alpha 9\beta 1$, and $\alpha v\beta 3$. The glandular epithelium expresses hormonally regulated ECM glycoproteins, such as osteopontin. Luminal cells exhibit cycle-dependent patterns of other $\alpha 2\beta 1$ and selectively express cytokeratin 13. Endometrial cancer cell lines have provided an excellent model to study normal endometrial epithelium. Of the many available cell lines, Ishikawa and ECC-1 cells maintain normal hormonal responsiveness to estrogen and progesterone, as well as to androgens. In this chapter, we review the potential of Ishikawa and ECC-1 cells as surrogate cells for normal endometrial epithelium and discuss their characteristics of glandular and luminal epithelia, respectively. Further characterization of these two cell lines may provide new insight into the mechanism of embryo implantation in humans and shed light on possible defects in endometrial receptivity.

Key words. Implantation, integrins, uterine receptivity, cell lines, endometrium

Introduction

The endometrium is a unique tissue, undergoing programmatic changes throughout each menstrual cycle in preparation for pregnancy. The developmental changes that ensue in response to estrogen, progesterone, and androgens are exceedingly complex [1]. Given the heterogeneous nature of this tissue and the number of different cell

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types that function within the endometrium, there remains much to learn about the regulatory mechanisms involved in gene expression and the temporal and spatial changes that lead to a receptive endometrium. One of the major contributions toward these efforts has been the development of endometrial cancer cell lines. Unlike ovarian cell lines, establishment of endometrial cell lines has been somewhat more difficult [2]. As shown in Table 1, many suitable cell line models for endometrial epithelium now exist, although their characteristics and hormone responsiveness vary significantly.

Using endometrial cancer cell lines, significant progress has been made during the past 20 years in our understanding of endometrial function. The purpose of this chapter is to review some of the progress as it relates to endometrial receptivity and infertility, and to suggest how new technologies, such as DNA microarray analysis, may provide a valuable new avenue of research using the hormone-responsive endometrial cell lines. It is clear that while much remains to be understood in the endometrium, a great deal has already been revealed using these valuable model systems.

Endometrial Cells to Study Regulation of Implantation-Specific Genes

The endometrium serves as the site of embryo implantation and provides the interface between the nascent embryo and mother. Tolerance of trophoblast in-growth, suspension of immunologic surveillance while controlling unrestricted access to tissues beyond the endometrium, requires carefully orchestrated patterns of gene expression. Under the influence of ovarian steroids, via endocrine, paracrine, and autocrine mechanisms, the epithelial compartment becomes "receptive" toward implantation for a narrow interval of time, occurring, in the human, around cycle day 20–24 [10]. The endometrium is one of the few tissues into which the embryo will not normally implant, except for this transient "window of implantation" [11]. Glandular epithelium may have the initial role of nurturing the tenuous embryo, whereas the luminal epithelium prepares for initial adhesion. Stromal/decidual responsibility comes later. We can see this temporal pattern of responsibility played out in the order of cell-specific gene expression [12].

There are relatively few good in vitro models to study endometrial receptivity. Animal models have certainly played a leading role in the study of implantation [13], but species differences may limit their applicability. Primary endometrial cells have proven useful, but can be cumbersome to set up and maintain, and are limited by the potential number of cell divisions and hormone responsiveness [3].

Endometrial cancer cell lines have been used extensively to investigate regulation of specific target genes by steroid and peptide hormones and growth factors [14–16], and their use has been extended to the study of epithelial–trophoblast interaction as well [17–19]. The Ishikawa cell line represents one of several endometrial cell lines that maintain steroid receptors, coactivators, and responsiveness to ovarian steroids. This cell line, first developed by Nishida et al. [9], contains both a functional estrogen receptor (ER) α [21,21] and estrogen-inducible progesterone receptors (PR) [21,22], as well as androgen receptors (AR) [23]. We have since shown that the PR is func-

TABLE 1. Established endometrial cell lines

Tumor	Origin	Steroid responsiveness	Reference
HEC-1A	Moderately differentiated endometrial adenocarcinoma from primary tumor	Nonresponsive	3
HEC-1B	Sub-strain from HEC-1A	Nonresponsive	4
SCRC	Moderately differentiated endometrial adenocarcinoma	Nonresponsive	4
AN3CA	Poorly differentiated endometrial carcinoma	Nonresponsive	5
RL95-2	Endometrial adenosquamous carcinoma from primary tumor	Estrogen receptor variable	6
KLE	Endometrial adenocarcinoma from undifferentiated primary tumor	Nonresponsive	7
Ishikawa	Well-differentiated endometrial adenocarcinoma from primary tumor	Estrogen, progesterone, and androgen responsive	8
ECC-1	Well-differentiated endometrial adenocarcinoma from primary tumor	Estrogen, progesterone, and androgen responsive	9

tional in these cells with induction of specific progesterone-responsive genes, including the α1 integrin subunit and the ECM molecule osteopontin (OPN) [24,25]. Characterization of integrin expression [25] has demonstrated that similar complements of epithelial integrins present on normal epithelium are present on Ishikawa cells (Fig. 1). HOXA gene regulation [27] and the induction of secretory products, such as OPN [28], by estrogen and progesterone have also been demonstrated using these cells. Further usefulness of these and other cell lines is increasing as we identify features and characteristics that make them individually useful for the study of specific aspects of endometrial function.

Hormone Responsiveness in Endometrial Cancer Cell Lines

We and others have studied hormone-responsive and non-responsive endometrial cancer cell lines [16,20–24,29]. Whereas most can be induced to respond to ovarian steroids by transfection of full length ER, PR, or AR, a few have maintained endogenous hormone responsiveness. As shown in Fig. 2, of the many available endometrial cancer cell lines, two exhibit consistent estrogen and progesterone responsiveness. Using ER- and PR-response elements (ERE and PRE, respectively) in the chloramphenicol acyltransferase (CAT) reporter gene system, we were able to demonstrate

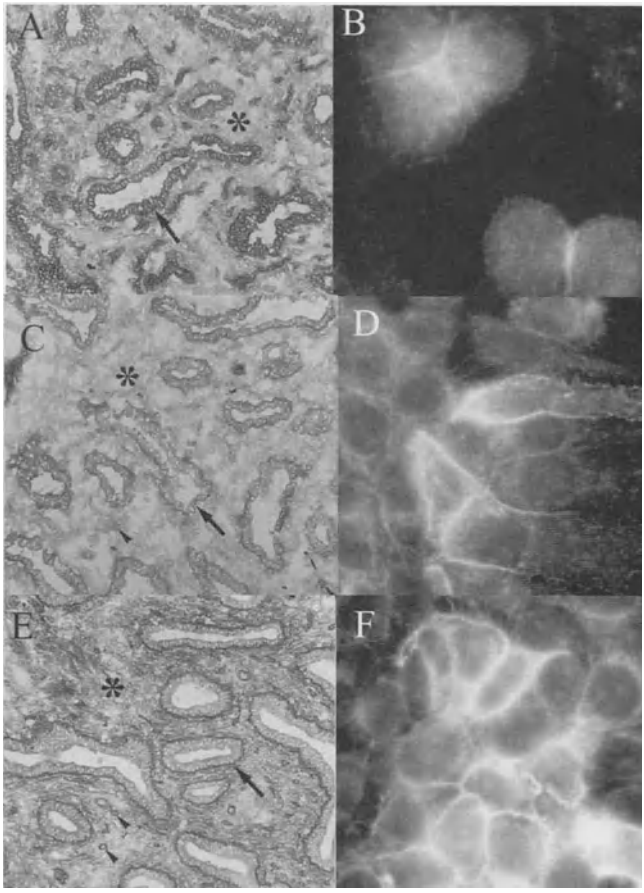

FIG. 1. Ishikawa cells and normal epithelium share many of the same integrins. Based on comparisons between normal endometrial epithelium and Ishikawa cells, many of the normal integrins expressed on glandular epithelium are also expressed on Ishikawa cells [42]

that both Ishikawa cells and ECC-1 cells exhibit activation of ERE-CAT promoter activity in response to estradiol (E2; 10^{-8} M) treatment. Likewise, in E2-primed cells, activation of PRE-CAT activity demonstrates a similar responsiveness to progesterone (P4) in both cell lines that is dependent on the induction of PR. The results of these studies are in keeping with those of previous reports demonstrating E2 and P4 responsiveness in these cell lines [22,30,31]. This retention of steroid responsiveness in these cell lines may reflect their well-differentiated phenotype compared with the other less-responsive cell lines that demonstrate a reduced steroid response.

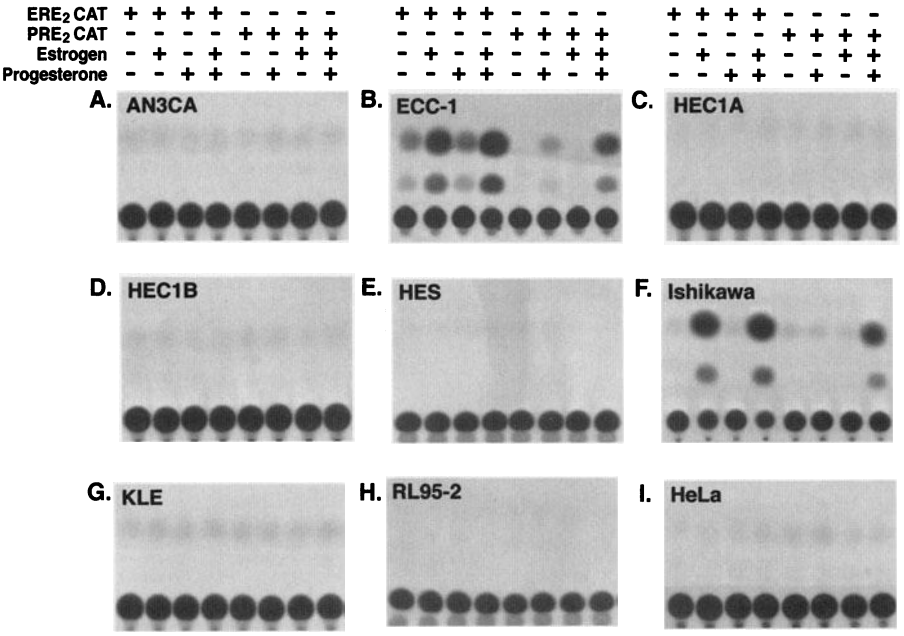

FIG. 2. Relative estrogen and progesterone responsiveness in various endometrial cell lines. Using a chloramphenicol acyltransferase reporter gene with estrogen- and propesterone-response elements (ERE and PRE, respectively), we compared the pattern of promoter activation in cells that were either untreated or treated with estradiol (E2; 10^{-8} M) plus or minus progesterone (P4; 10^{-6} M). Activity was noted by conversion of chloramphenicol into its metabolite. We noted that two of the nine cell lines tested (Ishikawa and ECC-1 cells) maintained estrogen responsiveness, as well as E2-inducible progesterone responsiveness. Whereas the other cell lines may harbor either estrogen or progesterone receptors, it would appear that the Ishikawa and ECC-1 cell lines maintain a robust response to both E2 and P4, similar to normal endometrial epithelium

Phenotypic Differences Between ECC-1 and Ishikawa Cells

Endometrial epithelium derives from the basalis and superficialis layers of the endometrium. Within the upper layer, both surface epithelium (luminal epithelium) and glandular epithelium can be identified. Each of these two epithelial subtypes exhibits similar but distinct cellular phenotypes. Using integrins as biomarkers, for example, we demonstrated distinct immunohistochemical properties for these two types of endometrial epithelium [32]. We recently suggested that ECC-1 and Ishikawa cells manifest characteristics that resembles a luminal (ECC-1) and glandular (Ishikawa) phenotype, respectively [33]. Olson et al. reported on how the differential expression of certain cytokeratins may differentiate luminal from glandular endometrial epithelium in both rabbit and human endometrium [34]. One cytokeratin,

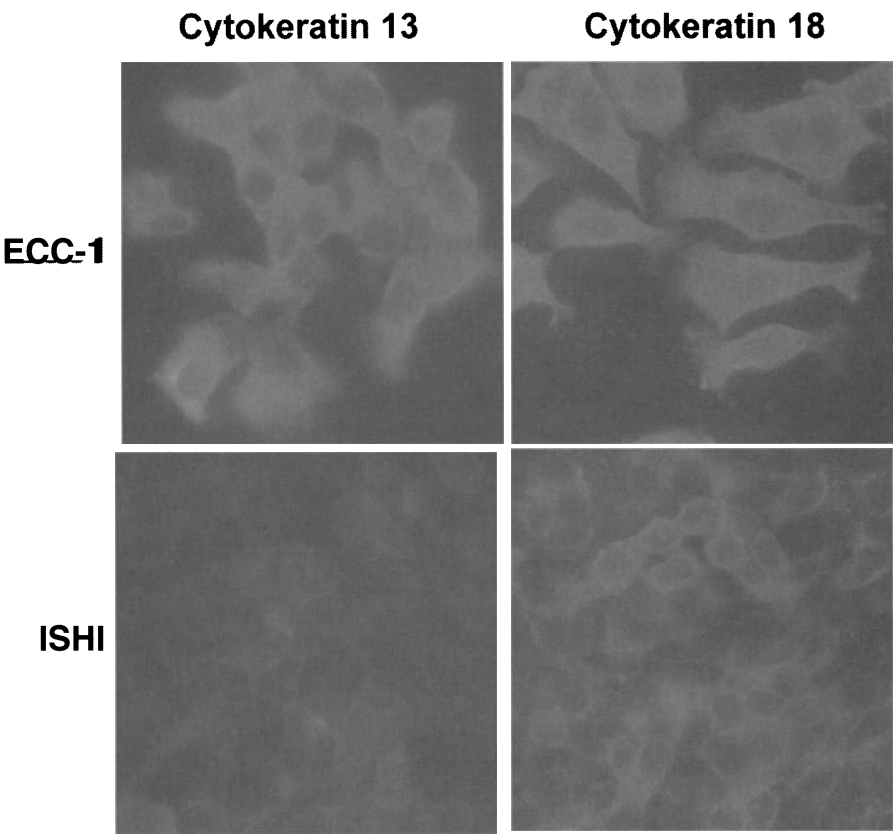

FIG. 3. Cytokeratin 18 and 13 expression in Ishikawa (*ISHI*) and ECC-1 cells. Using immunofluorescence and specific monoclonal antibodies to each intermediate filament protein, we demonstrate that ECC-1 cells maintain a higher expression of cytokeratin 13 compared with Ishikawa cells, whereas both cell types express cytokeratin 18

cytokeratin 18, appears to be expressed by both luminal and glandular cells, whereas cytokeratin 13 is generally confined to the surface epithelium. As shown in Fig. 3, the differential expression of cytokeratins 13/18 in two endometrial cell lines suggests that ECC-1 cells bear a phenotype more closely aligned with luminal epithelium, whereas Ishikawa cells appear more similar to glandular epithelium.

