Extracellular Matrix and the Patterns of Differentiation of Human Endometrial Carcinomas in Vitro and in Vivo

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ABSTRACT

Adenocarcinomas differ in their ability to form glandular structures, and the mechanism regulating this architectural differentiation is unknown. In the present study, the patterns of differentiation of two human endometrial carcinomas that differed with respect to their ability to form glands in their original host were studied in monolayer and three-dimensional cultures as well as in xenografts in athymic mice. A moderately differentiated adenocarcinoma of human endometrium, EnCa101, transplanted into nude mice formed tumors indistinguishable from the original neoplasm and secreted mucin. A cell line derived from this tumor, ECC-1, formed monolayers on tissue culture substratum and lost the ability to secrete mucin. However, upon culture within Matrigel, the ECC-1 cells formed glandular structures and secreted mucin. Ultrastructural examination revealed morphological polarity, as evident by intraluminal microvilli and characteristic adhesion structures composed of tight, gap, and desmosomal junctions adjacent to the lumen, and secretory activity. Whereas basal lamina was observed in vivo around glandular cells, epithelial cells were not tethered in vitro with this structure. In contrast, the epithelial cells of a poorly differentiated human endometrial adenocarcinoma, AN3, failed to form glands in nude mice or in Matrigel in vitro. These findings illustrate that gland-forming ability is an intrinsic property of well to moderately differentiated adenocarcinoma cells and that only cells with this inherent potential can be induced to form glands in response to appropriate extracellular signals.

MATERIALS AND METHODS

Materials. Athymic male NCr-nu mice were obtained from the National Cancer Institute (Bethesda, MD). 17β-Estradiol constant release pellets were purchased from Innovative Research of America (Toledo, OH), AN3 cells were obtained from the American Type Culture Collection (Rockville, MD), and Matrigel is a product of Collaborative Research, Inc. (Bedford, MA).

Growth of Endometrial Carcinoma Cells in Nude Mice. EnCa-101 was established by the subcutaneous growth of a moderately differentiated adenocarcinoma from human endometrium (15). It was maintained by serial transplantation in castrated male nude mice in the sustained presence of 17β-estradiol. ECC-1 cells were established in monolayer culture from EnCa101, a moderately differentiated transplantable human endometrial carcinoma grown in athymic mice (15, 16). AN3 was established in continuous culture in vitro and was derived from a lymph node metastasis of a poorly differentiated adenocarcinoma from human endometrium (17). AN3 and ECC-1 cells (10⁶) were injected s.c. into both flanks of surgically castrated, male, athymic 5- to 7-wk-old mice. The animals were given injections in the left flank in the absence of and the right flank in the presence of 0.2 ml of Matrigel. Tumor growth was monitored at weekly intervals by measuring the tumors in three dimensions with vernier calipers. When the tumors reached about 3 to 10 mm in GDD, they were excised and processed for histological and ultrastructural examinations.

Culture of ECC-1 and AN3 Cells in Vitro. The ECC-1 cells were grown as monolayers in Ham's F-12 medium containing 10% heat-inactivated, Mycoplasma- and virus-tested fetal calf serum, antibiotic-antimycotic mixture (GIBCO), human insulin (0.2 units/ml), human transferrin (25 µg/ml), and glucose (0.4 mg/ml). AN3 medium consisted of Eagle's minimal essential medium, Earle's balanced salt solution with nonessential amino acids, 10% fetal calf serum, antibiotic-antimycotic mixture, human insulin (0.2 units/ml), human transferrin (25 µg/ml), sodium pyruvate, and glucose (0.4 mg/ml). The cells were cultured in a humidified 95% air-5% CO₂ atmosphere at 37°C. For the ECM studies, the monolayer cells were trypsinized and grown at a density of 150,000 cells in each well of a 24-well plate in 0.5 ml of the appropriate medium. Frozen Matrigel was thawed overnight at 4°C, and a 0.2-ml aliquot was initially layered in the plate and then kept at 37°C for 10 min. Thereafter, 0.2 ml of Matrigel were mixed with 150,000 cells, placed in each well, and covered with 0.5 ml of appropriate medium.

Ultrastructural and Immunohistochemical Examinations of Cultured Cells and Nude Mouse-grown Tumors. The cultured cells were examined daily with phase-contrast microscopy. Both the cultured cells and the tumors grown in the nude mice were removed at various intervals and have been routinely observed when cells were grown as monolayers. Growth of epithelial cells from a variety of tissues on ECM components resulted in the reemergence of the original morphological and functional characteristics which were lost during their culture as monolayer cells on plastic (9-14). Most of these studies, especially those on human endometrium, focused on the maintenance of glandular morphology, lumen formation, and epithelial cell polarization in normal tissues. In this investigation, we have examined the gland morphogenesis of two established cell lines derived from endometrial carcinomas with and without the ability to form glands in different microenvironments.

INTRODUCTION

Human adenocarcinomas differ in their ability to form glands and, based on this characteristic, are defined as well, moderate, or poorly differentiated. This single histological feature generally serves as an important prognostic indicator of tumor behavior. Poorly differentiated tumors generally grow rapidly, metastasize, and carry a poor prognosis, while differentiated tumors have a better prognosis. The relation between histological grade and biological behavior is especially applicable to human endometrial adenocarcinomas, and these characteristics are maintained in various human endometrial carcinomas during subcutaneous growth in athymic mice (1, 2).

The mechanism regulating the development of glandular structures and the loss of this histological characteristic in anaplastic tumors are unclear. Whether this is an intrinsic property of tumor cells or is the outcome of the interaction of tumor cells with their microenvironment remains unanswered. Emerging evidence suggests that the growth and differentiation characteristics of epithelial cells may at least, in part, be influenced by the stromal cells and the ECM components involved in their proliferation/differentiation characteristics.

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3 The abbreviations used are: ECM, extracellular matrix; GMD, geometric mean diameter.
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Fig. 1. In A, cells of EnCa-101 grown in the nude mouse are positively stained for mucin (arrowheads) (× 200). EnCa-101 cells, grown in the nude mouse form glandular structures. B, 1-μm-thick section stained with toluidine blue (× 300). In C, glands formed by EnCa-101 cells in the nude mouse are tethered by a basal lamina (arrowheads) (× 5000). In D, glands express adhesion junctions including desmosomes (arrowheads) (× 9000). In E, some glands show secretory activity (arrow) (× 8000). In F, ECC-1 cells grown in the nude mouse are positively immunostained for cytokeratin (× 400). L, lumen.

fixed at 4°C in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for a minimum of 24 h. The samples were embedded in epoxy resin as described (18–20). One-μm sections were stained with toluidine blue, and thin sections were examined in a Hitachi Model H-7000 electron microscope at 75 kV. The cultured cells or tumors were also fixed in freshly prepared 4% paraformaldehyde, dehydrated in an ascending series of alcohol, and embedded in paraffin. Paraffin sections were stained by hematoxylin and eosin and by mucicarmine. These sections were also stained for cytokeratin by immunohistochemistry as previously described (21, 22). Briefly, paraffin-embedded sections were deparaffinized in xylene and ascending series of alcohol. The immunostaining consisted of incubation with normal horse serum, the mono-
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Fig. 2. In A, AN3 cells form sheets of poorly differentiated adenocarcinoma in the nude mouse. Mucin stain is not observed (x 200). In B, a 1-μm-thick section of AN3 tumor shows sheets of epithelial cells, supporting nude mouse tissue and Matrigel (M) (x 300). In C, neoplastic cells (arrows) have large nuclei and prominent nucleoli characteristic of neoplastic growth. Desmosomes or other types of junctions and basal lamina are absent in the AN3 tumor grown in the nude mouse (x 4000).

clonal antibody to cytokeratin (Catalogue Nos. 8, 18, and 19; Becton Dickinson, Mountain View, CA), biotinylated horse anti-mouse immunoglobulin G, and avidin-biotin complex and then development in a mixture of 4-chloro-1-naphthol (CN)-H2O2, resulting in a blue reaction product. Primary antibody was used at 1 to 2 mg/liter, and secondary antibody was used at 1/1000 dilution. Negative controls for immunostaining consisted of the substitution of the primary antibody by phosphate-buffered saline.

RESULTS

Growth of Carcinoma Cells in the Nude Mouse. The AN3 cells grew rapidly as solid tumors in the subcutis of the nude mice. Interestingly, AN3 cells administered with Matrigel grew relatively faster and reached 1-cm GMD within 4 wk (average of 3 tumors) compared with 7 wk (average of 2 tumors) needed for cells injected without Matrigel to reach the same size. The effect of Matrigel on the increased rate of growth was also observed with the ECC-1 cells. In the presence of Matrigel, the tumors reached 0.5-cm GMD by 72 days (average of 3 tumors), whereas the tumors growing in the absence of Matrigel were only 0.2 cm (average of 2 tumors).