Other biologic markers appear to be differentially expressed between luminal versus glandular epithelium of normal healthy endometrium (Table 2; Figs 4, 5). We have demonstrated previously that constitutive and cycle-dependent patterns of integrin expression were present in normal endometrial epithelium [35] and that the $\alpha v\beta 3$, $\alpha 4\beta 1$, $\alpha 9\beta 1$, and $\alpha 2\beta 1$ integrins were present and differentially regulated on the glandular and luminal epithelium during the menstrual cycle [31,36]. Differential regulatory mechanisms have been proposed based on studies involving endometrial cell lines. A direct action of P4 has been demonstrated for certain epithelial proteins, such as the $\alpha 1$ integrin subunit [25] and the ECM molecule [28], whereas paracrine mech-

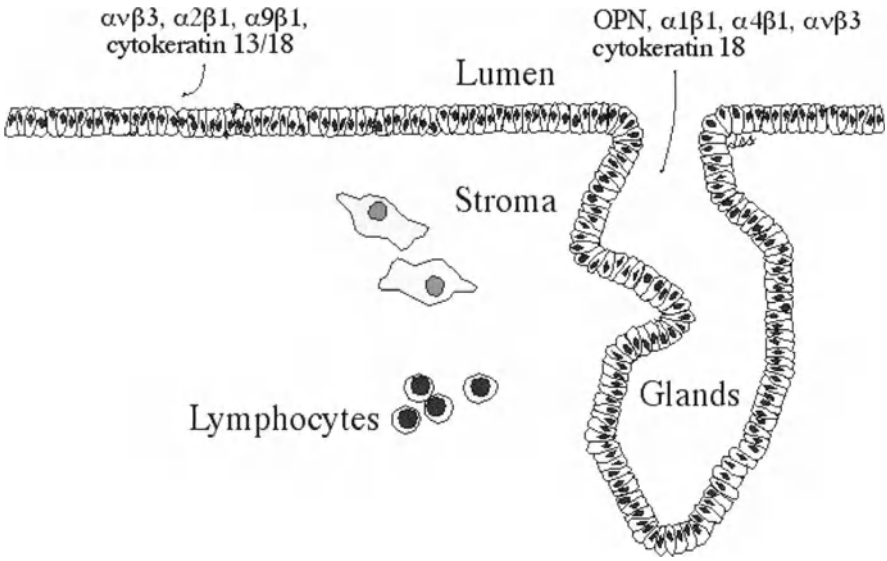

FIG. 4. Differential expression of endometrial biomarkers on luminal (surface) and glandular epithelium. Note that subtle differences exist that may provide clues to the segregation of function between secretory glandular epithelium and surface epithelium. *OPN*, osteopontin

TABLE 2. Differential expression of surface markers on luminal versus glandular epithelium in normal endometrium

	Proliferative		Secretory		Ishikawa	ECC-1
	Glands	Lumen	Glands	Lumen	(gland)	(lumen)
α1	–	–	+	–	σ (P4)	–
α2	+	+	+	–	+	τ (P4)
α4	–	–	+	–	σ (P4)	–
α6	+	+	+	+	+	+
αvβ3	–	–	+	+	σ (EGF)	σ (EGF)
DAF	–	–	+	+	σ (EGF)	σ (EGF)
OPN	–	–	+	(+)	σ (P4)	–

DAF, decay accelerating factor; *OPN*, osteopontin; *P4*, progesterone; *EGF*, epidermal growth factor

anisms also appear to regulate other genes, such as αvβ3 and decay accelerating factor (DAF) [37,38]. The expression of several integrin subunits, including β3, α6, and α2, are positively regulated by epidermal growth factor (EGF) or EGF-like growth factors [15,39] and the appearance of the αvβ3 integrin on glandular and luminal epithelium corresponds with the mid-secretory phase disappearance of the endometrial ER-α [32]. As shown in Fig. 6, using flow cytometry, the regulation of certain integrins can be effectively studied and compared between Ishikawa and ECC-1 cells, providing further evidence that their phenotypes are slightly different. Such differences provide

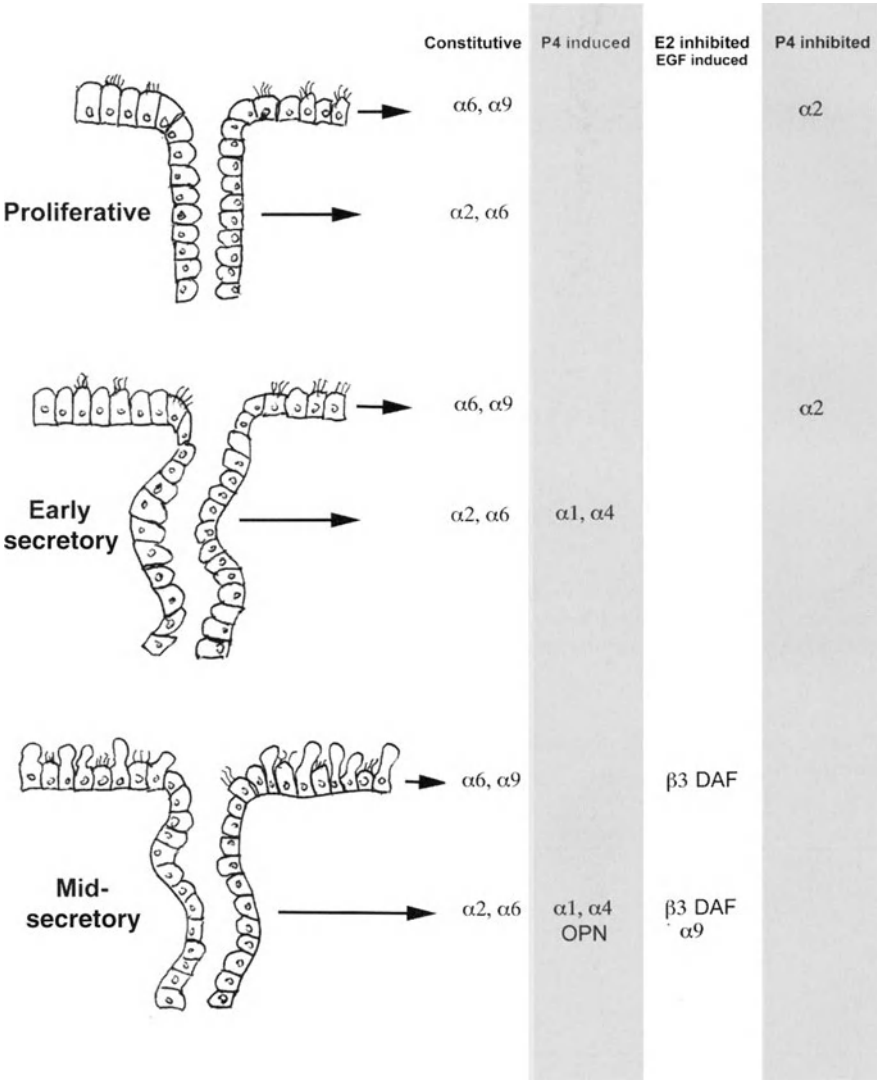

FIG. 5. Menstrual cycle-specific changes in biomarkers in luminal versus glandular epithelium may be useful to study endometrial cell lines as functional surrogates for these subtypes of endometrial epithelium. Using endometrial cancer cell lines, different regulatory pathways have been suggested. *OPN*, osteopontin; *DAF*, decay accelerating factor; *P4*, progesterone; *E2*, estradiol; *EGF*, epidermal growth factor

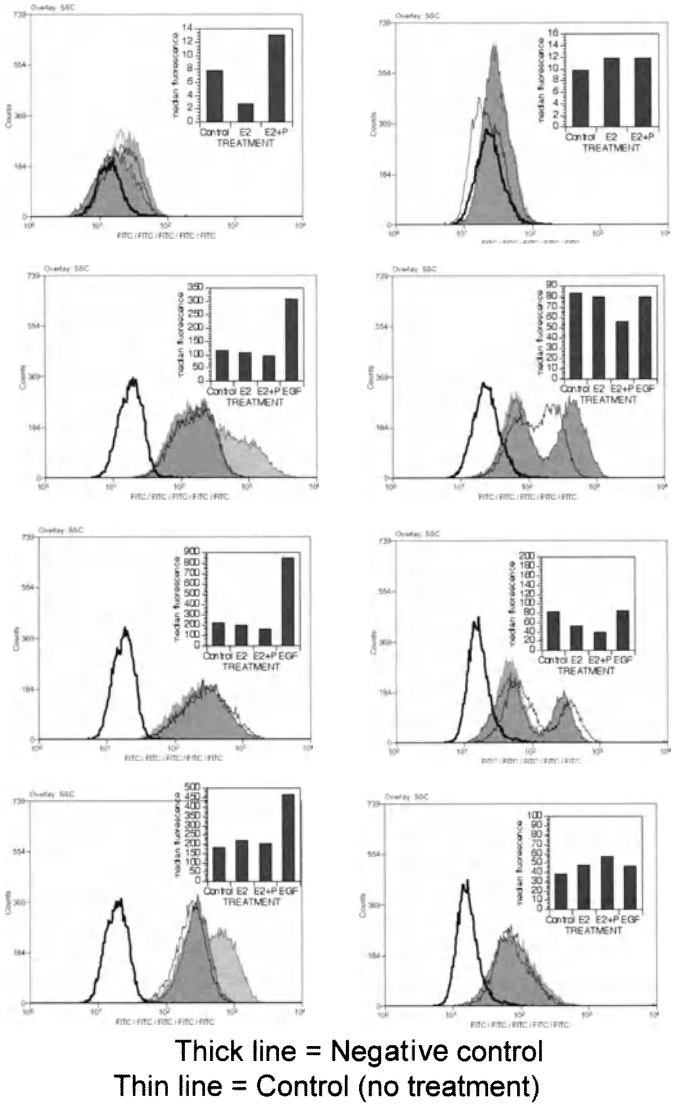

FIG. 6. Flow cytometry in treated and untreated Ishikawa and ECC-1 cells demonstrating regulated expression of several integrin subunits by both ovarian steroids, as well as epidermal growth factor (*EGF*). *P*, progesterone; *E2*, estradiol

models to gain a better understanding about the regulated expression of genes in luminal versus glandular epithelium during the normal menstrual cycle and, perhaps, in certain types of infertility.

DNA Microarray: The Future of Gene Discovery Using Endometrial Cell Lines

With the completion of the human genome project and the availability of all human gene sequences, researchers are able to probe complex tissues for the presence or absence of thousands of genes simultaneously. In the endometrium, which has a well-defined cyclic pattern of development each month, the use of DNA microarray technology provides an exciting new avenue for gene discovery. In the human, differential patterns of gene expression between the proliferative and mid-secretory phase [1] and between the early and mid-secretory transition [40] have been published. Such exercises have provided an enormous wealth of information, although the volume of new genes requiring validation can be quite large. In these two recent publications cited above, we were able to validate only a small subset of the many interesting genes discovered. Using tissue microarray, with multiple samples of endometrium on a single sectioned paraffin block (tissue microarray), we hope to more rapidly evaluate and validate new genes using in situ or immunohistochemistry to establish with greater certainty the temporal and spatial distribution of these new genes during the menstrual cycle.

There are potential pitfalls of mass DNA screening. One problem is the heterogeneity of the tissue itself. The endometrium consists of epithelial, mesenchymal, endothelial, and leukocyte populations with gene expression patterns that shift during the course of a menstrual cycle. A more direct and satisfying method may include the use a defined (homogeneous) endometrial cell set as the starting point for mRNA preparation. Given the complexity of resident cell types, and their tendency to shift during the cycle, the use of a purified, relatively homogeneous cell line would have great potential benefits of improved cellular specificity for the gene discovery process. Furthermore, in culture, these cells could be treated with any number of hormones or cytokines to assess regulation of gene expression.

For endometrial epithelial cells, the use of available endometrial cancer cells could also be useful. Success of this approach is based on the assumption that endometrial cancer cells (or even normal primary cells) taken out of context of the entire tissue still exhibit "normal" patterns of gene expression. Of course, by definition, cancer cells are not normal and, within the cell population, heterogeneity still exists. Whereas the well-differentiated Ishikawa cells have, to a large measure, been found to closely mimic the normal endometrial epithelium [15,21,22,25,41], this may not be true in every instance. In addition, the loss of ECM or paracrine interactions may alter, reduce or accentuate gene expression. In a recent study, we showed that Ishikawa cells, which do not normally express the secretory product glycodelin (PP14), expressed this gene product in a hormone-responsive manner when cultured with human stromal cells [23]. Likewise, normal endometrial epithelium, which loses hormone responsiveness when dissociated in culture, regains a more normal phenotype in the presence of ECM and endometrial stromal cells under certain conditions [42]. In addition, both

primary and neoplastic cell lines exhibit morphologic changes reminiscent of glandular structures when cultured in contact with ECM.

Summary and Conclusions

The advent of well-characterized human cell lines has provided an avenue for research in many areas of medicine. In the female reproductive tract, the development of endometrial cell lines with varying characteristics has greatly enhanced our ability to identify and study new gene products. Of the many cell lines that have been developed, only a handful of retain normal hormone responsiveness and functional ER, PR, and AR. In two cell lines, namely ECC-1 and Ishikawa, cells derive from well-differentiated endometrial adenocarcinoma and maintain hormonal characteristics that are reminiscent of luminal and glandular epithelium, respectively. Ishikawa cells have been very well characterized by multiple investigators, whereas ECC-1 cells have been less well studied. Given the models that are currently being developed using embryos or surrogate embryo spheroids to study implantation, such differentiation of cell types into glandular or luminal cells may prove a useful solution for the construction of an artificial endometrial model. The future appears bright for this direction of investigation.

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Part 10
Chemo-resistance and -sensitivity

Steroid and Xenobiotic Receptor (SXR) is a Key System for the Acquisition of cis-Platinum Resistance in Endometrial Cancer Cells

Hideki Sakamoto, Noriko Takami, and Tatsuo Yamamoto

Summary. A novel steroid and xenobiotic receptor (SXR) may be involved in chemoresistance. The role of the SXR in chemoresistance was studied in endometrial cancer cell lines. Ishikawa (cisplatin (CDDP) sensitive) and the CDDP-selected subclone ISIW$^+$ (resistant) were used. The ISIW$^+$ subclone showed much higher SXR expression. When Ishikawa cells were cultured with antisense oligonucleotides (AS), the cells failed to pass crisis and did not gain CDDP resistance. Scid mouse experiments showed AS-treated animals all survived, whereas control animals had a 50% survival at 35 days ($P < 0.0001$). The present data indicate that SXR is a key system to induce, maintain, and reverse the CDDP-resistant phenotype in endometrial cancer cells.

Key words. Steroid and xenobiotic receptor (SXR), drug resistance, endometrial cancer, CDDP

Introduction

Platinum-based multidrug chemotherapy is the first choice of treatment for advanced ovarian, endometrial, and cervical cancers in either adjuvant or neoadjuvant settings [1]. This treatment has been shown to increase the cancer progression-free intervals, but does not significantly prolong survival. Eventually, patients with advanced-stage disease develop chemoresistance and succumb to the disease [2,3]. Therefore, preventing cancer recurrence by suppressing the development of chemoresistance is the most important objective to successfully control the disease. Several factors contribute to the development of chemoresistance [4,5]. Aneuploidity and the expression of MDR, glutathione S-transferase pi (GSTpi), and excisional repair genes have been shown to confer chemoresistant phenotypes. In a recent study, we mapped gene expression in a cis-platinum-sensitive cell line, as well as in a resistant subclone, using gene array analysis [6] and showed clear differences in GSTpi expression in resistant cells by means of an unknown mechanism. A potential induction pathway of GSTpi

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expression may involve the steroid and xenobiotic receptors (SXR), which have been implicated in the control of GST family expression [7]. However, the impact of the SXR and GSTpi on chemoresistant phenotypes in endometrial cancers is not understood. Thus, in the present study, we explored the control mechanisms of SXR-GSTpi expression in endometrial cancers.

Methods

Cell Lines and Animals

Ishikawa human endometrial cells and the CDDP-resistant subclone ISIW$^+$ were used in in vitro studies. The establishment and characteristics of the ISIW$^+$ cell line and the culture conditions have been reported previously [6]. Briefly, cells were cultured in Eagle's MEM medium (Nissui) supplemented with 10% fetal bovine serum (FBS; GIBCO). We used a medium without phenyl red and estrogen levels in the final incubation medium were adjusted to less than 10^9 M using a specific radioimmunoassay. ISIW$^+$ cells (10^5 cells; Falcon 35 mm, 3002) were then incubated with 1.86 μM cisplatinum (Billeting, Bristol Pharmaceuticals, K.K.) and Ishikawa cells were incubated with the standard vehicle. Both cell lines were transplanted every 7 days. The number of viable cells in culture plates was counted using the WST-1 assay (Premixed WST-1 cell proliferation assay system; TAKARA) using a microplate recorder (MPR A4i; TOSO).

Six-week-old CB-17/lcr-scid Jcl mice were used for the in vivo study. Animals were kept in a clean room with a 12 h light–dark cycle. Water and pellets were given ad libitum. Animals ($n = 10$ per group) were injected intraabdominally with Ishikawa or ISIW$^+$ cells (10^6 cells) and their survival was monitored.

Effects of an SXR Ligand on CDDP Resistance

The SXR is known to bind with dexamethasone (DEX) and activates CYP3A4 or GST A2, which are important in the metabolism of many drugs. In order to study the direct effects of DEX binding and activation of the SXR on CDDP resistance, Ishikawa and ISIW$^+$ cells (3000) were cultured in microplates (Corning 25820) with between 10 nM and 10 μM DEX for 48 h. Cells were then exposed to 10 μM CDDP for 48 h to determine whether the cellular sensitivity to CDDP was altered.

Gene Expression Study

SXR and GSTpi expression in cells was measured by reverse transcription–polymerase chain reaction (RT-PCR). Cells were homogenized quickly with a Polytron homogenizer in 1 mL ice-cold Zol B solution (RNA Zol B Isolation Mixture; Tel-Test, USA). The mixtures were further incubated at room temperature for 5 min to dissolve the cells. Whole RNA from the lysates was extracted with chloroform and isopropanol. The extracted RNA was precipitated with ethanol and then dissolved in 20 μL water. The reverse transcription, with approximately 5 μg RNA templates, was performed using the First-strand cDNA synthesis Kit (Pharmacia Biotech). The synthesized cDNA was dissolved in 20 μL water containing 0.2% DEPC and stored at −80°C

TABLE 1. Primer sequences used in the present study

	Primer sequence	Annealing temperature (°C)	No. cycles
SXR	5′-CCC CAC CCC CAG TTC AGT CT-3′ 5′-CCT GGA GCC CAC AAA GCA GC-3′	61.6	30
GSTpi	5′-ATG GGA AGG ACC AGC AGG AGG CAG-3′ 5′-GAG AGC AGG GGG AAC GCA TCC AGG-3′	58.3	30
β-Actin*	5′-CTC CTG CTT GCT GAT CCA CAT-3′ 5′-AAC CGC GAG AAG ATG ACC CAG-3′	55	35

* From Takemura et al. [18]

TABLE 2. Steroid and xenobiotic receptor antisense and nonsense oligonucleotide

Antisense	CTCCTGTCCTGAACAAGGCAGCGGCTCCTTGGTAAAGCTACTCCTTGATC
Nonsense	GATCAAGGAGTAGCTTTACCAAGGAGCCGCTGCCTTGTTCAGGACAGGAG

until use. SXR and GSTpi expression was evaluated using RT-PCR. The human SXR and GSTpi sequences were obtained from recent reports [8]. β-Actin was also amplified to ensure an accurate mRNA/cDNA preparation. Briefly, 1 μg/μL cDNA template was added to the PCR mixture containing 200 nM ATP, TTP, CTP, and GTP, 1 μM primers, 1.25 mM $MgCl_2$, 1.25 mM KCl, and 2 U recombinant Taq polymerase (Takara) in 20 μL Tris-HCl buffer (pH 8.2). The PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and the density of the corresponding bands was measured using image-processing software (NIH Image 6.0). The primers used in the present study and their amplification conditions are shown in Table 1.

Gene expression was measured by constructing a dose-response curve using cDNA from Ishikawa cells. A series of diluted standard cDNA (6 μg cDNA from Ishikawa cells) solutions (1/2, 1/4, 1/8, and 1/10 dilutions) were prepared. The standard was run together with the RT-PCR. The intensity of the gene expression in samples was arbitrarily compared with the intensity (normalized over the β-actin intensity) of the nondiluted Ishikawa cDNA.

SXR Antisense Oligonucleotide Experiments

SXR antisense oligonucleotides (AS) against the DNA-binding domain were constructed and the effects were studied both in vitro and in vivo. The AS and nonsense oligonucleotides (NS) sequences are shown in Table 2. Ishikawa and $ISIW^+$ cells were cultured with 132 μg/mL AS and NS for 48 h. Cells were then incubated with 10 μM cisplatinum for 48 h. After the treatment, approximately 3000 cells/150 μL were placed in the wells of microtest plates (96-well plates; Falcon), 10 μL WST-1 mixture was added to the wells, and cells were stained for 2 h at 37°C. Viable cell numbers were estimated by reading the optical density (OD) at 450 nm using an ELISA plate reader.

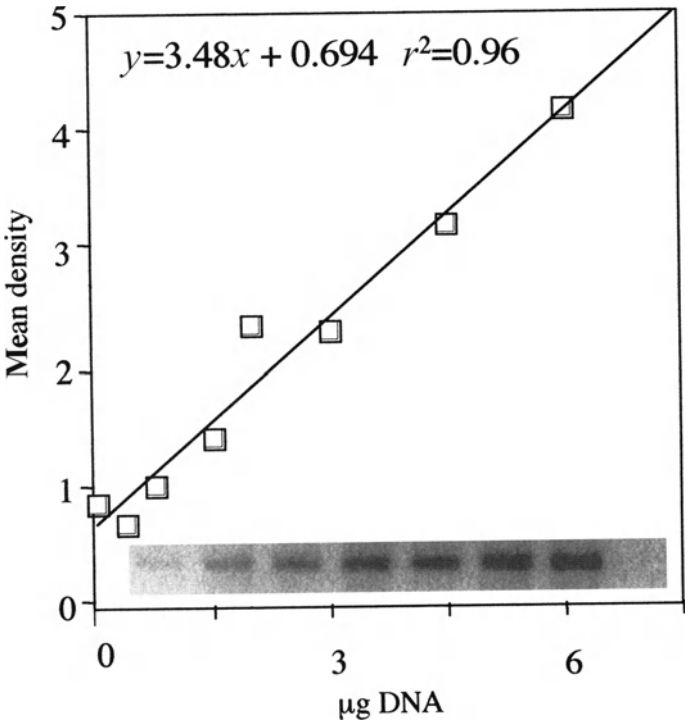

FIG. 1. To measure steroid and xenobiotic receptor (SXR) expression, cDNA from Ishikawa cells was serially diluted and the *SXR* gene was amplified in 30 rounds. The relative mean density was measured and the values were normalized by β-actin density. The mean density was then plotted to ensure linearity. The representative curve shown here had good linearity of $r^2 = 0.96$. When r^2 was lower than 0.80, the experiment was considered noninformative and a new set of experiments was performed

Statistical Analysis

Scid mouse survival was tested by Kaplan–Meier analysis, run with an SPSS package. Differences in gene expression were tested with unpaired Student's *t*-test (SPSS). Unless indicated otherwise, data are the mean ± SEM.

Results

SXR expression was initially studied in Ishikawa and ISIW$^+$ cells. The standard curve generated from the serial dilution showed linearity (Fig. 1) with amplification of less than 30 cycles. The SXR was expressed in both Ishikawa and ISIW$^+$ cells. SXR expression measured by RT-PCR was estimated at 2.00 ± 0.25 and 8.00 ± 0.27 in Ishikawa and ISIW$^+$ cells, respectively. SXR expression in ISIW$^+$ cells was much higher than in Ishikawa cells (Student's *t*-test $P < 0.004$; Fig. 2), suggesting that SXR interaction with

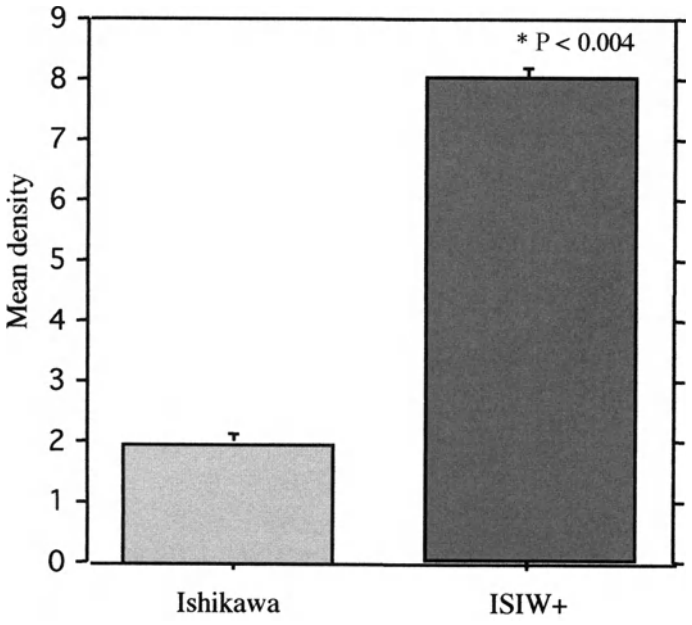

FIG. 2. Steroid and xenobiotic receptor expression in ISIW$^+$ cells showed a fourfold increase over the mother clone, Ishikawa cells. The difference was statistically significant ($P < 0.004$, unpaired Student's t-test)

a ligand may induce CDDP resistance. Therefore, we tested the two cell lines for CDDP resistance after exposure to between 10 nM and 10 μM DEX for 48 h. Figure 3 shows that both Ishikawa and ISIW$^+$ cell lines showed no apparent alteration in sensitivity to CDDP after DEX treatment.