Morphology of Carcinomas Growing s.c. in the Nude Mouse. The ECC1 cells grown in the nude mouse were morphologically similar to EnCa-101 cells and were reminiscent of a moderately differentiated adenocarcinoma (Fig. 1, A–C). They consisted of glands of various configurations and sizes with some showing a back-to-back appearance. Commonly, a central lumen was bordered by one to multiple layers of epithelium. The cells showed nuclear atypia characterized by various sizes and shapes and nuclear hyperchromasia. Electron microscopy revealed that glands rested over a well-defined basement membrane (Fig. 1C). Polarity was evident by the presence of microvilli protruding into the lumen and the presence of junctions, including gap, tight junctions, and desmosomes (Fig. 1D). Some cells exhibited presence of secretory granules (Fig. 1E). Intracellular lumina could also be identified within the cytoplasm of some cells. These glandular structures were embedded in a stroma consisting of cells with morphological configurations of fibroblasts and vessels originating from the host. The tumors that were derived from suspension of tumor cells in Matrigel prior to inoculation into nude mice showed an identical morphology to those grown directly in nude mice. However, remaining Matrigel could still be identified surrounding the nests of tumor cells. The majority of cells expressed mucin in their cytoplasm and were positive for cytokeratin (Fig. 1F). In contrast, the tumor that grew from AN3 cells formed islands and sheets of cells without any evidence of formation of glands. The AN3 cells expressed cytokeratin and were devoid of mucin (Fig. 2, A and B). At the ultrastructural level, epithelial cells did not surround lumina as seen with EnCa-101 and ECC1 tumors, and basal lamina, desmosomes, and other junctions or secretory activity were lacking (Fig. 2C).

Morphology of Carcinoma Cells Grown in Two- and Three-dimensional Cultures. The cultured ECC1 and AN3 cells, adherent to the plastic substratum as monolayers, had polygonal morphology and formed sheets that gradually coalesced as the cells proliferated. Both cell types were positive for cytokeratin. The ECC1 cells rarely expressed mucin (Fig. 3A), while AN3 cells were negative for mucin. Ultrastructural examination of ECC-1 (Fig. 3A) and AN3 (not shown) monolayers revealed a single layer of flat cells that showed neither secretory activity nor junctional complexes. As evaluated by phase-contrast microscopy, the morphology of epithelial cells of the ECC-1 cell line, bound to the surface of Matrigel, did not significantly differ from that seen in monolayer cultures. Ultrastructural studies revealed the presence of one to several layers of epithelial cells that exhibited formation of junctions and desmosomes (Fig. 3B). However, no evidence for the formation of glands,
secretory activity, or basal lamina was observed. The ECC-1 cells grown within Matrigel exhibited a distinctly different morphology. The cells did not form monolayers. Rather, they aggregated and formed gland-like structures that expanded with time. These were initially formed by two to several cells surrounding a central lumen and had a ball-like structure. However, within 5 to 10 days they developed various configurations and became sausage shaped (Fig. 3C). Electron microscopy showed that these structures are virtually indistinguishable from the glandular structures formed in the nude mice with the exception of the absence of the basal lamina. Glands consisted of one to several layers of cells with their microvilli projecting into a central lumen (Fig. 3D). Well-developed junctional complexes and desmosomes were readily evident (Fig. 3D). Similar to the tumors grown in nude mice, some intracellular lumen formation and secretory activity were observed. All cells expressed cytokeratin, and the majority of cells expressed mucin in their cytoplasm (not shown). AN3 cells grown within Matrigel in vitro, similar to those grown in nude mice, did not exhibit features of differentiation at the light or ultrastructural level. Cells grew as aggregates of a few to several cells with irregular borders and without any evidence of the formation of glandular structures or junctions (Fig. 4). However, pseudolumina were formed as a result of the degeneration of cells (Fig. 4B). The AN3 cells grown in vitro were positive for cytokeratin, but did not show positive staining for mucin (data not shown).

**DISCUSSION**

Results of the present study suggest that the gland-forming ability may be an inherent property of the well to moderately differentiated adenocarcinoma cells. The ECC-1 cells, derived
from a moderately differentiated endometrial carcinoma, which formed glands when grown as a solid tumor in nude mice, retained the ability to interact with each other and the ECM in vitro and organized into glands consisting of polarized columnar epithelia surrounding a lumen. In contrast, cells derived from a poorly differentiated endometrial carcinoma, AN3, lacked the gland-forming ability when grown subcutaneously as solid tumors in athymic mice. Providing ECM components, in vitro, also did not induce the organization of these cells into glands. It is, indeed, likely that other tumor cells may lack such a correlation as observed with these two cell lines, and alternative approaches of differentiation induction may elicit different responses.

ECC-1 cells showed a remarkable resemblance to the EnCa-101 tumor and exhibited features of a neoplastic epithelium when regrown as a solid tumor in vivo or in vitro within Matrigel. This was evident from the heterogeneity in the size and shape of cells, their nuclear morphology, hyperchromasia, and the presence of prominent nucleoli. The similarity was striking when the cells were grown within Matrigel, with the exception that the ECC-1 cells were not mantled by basal lamina as seen in vivo.