In vitro treatment with the AS inhibited SXR expression in both Ishikawa and ISIW$^+$ cells. The inhibition in Ishikawa cells was estimated to be 2.00 ± 0.25 vs. 1.10 ± 0.21, whereas inhibition in ISIW$^+$ cells was estimated at 8.00 ± 0.27 vs. 2.47 ± 0.20 (measurement in quadruplicate, three experiments repeated; $P < 0.01$ for Ishikawa cells and $P < 0.0001$ for ISIW$^+$ cells; Fig. 4). GSTpi expression was also measured after AS treatment. The AS inhibited GSTpi expression in Ishikawa cells (0.71 ± 0.68 vs. 0.003 ± 0.006; $P < 0.0001$), but not in ISIW$^+$ cells (2.63 ± 0.33 vs. 2.56 ± 0.14; Fig. 5). When ISIW$^+$ cells were cultured with the AS or NS (135 μg/mL) before treatment with CDDP, they showed no resistance to CDDP (Fig. 6). In addition, when Ishikawa cells were chronically cultured with the AS, cells failed to pass the crisis and did not develop CDDP resistance (Fig. 7).

The in vivo effects of the AS were also evaluated using CB-17/lcr-scid Jcl mice. Scid mice ($n = 10$/group) were injected with 10^6 cells pretreated with CDDP (7.46 μM) and either the AS or NS (135 μg/mL). Animals were killed on day 14 and implants found in the abdominal cavity were counted. All 10 animals injected with ISIW$^+$ cells pretreated with the AS plus CDDP showed no visible intraperitoneal implants. In contrast, six of 10 animals that received the NS with CDDP had intraperitoneal implants

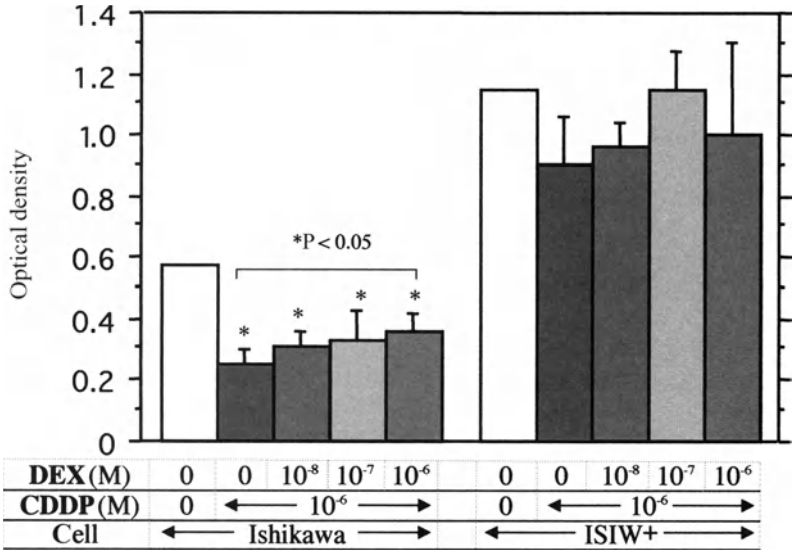

FIG. 3. Ishikawa and ISIW$^+$ cells were treated with the steroid and xenobiotic receptor ligand dexamethasone (*DEX*) and cisplatin (*CDDP*). Ishikawa cells were sensitive to CDDP ($*P < 0.05$), whereas ISIW$^+$ cells remained resistant to CDDP

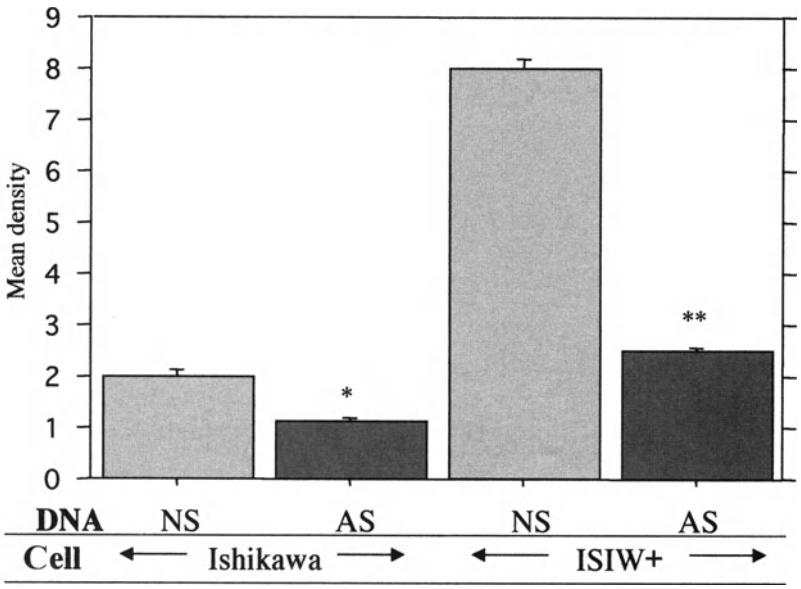

FIG. 4. When Ishikawa and ISIW$^+$ cells were cultured with antisense oligonucleotides (*AS*) or nonsense oligonucleotides (*NS*), steroid and xenobiotic receptor expression was significantly decreased ($*P < 0.001$, $**P < 0.0001$). The magnitude of the decrease appeared much higher in ISIW$^+$ cells

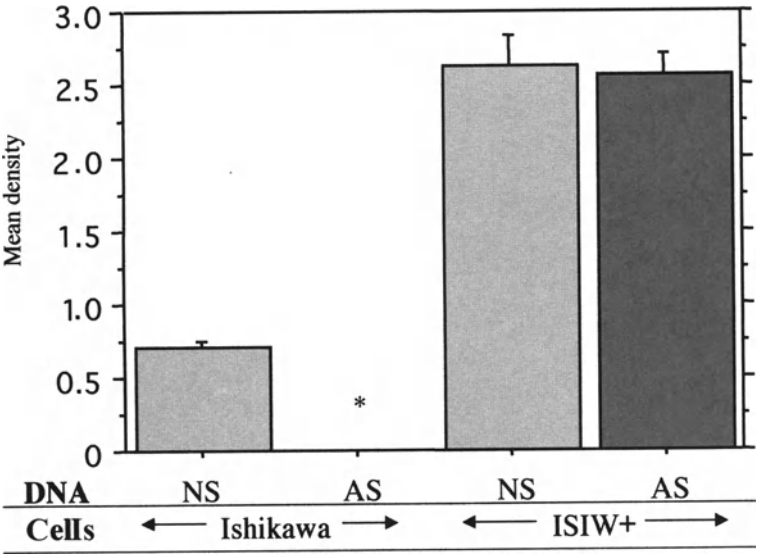

FIG. 5. Glutathione S-transferase pi expression after treatment with antisense oligonucleotides (*AS*) or nonsense oligonucleotides (*NS*) was significantly decreased in Ishikawa cells (*$P <$ 0.0001), but was not changed in ISIW$^+$ cells

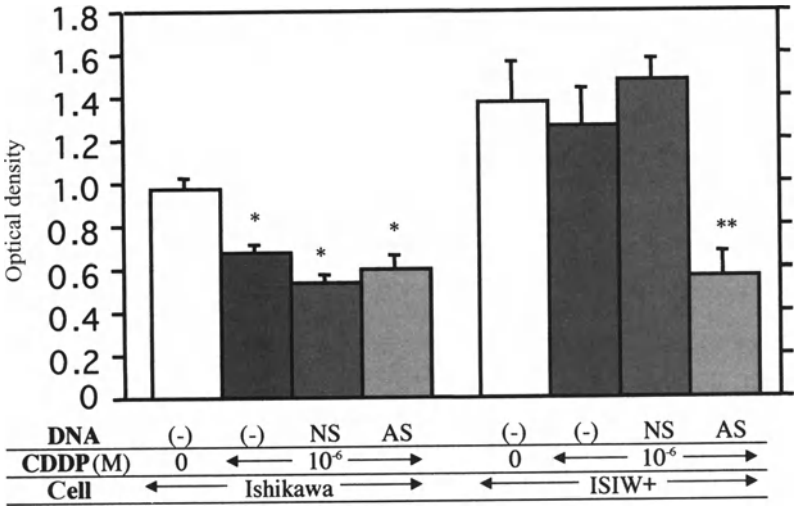

FIG. 6. When Ishikawa and ISIW$^+$ cells were acutely cultured (48 h) with the antisense oligonucleotides (*AS*) or nonsense oligonucleotides (*NS*; 135 μg/mL) before treatment with cisplatin (*CDDP*), Ishikawa cells showed normal sensitivity to CDDP (*$P < 0.05$). CDDP resistance was reversed by AS treatment in ISIW$^+$ cells (**$P < 0.005$)

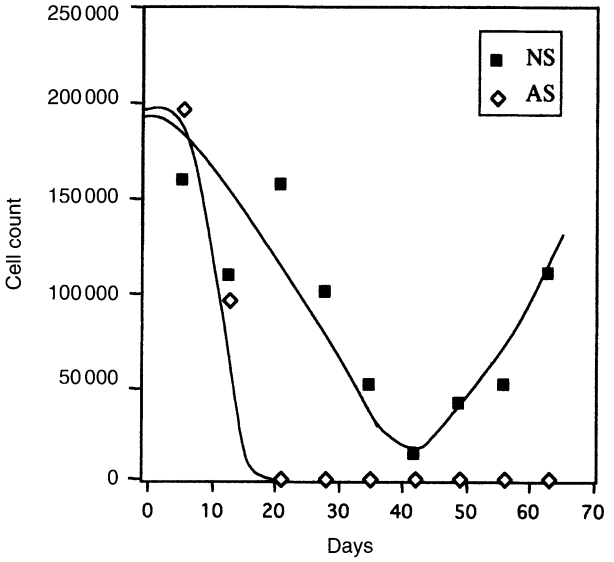

FIG. 7. To determine whether the antisense oligonucleotide (*AS*) inhibits the Ishikawa to ISIW$^+$ transformation, the two cell lines were cultured chronically with 1.86 μM cisplatin with the AS or nonsense oligonucleotide (*NS*). Ishikawa cells cultured with the AS failed to pass the crisis point and died 3 weeks later, whereas Ishikawa cells cultured with the NS survived the crisis and transformed into ISIW$^+$ cells

(Fig. 8). AS treatment significantly inhibited implant formation ($P < 0.02$, Fisher's exact test).

In a separate set of experiments, 20 animals (10 per group) received the treatments described above and animals were observed for 50 days. No animals died in the AS with CDDP treatment group, whereas 50% of mice treated with the NS and CDDP died by day 37. All animals in the NS and CDDP treatment group died by day 50 post-injection and the difference in survival between the two groups was highly significant ($P << 0.0001$, log-rank test; Fig. 9).

Discussion

The novel orphan receptor pregnane X receptor (PXR) is activated by pregnanes and was originally cloned from a mouse liver cDNA library [9]. The human homolog (SXR) was then cloned from human liver cDNA [10]. The SXR is activated by the pregnenolone derivatives glucocorticoid and RU-38486. Activation of SXR results in transcription of CYP3A enzymes that are important in the detoxification/biotransformation of many drugs [11], including anticancer agents, such as CDDP. In addition, recent studies have shown that the SXR directly binds with a promoter sequence of the glutathione S-transferase gene [7] or P-glycoproteins [12]. Therefore, we tested the hypothesis that the SXR system plays an important role in the drug resistance of endometrial cancer.

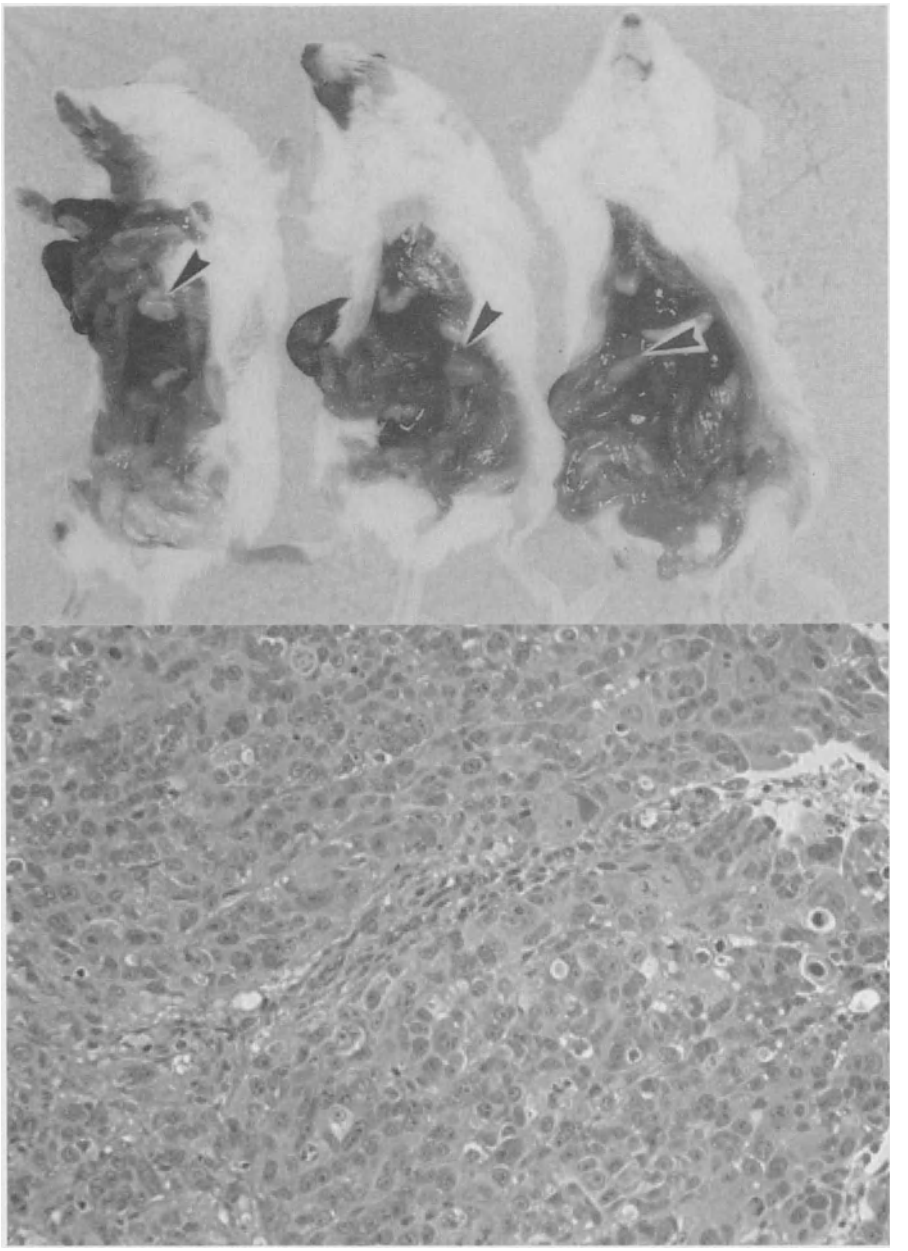

FIG. 8. Scid mice injected with ISIW^{+} cells pretreated with nonsense steroid and xenobiotic receptor oligonucleotides developed peritoneal implants by day 14 postinjection. The implants were poorly differentiated tumor cells with high mitotic indices and atypical nuclei

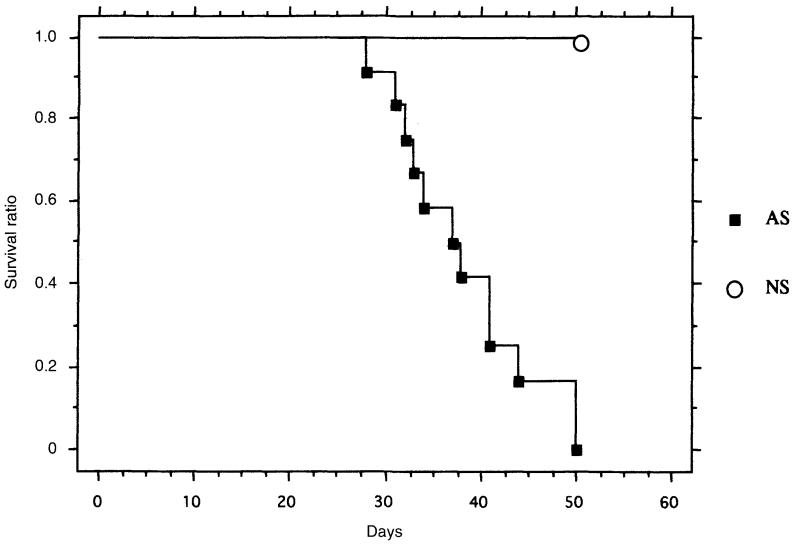

FIG. 9. Scid mice (n = 10 per group) were injected with 10^6 cells pretreated with cisplatin (7.46 μM) with antisense oligonucleotide (*AS*) or nonsense oligonucleotide (*NS*; each 135 μg/mL). During the 50-day obserbation period, no animals died in the AS-treated group, whereas all animals died in the NS-treated group. The 50% survival period for the NS group was 37 days

First, we established a CDDP-resistant subclone of the Ishikawa cell line, namely ISIW$^+$ [6]. Both Ishikawa and ISIW$^+$ cell lines expressed the SXR, indicating that these cells are able to operate the SXR-mediated system. To the best of our knowledge, this is the first description of the SXR in human endometrial cancer cells.

Interestingly, ISIW$^+$ cellular expression of the SXR was approximately fourfold higher than in the mother clone, suggesting that SXR overexpression may be related to CDDP resistance. Therefore, we tested whether SXR ligand binding promotes CDDP resistance. Although the SXR has specificity for multiple drugs and xenobiotics, such as RU 486, rifampicin, phenobarbital, and paclitaxel, we selected DEX because DEX is used frequently in CDDP—taxol-based chemotherapy. CDDP sensitivity was unchanged within the range tested and we concluded that the acute use of DEX, such as during chemotherapy, may not induce CDDP resistance. However, SXR-mediated CDDP resistance may develop over a period of weeks or months.

In order to determine the function of the SXR in both Ishikawa and ISIW$^+$ cells, we constructed an AS against the DNA-binding domain of the SXR. The AS, but not the NS, successfully inhibited SXR expression in vitro. In addition, when Ishikawa or ISIW$^+$ cells were cultured with the AS or NS, only the AS reversed the CDDP resistance. Furthermore, the SXR-AS blocked the mechanism by which Ishikawa cells become ISIW$^+$ cells through chronic exposure to CDDP in vitro. Thus, the in vitro data strongly suggest that the SXR is key in the development of CDDP resistance.

Studies have shown that CDDP induces GSTpi and protects DNA from anticancer agents [13,14]. GSTpi expression correlates with a clinical resistance to chemotherapy

in ovarian cancers [15]. However, in endometrial cancer the role of GSTpi is unknown. Barnette et al. have shown the presence of GSTpi in normal, as well as cancerous, endometrium [16]. GSTpi expression was lower in cancer patients who smoked, but no relationship between grade, outcome, or chemoresistance was demonstrated. In the present study, we confirmed the expression of GSTpi in established endometrial cancer cells. In addition, we have shown that GSTpi expression is approximately four-fold higher in ISIW$^+$ cells. Thus, GSTpi may play a role in CDDP resistance in the ISIW$^+$ cell line. However, the SXR-AS failed to inhibit GSTpi in ISIW$^+$ cells. The altered response to the SXR-AS in ISIW$^+$ cells may be due to promoter methylation, as seen in prostate adenocarcinoma [17]. Thus, the SXR may cross-talk with GSTpi in cells sensitive to CDDP, such as Ishikawa cells. Alternatively, CDDP-resistant cells, such as ISIW$^+$ cells, may have a disrupted link. Nevertheless, the SXR appears to be the primary point for development of CDDP resistance, because SXR-AS + CDDP treatment in vivo prevented cancer implantation in and the demise of scid mice.

Finally, chronic exposure to CDDP induces resistance, but the process was blocked by the SXR-AS. Because the SXR-AS itself does not inhibit growth, it either augments the effects of CDDP or blocks the mechanism of acquisition of drug resistance. The mechanism of action requires further investigation, but may provide a novel method for controlling drug resistance in endometrial cancer treatment.

Acknowledgments. The work was supported, in part, by a grant (no. 11671654) from the Ministry of Health and Welfare to H. S. The editorial advice of Dr. Tom K. Fujii and the technical assistance of S. Ige are gratefully acknowledged.

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Relationship Between *HER-2/neu* Gene Status and Chemosensitivity of Human Endometrial Cancer Cell Lines

Hisakazu Hiramatsu, Sanshiro Okamoto, Tsunekazu Kita, and Yoshihiro Kikuchi

Summary. We have evaluated the growth inhibitory effects of various anticancer drugs on human endometrial cancer cell lines (Ishikawa, HEC-1A, HEC-50B, HEC-59, and HEC-108). When in vitro sensitivity was defined as an IC_{50} lower than 10% of the peak plasma concentration, all endometrial cancer cells were sensitive to paclitaxel (Tx). When the combined effects of Tx with another drug were determined by median-effect analysis, Tx followed by cisplatin resulted in synergistic effects on all cell lines. Tx followed by SN-38 (the active metabolite of irinotecan) and etoposide followed by Tx also had synergistic effects on four cell lines. Tx followed by pirarubicin (THP) and THP followed by Tx showed synergistic effects on three cell lines. We then evaluated the relationship between *HER-2/neu* gene status and the chemosensitivity of six endometrial cancer cells (SPAC-1-L was added to the cells described above). *HER-2/neu* overexpression, determined by immunohistochemistry (HercepTest; Dako, Carpinteria, CA, USA), was seen only in SPAC-1-L cells, which were the most resistant cells to Tx and doxorubicin (ADR) of the six cells. Induction of *HER-2/neu* overexpression by dexamethasone was seen in Ishikawa and HEC-1A cells. Furthermore, induction of *HER-2/neu* overexpression by Tx was seen in HEC-59 cells. Chemosensitivity of these *HER-2/neu*-overexpressing cells to Tx and ADR varied with the change of *HER-2/neu* gene status. However, there was no common relationship between *HER-2/neu* gene status and chemosensitivity in all cells. The IC_{50} values of herceptin (Trastuzumab Roche; Basel, Switzerland; humanized antiHER-2/neu monoclonal antibody) in these cells were not obtained at the micromolar level. To confirm the results, further studies to quantify *HER-2/neu* gene amplification are needed.

Key words. Endometrial cancer, paclitaxel, chemosensitivity, *HER-2/neu* overexpression, herceptin (Trastuzumab)

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Introduction

In Japan, postoperative adjuvant chemotherapy is commonly administered in high-risk endometrial cancer. The combination most widely used is the PAC regimen, which consists of cisplatin, doxorubicin, and cyclophosphamide [1]. However, it is controversial whether PAC chemotherapy is more effective than radiation therapy as an adjuvant therapy for high-risk endometrial cancer [2]. In our retrospective analysis, disease-free survival of patients with radiation after optimal surgery was significantly longer than that of patients with PAC chemotherapy [3]. Against this background, we first examined in vitro chemosensitivity tests of human endometrial cancer cell lines to determine a more effective combination than PAC. Then, as a second series of experiments, we evaluated the relationship between *HER-2/neu* gene status and the chemosensitivity of human endometrial cancer cell lines in order to clarify the role of herceptin in endometrial cancer, as well as to determine whether changes in *HER-2/neu* status can sensitize endometrial cancer cells to paclitaxel (Tx) and anthracycline because a relationship between *HER-2/neu* overexpression and sensitivity to anthracycline in breast cancer has been reported [4]. In addition to anthracycline, a trial to sensitize breast cancer cells to Tx by downregulating the *HER-2/neu* overexpression using adenovirus type 5 E1A has been reported [5]. However, in endometrial cancer, the relationship between *HER-2/neu* overexpression and chemosensitivity is unclear, despite the importance of anthracycline and Tx. Therefore, we have examined whether a relationship between *HER-2/neu* gene status and chemosensitivity to ADR and Tx exists.

Methods

Cell Lines and Cell Culture

Ishikawa 3-H-12 cells were kindly provided by Dr. M. Nishida [6]. HEC-1A, HEC-50B, HEC-59, and HEC-108 cells were kindly supplied by Dr. H. Kuramoto [7,8]. SPAC-1-L cells were kindly offered by Dr. Y. Hirai [9]. Ishikawa cells were derived from well-differentiated adenocarcinoma of the endometrium. HEC-1A, HEC-50B, and HEC-59 cells were established from moderately differentiated endometrial cancer. HEC-108 cells were derived from poorly differentiated adenocarcinoma of the endometrium. SPAC-1-L cells were derived from serous papillary endometrial adenocarcinoma (USPC).

In the second experiment, we used ovarian cancer cell lines established in our laboratory [10,11] and compared them with endometrial cancer cells. KF_{28} cells were derived from serous cystadenocarcinoma of the ovary. KF_{r13} is a cisplatin-resistant cell line induced from KF_{28} by continuous exposure to low concentrations of cisplatin. KF_{28}Tx and KF_{r13}Tx are paclitaxel-resistant cell lines induced from KF_{28} and KF_{r13} by continuous exposure to low concentrations of paclitaxel (Tx).

Cells were incubated in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U penicillin/mL, and 100 g streptomycin/mL (Gibco, Grand Island, NY, USA) at 37°C in a 5% CO_2 incubator. The medium was changed every 3 days and cells were passaged when they reached confluence.

Drugs

Cisplatin (CDDP), etoposide (VP-16), and paclitaxel (Tx) were obtained from Bristol-Myers Squibb (Tokyo, Japan). SN-38, which is an active metabolite of irinotecan (CPT-11), was supplied by Daiichi Pharmaceutical (Tokyo, Japan). 5-Fluorouracil (5-FU) and doxorubicin (ADR) were supplied by Kyowa Hakko Kogyo (Tokyo, Japan). Pirarubicin (THP), aclarubicin (ACR), and actinomycin-D (ACD) were obtained from Meiji Seika (Tokyo, Japan), Yamanouchi Pharmaceutical (Tokyo, Japan), and Banyu Pharmaceutical (Tokyo, Japan), respectively. Because ADR is not used frequently in Japan because of its cardiotoxicity, the efficacy of THP and ACR was examined instead of ADR in the first series of experiments. 4-Hydroperoxy ifosfamide (4-OHIFO), which is an active metabolite of ifosfamide (IFO), was provided by Shionogi Pharmaceutical (Osaka, Japan). In the second series of experiments, we used EGF, 17β-estradiol (E2), obtained from Sigma (St. Louis, MO, USA), and dexamethasone (DEX), supplied by Banyu Pharmaceutical (Tokyo, Japan), to change the *HER-2/neu* gene status.

In Vitro Growth Inhibition Test

To examine concentrations of anticancer drugs required for 50% inhibition of cell growth in vitro (IC_{50}), 3000 cells/well in 100 μL growth medium were seeded into 96-well flat-bottomed microtiter plates (Becton Dickinson, Franklin Lakes, NJ, USA). After incubation for 24 h, various concentrations of drugs diluted in 100 μL medium were added to each well and, after an additional incubation for 72 h, the cytotoxicity of various anticancer drugs on each cell line was determined using a crystal violet staining method [12]. The concentration that inhibited the growth of cells to the level of 50% of control growth (IC_{50}) was obtained from graphical plots. The efficacy of each drug used in the present study was defined as "sensitive" when the IC_{50} value was lower than 10% of the peak plasma concentrations (PPC), as described previously [13].