Epithelial cells exhibiting a polarized pattern of growth also appear to be functional. Rat uterine epithelial cells grown over Matrigel were shown to retain the ability to secrete proteins identified to be markers of estrogen sensitivity in the intact uterus (10). Recently, progesterin responsiveness of rabbit uterine epithelial cells polarized in vitro was also demonstrated (11). Epithelial cells of the thyroid gland grown within Matrigel also retained the ability to produce thyroid hormone (5). Differential regulation of β-casein and the patterns of interaction of basement membrane components and mouse mammary epithelial cells were demonstrated during the growth of these cells on various substrata (9). In this study, we have demonstrated the presence of mucins in the glands when ECC-1 cells were grown within Matrigel. This is indicative of the restoration of the secretory function in ECC-1 cells, a function which was lost when these cells were grown as monolayers.

The morphological characteristics of both AN3 and ECC-1 cells were similar when grown on a plastic surface. These cells adhered to plastic, growing as a flat monolayer of cells without exhibiting the characteristics of a polarized epithelium. Typically, a polarized epithelium consists of tall columnar cells with basally located nuclei, apical secretory activity, junctional complexes including tight, gap, and desmosome junctions, expression of uvomorulin at basolateral surfaces, apical microvilli, and vectorial transport (10, 12, 23). It is presumed that the loss of polarization in epithelial cells grown as monolayers is a reflection of the substrate on which the cells grow. The ECC-1 cells which grew as monolayers retained the intrinsic property to polarize when grown again in a suitable milieu. In contrast, AN3 cells did not manifest this characteristic despite their growth in extracellular matrix. When ECC-1 cells were grown over the Matrigel, many of the characteristics of a polarized epithelium were reexpressed, as was evident from the increased cell height, demonstration of junctional complexes, and secretory activity. Similar morphological polarization over Matrigel was shown in cultures of epithelial cells from immature rat uteri and glandular cells of human endometrium (7, 8, 10, 12, 13). Similarly, Sertoli cells grown over reconstituted ECM showed vectorial secretion of transferrin and androgen-binding protein (3). However, the growth of these cells in two-dimensional cultures does not allow full expression of the polarized potential of the epithelial cells.

Organization of carcinoma cells depends extensively on whether they are allowed to interact with the surrounding matrix on one or all dimensions. Endometrial epithelial cells, barring those covering the surface, are arranged around a central lumen, forming glandular structures that are separated from the surrounding stroma by a basal lamina. The ECC-1 cells reexpressed the ability to interact with each other and with the surrounding matrix to properly align themselves to form glandular structures only when grown “within” Matrigel. This observation is in agreement with several recent studies on the differential growth characteristics of epithelial cells grown “over” as compared to “within” the ECM. Thyroid epithelial cells form follicles when grown “within” the Matrigel (5). Growth of Sertoli cells “within” rather than “over” the reconstituted basement membrane resulted in the testicular cord formation and germ cell development (3). Rat mammary epithelial cell lines failed to form tubular structures when grown “over” floating gels, while tubules were formed upon folding the gel (4). The lack of morphological differentiation of the two human endometrial carcinoma cells reported by Boyd et al. (24) upon growth “over” the Matrigel may reflect either the intrinsic property of the carcinoma cells chosen or the fact that they were grown “over” rather than “within” the Matrigel.
In principle, the growth of epithelial cells may be divided into three patterns. In the first pattern, the cells proliferate in two dimensions over the stromal-extracellular matrix forming the surface epithelium. In the second pattern, growth of cells in three dimensions within the stroma causes gland formation, while cells which grow downward into deeper tissues exhibiting directional proliferation and migration may constitute the third type of growth. The first and second patterns of growth of cells can be simulated by growing cells "over" and "within" Matrigel, respectively. However, the third type of directional growth may be lacking in this system and, hence, the cells assume ball-like or sausage-shaped structures. The directional growth of gland structures may be modulated by the epithelial-stromal interaction. The stromal element, either by physical contact or through elaboration of cytokines, may modulate the directional growth. Several cytokines, including interleukins 1 and 6, γ-interferon, transforming growth factor α, and epidermal growth factor/transforming growth factor α, are known for their effects on proliferation, differentiation, and induction of morphogenesis.

The absence of differentiated properties in AN3 cells, under conditions where ECC-1 cells exhibit these characteristics, may be a consequence of an arrest of these cells in a genetically programmed pathway leading to the differentiated state. Such an arrest in differentiation may result from a lack of factor(s) essential for the progression to the differentiated state. Alternately, an inhibitor may prevent the progression through the differentiation pathway. Availability of an in vitro system where the differentiated states of varying carcinoma cells can be maintained and modulated will enable us to test the above predictions and allow examination of the effects of various chemical agents known to induce differentiation in other systems. These studies may provide clues to the mechanisms involved in the regulation of differentiation with the potential for designing rational treatment strategies in the control of growth of poorly differentiated tumors.

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