Median-Effect Analysis

Combined effects of Tx with other anticancer drugs were analyzed by median-effect analysis, as described previously [14]. Tx was added simultaneously with other drugs and cells were incubated for 72 h. In sequential exposure experiments, after or before cells were treated with Tx for 36 h, another second drug was added for 36 h. Regression lines were then fitted to growth inhibition data and the concentration of each drug that produced a given level of growth inhibition (fractional effect (F_a)), alone or in combination, was determined. The combination index (CI) for a given F_a (typically 0.5) was calculated as follows:

$$CI = d_1/D_1 + d_2/D_2$$

where D_1 and D_2 are the doses of drugs 1 and 2, respectively, which by themselves produce a given F_a (i.e. IC_{50}) and d_1 and d_2 are the doses that produce the same F_a in combination. CI = 1 indicates zero interaction (additive cytotoxicity), CI < 1 indicates synergy, and CI > 1 indicates antagonism. Results are expressed as the mean ± SD. Statistical significance of differences in mean values was determined by Student's *t*-test.

Immunohistochemistry

Immunohistochemistry was performed according to the protocol for the HercepTest (Dako, Carpinteria, CA, USA), an immunohistochemical detection and scoring system for the evaluation of *HER-2/neu* overexpression, which is approved by the US Food and Drug Administration (FDA) [15]. After cultivation on CultureSlide (Becton Dickinson Labware, Franklin Lakes, NJ, USA) for 72 h, cell lines were formalin fixed for 24 h. Cells were then washed three times with phosphate-buffered saline (PBS) and immersed in methanol with 0.3% H_2O_2. Cells were incubated with rabbit antiHER-2/neu polyclonal antibody (diluted at 1:200) for 30 min at room temperature. The immunoreaction was detected by incubating cells with goat antirabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer for 30 min and by immersing the cells in 3,3′-diaminobenzidine tetrahydrochloride (DAB) for 10 min. Nuclei were lightly counterstained with hematoxylin. As a control for the antibody, cells were treated with PBS and normal rabbit immunoglobulins in place of the primary antibody. No staining was observed in these cells.

Immunohistochemical expression of the HER-2/neu protein was evaluated according to the scoring guidelines of the HercepTest by an experienced pathologist. Briefly, the scores were allocated as follows: 0, specimens showing no staining or membrane staining in <10% of cells (Fig. 1); 1+, specimens showing faint or barely perceptible membrane staining in >10% of cells (Fig. 2); 2+, specimens showing weak to moderate complete membrane staining in >10% of cells (Fig. 3); and 3+, specimens showing

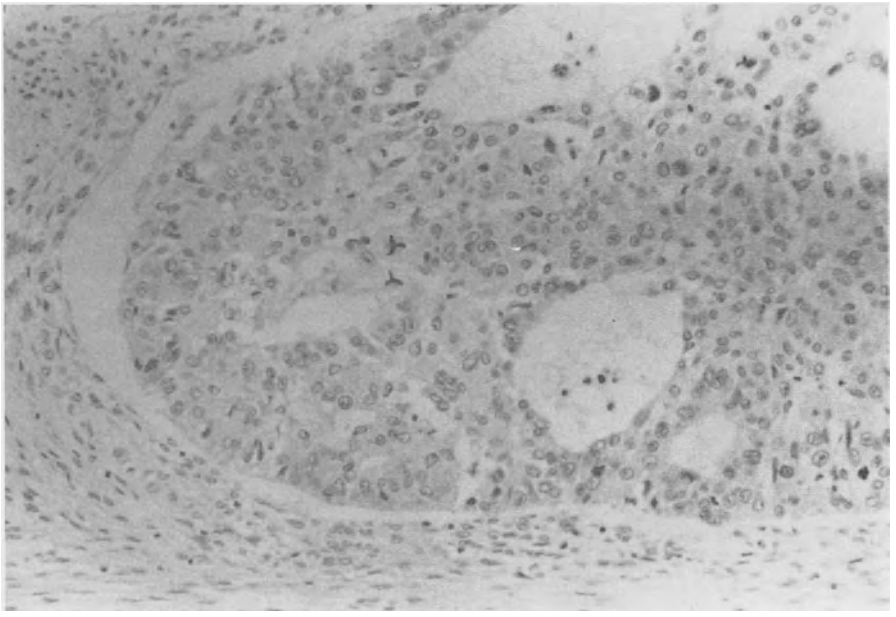

FIG. 1. A negative control specimen (score 0 on the HercepTest) derived from ovarian serous cyst adenocarcinoma shows no staining or membrane staining in <10% of cells

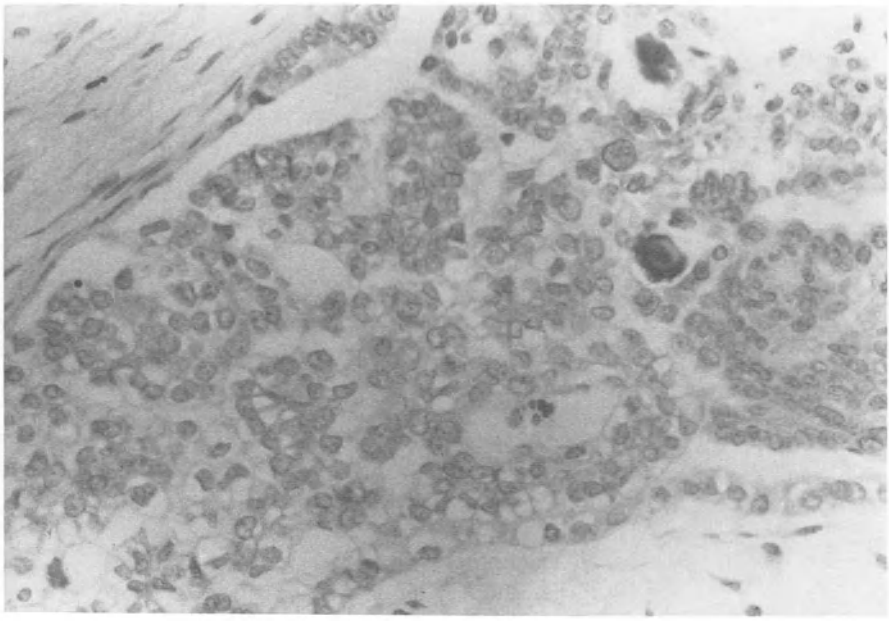

FIG. 2. A negative control specimen (score 1+ on the HercepTest) derived from ovarian serous cyst adenocarcinoma shows faint or barely perceptible membrane staining in >10% of cells

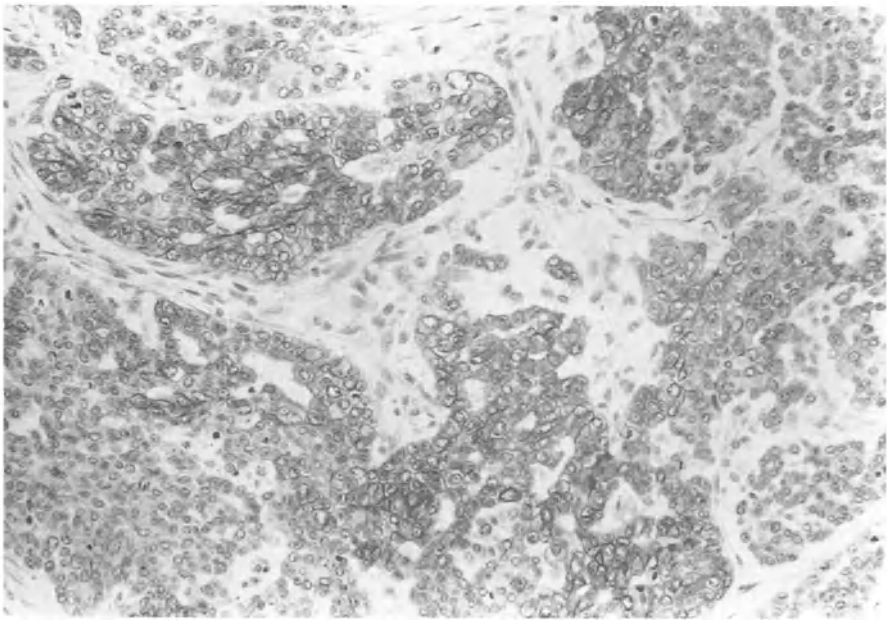

FIG. 3. A positive control specimen (score 2+ on the HercepTest) derived from ovarian serous cyst adenocarcinoma shows weak to moderate complete membrane staining in >10% of cells

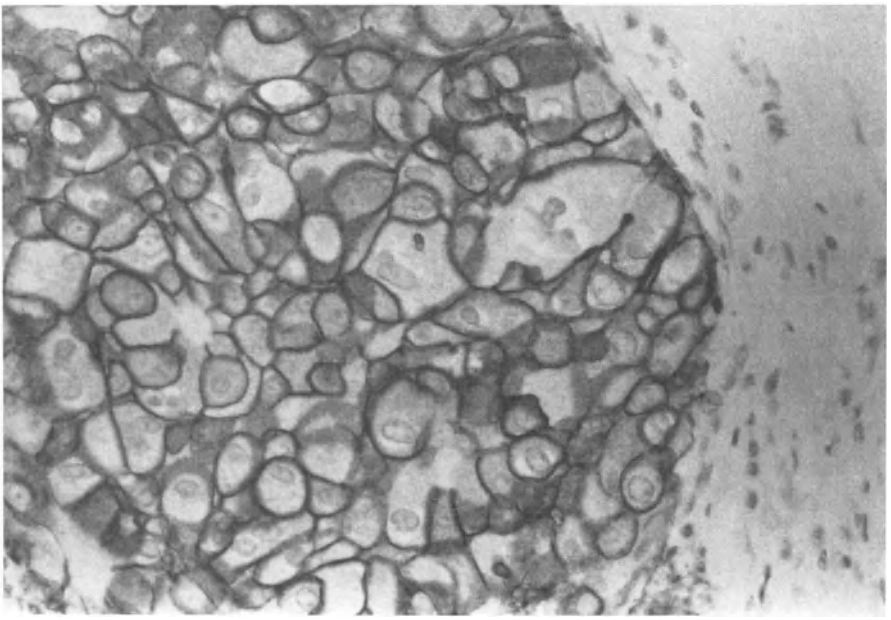

FIG. 4. A positive control specimen (score 3+ on the HercepTest) derived from breast cancer shows strong complete membrane staining in >10% of cells

strong complete membrane staining in >10% of cells (Fig. 4). Scores of 0 and 1+ were defined as negative for HER-2/neu overexpression, whereas scores of 2+ and 3+ were defined as positive for HER-2/neu overexpression.

In determining the change of *HER-2/neu* status after drug exposure, cells were preincubated with EGF, E2, DEX, and Tx, concentrated to one-tenth of the IC_{50}, for 72 h and then seeded onto the CultureSlide. Thereafter, the same procedures were performed as for Hercep test.

Results

Growth Inhibitory Effects of Anticancer Drugs in Endometrial Cancer Cell Lines

The effects of various anticancer drugs on the proliferation of five endometrial cancer cell lines are shown in Table 1. The IC_{50} values of Tx for all cell lines used in the present study were lower than one-tenth of the PPC, suggesting that all endometrial cancer cell lines were sensitive to Tx. Four cell lines were sensitive to ACR and ACD. Three cell lines were sensitive to SN-38. Two cell lines were sensitive to THP and VP-16. The Ishikawa cell line was sensitive only to 5-FU. However, none of the cell lines in the present study was sensitive to either CDDP or 4-OHIFO. Among all endometrial cancer cell lines, the Ishikawa cell line, derived from well-differentiated adenocarci-

TABLE 1. Growth inhibitory effects (IC_{50}) of various anticancer drugs in endometrial cancer cell lines

Cell lines	Tx (μM)	ACR (μM)	ACD (nM)	SN-38 (nM)	THP (nM)	VP-16 (μM)	5-FU (μM)	4-OHIFO (nM)	CDDP (nM)
Ishikawa	5.2	2.6	1.4	7.9	12.5	4.4	11.1	40.0	2.8
HEC-1A	1.1	1.3	0.8	8.7	10.0	2.1	36.7	40.0	8.2
HEC-50B	21.5	1.7	210.0	66.6	122.9	25.5	85.0	32.1	3.3
HEC-59	6.5	53.8	2.6	5.1	46.5	9.6	58.0	32.5	3.9
HEC-108	3.9	1.9	3.2	20.2	66.5	21.2	130.1	36.0	7.5
PPC	4680.0	235.8	477.9	124.9	159.3	51.0	117.6	90.1	5.0
Cut off level	468.0	23.6	47.8	12.5	15.9	5.1	11.8	9.0	0.5

Each value is the average from three independent experiments
The cut off level is defined as one-tenth of the peak plasma concentration (*PPC*) level, as described in Methods
Underlined data indicate sensitiveness
IC_{50}, drug concentration required for 50% inhibition of cell growth in vitro; *Tx*, paclitaxel; *ACR*, aclarubicin; *ACD*, actinomycin-D; *SN-38*, active metabolite of irinotecan (CPT-11); *THP*, pirarubicin; *VP-16*, etoposide; *5-FU*, 5-fluorouracil; *4-OHIFO*, 4-hydroperoxy ifosfamide; *CDDP*, cisplatin

noma, was the most sensitive to anticancer drugs. The HEC-1A cell line, derived from moderately differentiated adenocarcinoma, was the second most drug-sensitive cell line. Except for 5-FU, the sensitivity profile of the HEC-1A cell line was similar to that of the Ishikawa cell line.

Combined Effects of Tx with Other Anticancer Drugs

Combination effects of Tx with other anticancer drugs, as determined by median-effect analysis, are shown in Tables 2–4. In the simultaneous-exposure experiments shown in Table 2, except for SN-38, VP-16, and 5-FU, combinations of Tx with the other drugs produced antagonistic effects on all cell lines. As shown in Table 3, Tx followed by CDDP produced synergistic effects on all cell lines. Tx followed by SN-38 produced synergistic effects on four cell lines. Tx followed by THP, 5-FU, and 4-OHIFO exhibited synergistic effects on three cell lines. As shown in Table 4, VP-16 followed by Tx produced synergistic effects on four cell lines. THP followed by Tx exhibited synergistic effects on three cell lines. It is of note that CDDP followed by Tx produced antagonistic effects on all cell lines, whereas Tx followed by CDDP exhibited the greatest synergy.

Relationship Between HER-2/neu *Gene Status Determined by the HercepTest and Chemosensitivity*

At first, in order to compare results with those from endometrial cancer cells, we examined the *HER-2/neu* status and chemosensitivity to ADR in Tx-resistant ovarian cancer cells that were induced by continuous exposure of cells to low concentrations of Tx. As shown in Table 5, *HER-2/neu* status did not change and herceptin exhibited no growth-inhibitory effects on either primary or Tx-resistant cells. However, the

TABLE 2. Combination indices of paclitaxel with other drugs in endometrial cancer cell lines (simultaneous exposure)

Cell lines	ACR	ACD	SN-38	THP	VP-16	5-FU	4-OHIFO	CDDP
Ishikawa	1.71 ± 0.21**	1.91 ± 0.12**	1.37 ± 0.20	1.91 ± 0.03**	1.30 ± 0.21	1.08 ± 0.06	2.16 ± 0.46**	1.56 ± 0.07**
HEC-1A	1.93 ± 0.21**	1.95 ± 0.13**	0.95 ± 0.15	1.83 ± 0.31**	1.05 ± 0.05	1.21 ± 0.18	1.64 ± 0.05**	1.31 ± 0.17
HEC-50B	1.99 ± 0.03**	1.00 ± 0.01	0.88 ± 0.11	1.48 ± 0.34	0.93 ± 0.14	1.17 ± 0.17	1.19 ± 0.10	1.41 ± 0.36
HEC-59	1.53 ± 0.33	1.15 ± 0.25	1.17 ± 0.37	1.63 ± 0.02**	1.36 ± 0.27	0.68 ± 0.18*	1.14 ± 0.04	1.50 ± 0.25
HEC-108	1.92 ± 0.12**	1.08 ± 0.02**	1.25 ± 0.05**	1.39 ± 0.41	1.85 ± 0.09**	1.37 ± 0.02**	1.41 ± 0.18	1.48 ± 0.06**

Combination indices (CI) values were determined by median-effect analysis at fractional effect (F_a) = 0.5. CI < 1, CI = 1, and CI > 1 indicate synergism (underlined), additive effects, and antagonism, respectively

Data are the mean ± SD (n = 3)

* Significantly synergistic ($P < 0.05$, compared with CI = 1)

** Significantly antagonistic ($P < 0.05$, compared with CI = 1)

ACR, aclarubicin; *ACD*, actinomycin-D; *SN-38*, active metabolite of irinotecan (CPT-11); *THP*, pirarubicin; *VP-16*, etoposide; *5-FU*, 5-fluorouracil; *4-OHIFO*, 4-hydroperoxy ifosfamide; *CDDP*, cisplatin

TABLE 3. Combination indices of paclitaxel with other drugs in endometrial cancer cell lines (sequential exposure to paclitaxel followed by other drugs)

Cell lines	ACR	ACD	SN-38	THP	VP-16	5-FU	4-OHIFO	CDDP
Ishikawa	2.22 ± 0.06**	2.07 ± 0.14**	0.92 ± 0.28	1.73 ± 0.14**	1.37 ± 0.05**	1.69 ± 0.18**	2.20 ± 0.34**	0.98 ± 0.26
HEC-1A	1.55 ± 0.36	2.34 ± 0.25**	0.43 ± 0.07*	0.64 ± 0.17	1.10 ± 0.09	0.68 ± 0.06*	0.76 ± 0.24	0.75 ± 0.10*
HEC-50B	1.53 ± 0.16**	0.98 ± 0.15	0.40 ± 0.19*	0.50 ± 0.14*	0.53 ± 0.08*	1.11 ± 0.13	0.36 ± 0.14*	0.77 ± 0.25
HEC-59	1.85 ± 0.07**	3.29 ± 0.13**	1.14 ± 0.09	1.29 ± 0.12	1.50 ± 0.11**	0.77 ± 0.14	0.79 ± 0.29	0.95 ± 0.13
HEC-108	0.94 ± 0.16	2.89 ± 0.05**	0.66 ± 0.24	0.71 ± 0.13	1.12 ± 0.38	0.77 ± 0.21	1.90 ± 0.08**	0.60 ± 0.16*

Combination indices (CI) values were determined by median-effect analysis at fractional effect (F_a) = 0.5. CI < 1, CI = 1, and CI > 1 indicate synergism (underlined), additive effects, and antagonism, respectively

Data are the mean ± SD ($n = 3$)

* Significantly synergistic ($P < 0.05$, compared with CI = 1)

** Significantly antagonistic ($P < 0.05$, compared with CI = 1)

ACR, aclarubicin; *ACD*, actinomycin-D; *SN-38*, active metabolite of irinotecan (CPT-11); *THP*, pirarubicin; *VP-16*, etoposide; *5-FU*, 5-fluorouracil; *4-OHIFO*, 4-hydroperoxy ifosfamide; *CDDP*, cisplatin

TABLE 4. Combination indices of paclitaxel with other drugs in endometrial cancer cell lines (sequential exposure of other drugs followed by paclitaxel)

Cell lines	ACR	ACD	SN-38	THP	VP-16	5-FU	4-OHIFO	CDDP
Ishikawa	1.92 ± 0.11**	2.12 ± 0.04**	0.86 ± 0.13	1.96 ± 0.35**	0.75 ± 0.18	1.81 ± 0.18**	2.72 ± 0.66**	1.73 ± 0.16**
HEC-1A	2.15 ± 0.22**	1.33 ± 0.17	1.03 ± 0.13	0.81 ± 0.08	0.54 ± 0.17*	1.17 ± 0.13	2.49 ± 0.52**	2.25 ± 0.22**
HEC-50B	2.64 ± 0.23**	1.28 ± 0.14	0.80 ± 0.13	1.13 ± 0.16	0.75 ± 0.05*	1.83 ± 0.09**	1.24 ± 0.16	1.04 ± 0.15
HEC-59	1.15 ± 0.05	0.63 ± 0.14*	1.55 ± 0.07**	0.91 ± 0.12	0.94 ± 0.07	0.88 ± 0.11	1.26 ± 0.13	1.19 ± 0.39
HEC-108	1.43 ± 0.10**	1.52 ± 0.16**	1.22 ± 0.04**	0.87 ± 0.24	1.60 ± 0.08**	1.56 ± 0.05**	0.73 ± 0.10*	1.26 ± 0.14

Combination indices (CI) values were determined by median-effect analysis at fractional effect (F_a) = 0.5. CI < 1, CI = 1, and CI > 1 indicate synergism (underlined), additive effects, and antagonism, respectively

Data are the mean ± SD (n = 3)

* Significantly synergistic ($P < 0.05$, compared with CI = 1)

** Significantly antagonistic ($P < 0.05$, compared with CI = 1)

Tx, paclitaxel; *ACR*, aclarubicin; *ACD*, actinomycin-D; *SN-38*, active metabolite of irinotecan (CPT-11); *THP*, pirarubicin; *VP-16*, etoposide; *5-FU*, 5-fluorouracil; *4-OHIFO*, 4-hydroperoxy ifosfamide; *CDDP*, cisplatin

TABLE 5. IC_{50}, relative resistance values, *HER-2/neu* status, and sensitivity to herceptin in paclitaxel-resistant ovarian cancer cell lines

Cell lines	Tx IC_{50} (nM)	RRV	CDDP IC_{50} (μM)	RRV	ADR IC_{50} (nM)	RRV	*HER-2/neu*	Herceptin IC_{50}
KF_{28}	4.65	1.0	0.18	1.0	49.9	1.0	N(0)	NO
$KF_{28}Tx$	53.3	11.5	0.14	0.78	261.8	5.25	N(0)	NO
KF_{r13}	2.61	1.0	0.85	1.0	39.3	1.0	N(0)	NO
$KF_{r13}Tx$	12.7	4.88	0.53	0.62	193.4	4.92	N(1+)	NO

Each IC_{50} value is the average of three independent experiments
The relative resistance values (*RRV*) indicate how many fold the sensitivity changed after the induction of resistance
HER-2/neu status was determined by the HercepTest (Dako, Carpinteria, CA, USA)
(*0*), (*1+*), HercepTest score
Tx, paclitaxel; *ADR*, doxorubicin; *NO*, not obtained at the micromolar level; *N*, negative *HER-2/neu* overexpression

TABLE 6. Relationship between *HER-2/neu* gene status and chemosensitivity in endometrial cancer cell lines

Cell lines	*HER-2/neu* status	Tx IC_{50} (nM)	ADR IC_{50} (nM)	Herceptin IC_{50} (mM)
Ishikawa	N(1+)	5.2	80.0	NO
HEC-1A	N(1+)	1.1	9.9	NO
HEC-50B	N(1+)	21.5	76.3	NO
HEC-59	N(1+)	6.5	520	NO
HEC-108	N(1+)	3.9	61.5	NO
SPAC-1-L	P(2+)	40.1	6200	NO

Each IC_{50} value is the average of three independent experiments
HER-2/neu status was determined by the HercepTest (Dako, Carpinteria, CA, USA)
(*1+*), (*2+*), HercepTest score
Tx, paclitaxel; *ADR*, doxorubicin; *NO*, not obtained at the micromolar level; *N*, negative *HER-2/neu* overexpression; *P*, positive *HER-2/neu* overexpression

induction of Tx resistance resulted in resistance to ADR. $KF_{28}Tx$ and $KF_{R13}Tx$ became 5.25- and 4.92-fold resistant to ADR, respectively.

As shown in Table 6, *HER-2/neu* overexpression in endometrial cancer cells by the HercepTest was observed only in the SPAC-1-L cell line, which was derived from USPC (Fig. 5). The IC_{50} values for Tx and ADR were relatively higher than those in other cell lines with negative *HER-2/neu* overexpression. In particular, the SPAC-1-L cell line was approximately 100-fold more resistant to ADR than other cell lines. No growth-inhibitory effects of herceptin were observed in either HER-2/neu-negative cells or HER-2/neu-positive SPAC-1-L cells. Furthermore, when combined with Tx, there were no combination effects of herceptin observed.

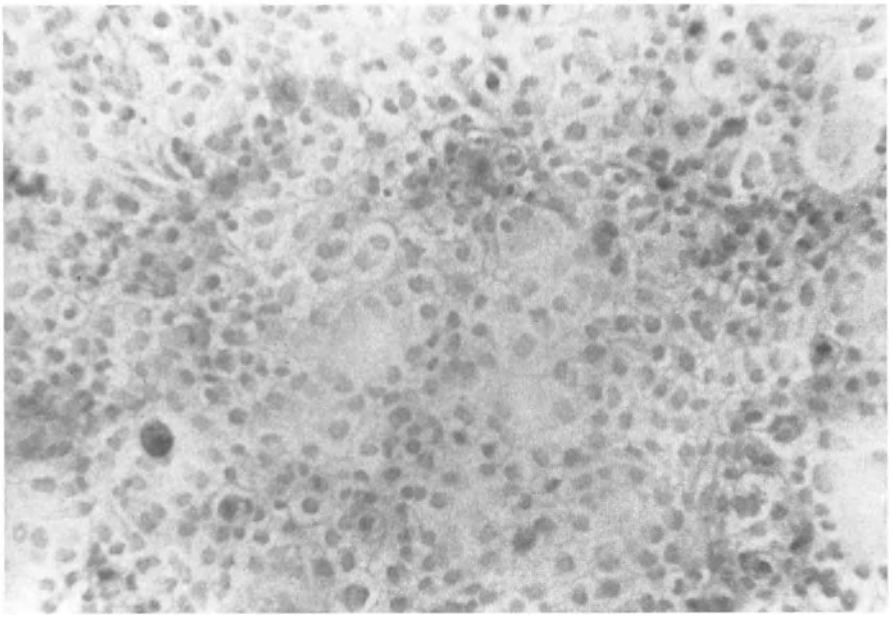

FIG. 5. SPAC-1-L cells were determined as positive for *HER-2/neu* overexpression by a score of 2+ on the HercepTest. SPAC-1-L cells show weak complete membrane staining in >10% of cells

Induction of HER-2/neu *Overexpression and Change of Chemosensitivity to Tx and ADR*

In the present study, only one of six endometrial cancer cell lines showed *HER-2/neu* overexpression. Thus, we attempted to induce *HER-2/neu* overexpression by drug exposure. 100 nM E2, 100 nM DEX, 1 nM EGF, and low concentrations (one-tenth of the IC_{50} values) of Tx were tested, as shown in Table 7. Changes in the *HER-2/neu* status after 72 h drug exposure occurred in three cell lines. Exposure of Ishikawa and HEC-1A cells (Fig. 6) to DEX and exposure of HEC-59 cells (Fig. 7) to Tx induced *HER-2/neu* overexpression. However, in SPAC-1-L cells, *HER-2/neu* status did not change. Moreover, the influence of the *HER-2/neu* status on chemosensitivity was examined. As shown in Table 8, induction of *HER-2/neu* overexpression in Ishikawa cells caused a 16.3-fold sensitization to Tx, but sensitivity to ADR was not changed. Induction of *HER-2/neu* overexpression in HEC-1A cells by DEX resulted in a 4.2-fold increase in resistance to ADR, but sensitization to Tx was not observed. Induction of *HER-2/neu* overexpression in HEC-59 cells by Tx resulted in a 1.7-fold sensitization to ADR.

TABLE 7. Induction of *HER-2/neu* overexpression by exposure to drugs in endometrial cancer cell lines

		HER-2/neu overexpression			
		After exposure to			
Cell lines	Before exposure	E2	DEX	EGF	Tx
Ishikawa	N	N	P(2+)	N	N
HEC-1A	N	N	P(2+)	N	N
HEC-50B	N	N	N	N	N
HEC-59	N	N	N	N	P(2+)
HEC-108	N	N	N	N	N
SPAC-1-L	P(2+)	P(2+)	P(2+)	P(2+)	P(2+)

Results were obtained from three independent experiments
Cells were exposed to 100 nM 17β-estradiol (E_2), 100 nM dexamethasone (*DEX*), 1 nM epidermal growth factor (*EGF*), and one-tenth of the IC_{50} of paclitaxel (*Tx*). Then, the *HER-2/neu* status of the cells was determined by the HercepTest (Dako, Carpinteria, CA, USA)
(2+), HercepTest score
N, negative *HER-2/neu* overexpression; *P*, positive *HER-2/neu* overexpression

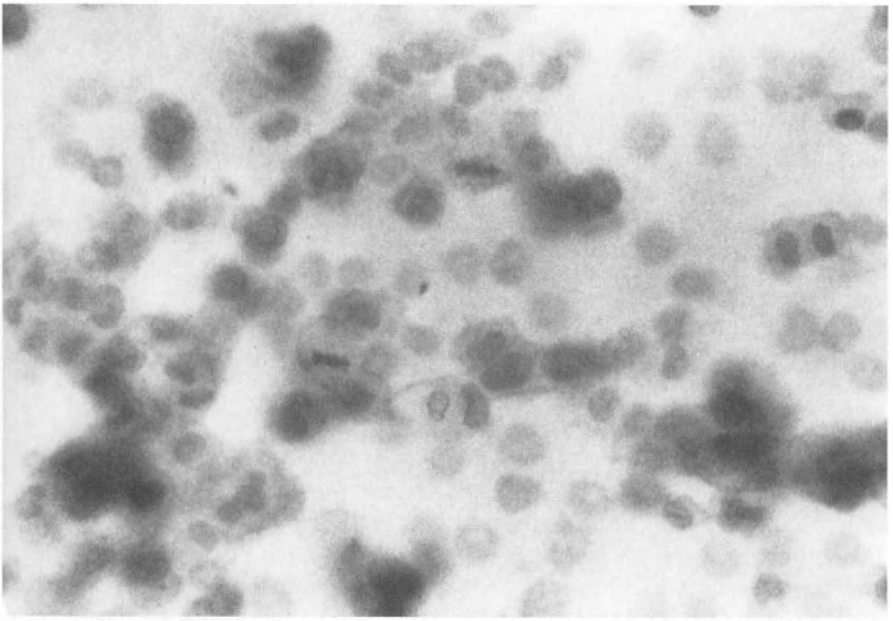

FIG. 6. After 72 h exposure to 100 nM dexamethasone, HEC-1A cells were determined as positive for *HER-2/neu* overexpression by a score of 2+ on the HercepTest. The HEC-1A cells show moderate complete membrane staining in >10% of cells

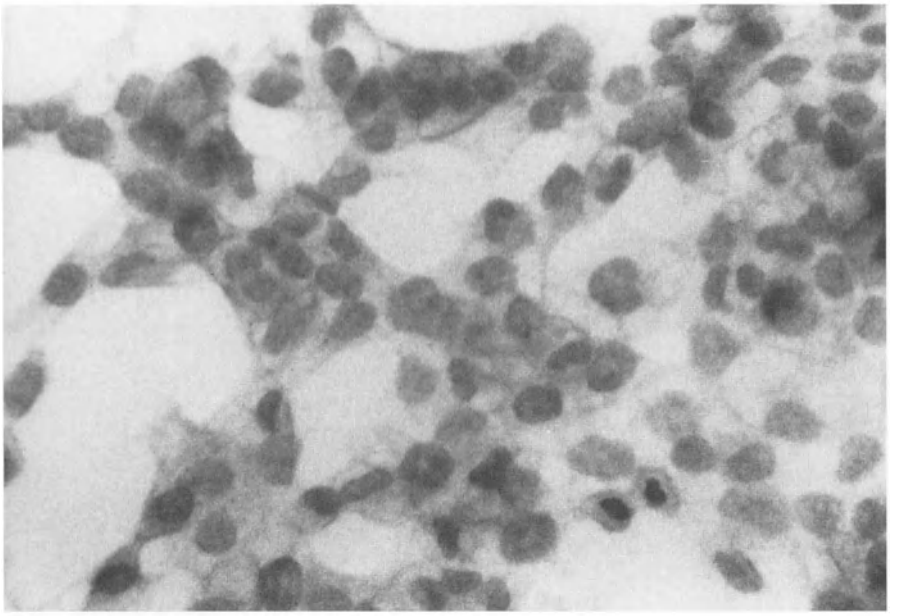

FIG. 7. After 72 h exposure to one-tenth of the IC_{50} of paclitaxel, HEC-59 cells were determined as positive for *HER-2/neu* overexpression by a score of 2+ on the HercepTest. The HEC-59 cells show moderate complete membrane staining in >10% of cells

TABLE 8. Chemosensitizing effects of the induction of *HER-2/neu* overexpression

Cell lines	*HER-2/neu* status			Tx IC_{50} B/A (nM)	ADR IC_{50} B/A (nM)	Herceptin IC_{50} B/A (nM)
	Before	After DEX	After Tx			
Ishikawa	N(1+)	P(2+)	N(1+)	5.2/0.32	80.0/72.1	NO/NO
HEC-1A	N(1+)	P(2+)	N(1+)	1.1/1.3	9.4/41.5	NO/NO
HEC-59	N(1+)	N(1+)	P(2+)	ND	520/310	NO/NO

Each IC_{50} value is the average of three independent experiments
(*1+*), (*2+*), HercepTest score
B, before induction of *HER-2/neu* overexpression; *A*, after induction of *HER-2/neu* overexpression; *N*, negative; *P*, positive; *NO*, not obtained at the micromolar level; *ND*, not determined

Discussion

In the present study, we have demonstrated that the most active single agent in endometrial cancer cell lines is Tx. Tx was active against all endometrial cancer cell lines, including not only grade 1, but also grade 2 and 3 cells (Table 1). These results are in agreement with results of clinical trials [16,17]. Those authors reported that Tx was active against platinum-resistant endometrial cancer with histologic grade 2 or 3. Therefore, it is possible that Tx is promising not only in advanced endometrial

cancer patients, but also in high-risk endometrial cancer patients. ACR was active against four endometrial cancer cell lines, whereas THP and VP-16 were active against two cell lines. Although ACR and THP were used instead of ADR in the present study, ADR showed a 26% response rate in a previous report [16] when it was used as a single agent. Anthracyclines seem to be the key drugs in endometrial cancer. ACD was active against four cell lines and SN-38 was active against three cell lines. These should also be considered as key drugs. In the present study, the results obtained by combining these drugs are not shown because of space limitations. Further details can be found in our previous report [18]. However, in the present in vitro study, CDDP and 4-OHIFO did not show activity against any endometrial cancer cell lines examined. Although these results are contradictory to previous reports concerning the clinical use of these drugs [16], most such clinical studies have shown results obtained by combinations of CDDP or IFO with other drugs, but not the use of single agent [19,20].

When simultaneously combining Tx with CDDP, THP, ACR, or 4-OHIFO, antagonistic effects rather than additive effects were observed, as analyzed by median-effect analysis (Table 2). Synergistic effects were observed in two endometrial cancer cell lines by simultaneous combination of Tx with SN-38. Simultaneous combination of Tx with VP-16 or 5-FU resulted in synergistic effects in only one cell line. In the sequential combination (Table 3) of Tx and CDDP, when Tx was followed by CDDP, synergistic effects were observed in all endometrial cancer cells, as reported for ovarian cancer cells [21]. However, in the reverse sequence (Table 4), with CDDP followed by Tx, antagonistic effects were observed in all cell lines. These results can be explained by an elevation of CDDP uptake by Tx and blockade of Tx-induced apoptosis by CDDP [11,22]. Similarly, Tx followed by SN-38 had synergistic effects in four cell lines, especially in the two (HEC-50B and HEC-108) cell lines with IC_{50} values for SN-38 higher than one-tenth of the PPC, suggesting sensitization of SN-38 by Tx. Moreover, VP-16 followed by Tx exhibited synergistic effects in four cell lines and Tx followed by 5-FU or 4-OHIFO showed synergistic effects in three cell lines. Regardless of whether THP was administered before or after Tx, synergistic effects were obtained in three cell lines. The sensitization of THP by antecedent Tx may be caused by topoisomerase IIα amplification, which occurs with the induction of *HER-2/neu* amplification by Tx [23]. Combination of Tx with the other drugs was less effective. Because these results were obtained in an in vitro study, further studies are necessary to confirm these results in the clinical setting.

In the second series of experiments, we evaluated the relationship between *HER-2/neu* gene status and chemosensitivity in endomatrial cancer cell lines. *HER-2/neu* overexpression has been reported to be associated with poor overall survival [24] while causing an improvement by adjuvant chemotherapy or radiation [25] in endometrial cancer. In breast and ovarian cancer cells, *HER-2/neu* overexpression has been reported to induce chemoresistance to Tx [26] and a trial to sensitize breast cancer cells to Tx by downregulating the *HER-2/neu* overexpression using adenovirus type 5 E1A has been performed [5]. However, the results were contradictory. Our results showed no relationship between sensitivity to Tx and *HER-2/neu* overexpression in ovarian cancer cells (Table 5). In a recent study, we analyzed genes related to Tx resistance in ovarian cancer cells using cDNA microarray analysis. The results suggested that *IGFBP3* and *Rh_0-GDI* genes would be related to Tx resistance, but not *HER-2/neu* (T. Goto et al., unpublished data, 2001). In breast cancer, a relationship between

HER-2/neu overexpression and sensitivity to anthracycline has been suggested [27]. Although Tx and anthracyclines are the most effective drugs in endometrial cancer, as shown in the results of the first series of experiments, the relationship between *HER-2/neu* overexpression and chemosensitivity to both drugs remains unclear. Therefore, we examined whether the relationship exists in endometrial cancer cell lines.

The SPAC-1-L cell line derived from USPC was used in the second series of experiments because USPC has been reported to have a high frequency (80%) of *HER-2/neu* overexpression [28]. When determined by the HercepTest, *HER-2/neu* overexpression was observed only in the SPAC-1-L cell line (Table 6). Furthermore, the SPAC-1-L cell line was the most resistant to both Tx and of all the endometrial cancer cell lines used in the present study. Resistance of *HER-2/neu*-overexpressing cells to ADR is contradictory to previous results in breast cancer [4]. Efficacy of herceptin in USPC cells with *HER-2/neu* overexpression, as reported recently [28], was not demonstrated in the present study. Further studies are necessary to clarify the role of herceptin (Trastuzumab Roche; Basel, Switzerland; humanized antiHER-2/neu monoclonal antibody) in gynecologic malignancies, including endometrial cancer. In combination with Tx, herceptin showed no effects. Synergistic combined effects of herceptin with docetaxel and vinorelbine have been reported in breast cancer [29]. An effective combination of herceptin with other drugs in endometrial cancer should also be clarified by further studies.

E2 and DEX have been reported to be able to induce *HER-2/neu* expression in the Ishikawa cell line [30]. Thus, we tried to induce the overexpression of *HER-2/neu* using E2, DEX, EGF, and Tx (Table 7). A change in the *HER-2/neu* status occurred in Ishikawa cells exposed to DEX, as reported previously [30], and a change in *HER-2/neu* status was also observed in HEC-1A cells without hormone receptors exposed to DEX. We selected the HercepTest as the method to detect *HER-2/neu* overexpression because of its simplicity. Discrepancy between *HER-2/neu* overexpression detected by immunohistochemistry and *HER-2/neu* amplification detected by fluorescence in situ hybridization has been reported [31]. *HER-2/neu* gene amplification in HEC-1A cells detected by Western blot analysis has been reported previously [32]. Moreover, changes in the *HER-2/neu* status occurred in HEC-59 cells exposed to one-tenth of the IC_{50} of Tx. After exposure to Tx, HEC-59 cells became approximately 1.7-fold more sensitive to ADR (Table 8). Such sensitization may be caused by topoisomerase IIα amplification concurrent with *HER-2/neu* amplification [27]. The topoisomerase IIα gene is located next to the *HER-2* gene on chromosome 17q12–q21 and its amplification seems to occur with the induction of *HER-2* amplification [33]. The change in *HER-2/neu* status sensitized Ishikawa cells to Tx, but not to ADR. If chemosensitizing effects of steroids, such as DEX, to Tx are proven to be common, premedication routinely used to prevent allergic reactions will be proven as rational. In contrast, the change in *HER-2/neu* status in HEC-1A cells resulted in a 4.2-fold resistance to ADR, but not Tx. It is difficult to generalize data from one type of tumor cells to another.

Conclusions

Tx is the most effective drug in endometrial cancer. However, when combined with other drugs, sequential effects must be taken into consideration, as described in the present study.

Herceptin seems to have less of a role in the treatment of endometrial cancer.

There was no common relationship between *HER-2/neu* gene status and chemosensitivity in endometrial cancer, but *HER-2/neu* overexpression may sensitize endometrial cancer cells to Tx or ADR.

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