Cancer-associated Myofibroblasts Possess Various Factors to Promote Endometrial Tumor Progression

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ABSTRACT

Myofibroblastic invasion associated with malignant epithelial cells of endometrial cancer as well as other cancers is often found in the interstitium. To assess the myofibroblastic-epithelial interaction, frozen sections from a total of 10 endometrial cancers with or without invasive myofibroblasts were immunohistochemically examined. Interestingly, the invasive myofibroblasts adjacent to malignant epithelial cells showed frequently intense positive staining of several growth factors such as vascular endothelial growth factor (VEGF), insulin-like growth factor I, and epidermal growth factor, the cognate receptors such as Fetal liver kinase-1/kinase Insert Domain-containing receptor/VEGF receptor-2, fms-like tyrosine kinase-1/VEGF receptor-1, and epidermal growth factor receptor, several cell cycle regulators such as cyclins and cyclin dependent kinases, and estrogen receptor α. Moreover, we indicated that the majority of the myofibroblasts as well as cancer epithelial cells are proliferating because of their positive staining of proliferating cell nuclear antigen and Ki-67. Furthermore, the myofibroblasts were also positive of hypoxia-inducible factor 1α, which is a marker protein of hypoxia, probably followed by activation of VEGF-Flik-1 and VEGF-fms-like tyrosine kinase-1 signals, which could initiate angiogenesis. These findings suggest directly that the myofibroblasts might participate in the progression of tumor cells in terms of cancer cell growth stimulation and also activated initiation of angiogenesis.

INTRODUCTION

Tumorigenesis is a multistep process accompanied by genetic alterations of precancerous cells and simultaneously by building up the microenvironment that promotes transformation (1–3). Consequently, epigenetic contributions from stroma cells surrounding cancer cells play important roles for formation of progressive neoplasm (4–6). The features of stromal invasion in carcinomas are frequently shown in many cancers including breast, endometrium, prostate, lung, colon, and stomach. It is also known that the stroma is histopathologically composed of several supportive coconspirators such as fibroblasts, inflammatory cells, immune cells, smooth muscle cells, and endothelial cells (3, 4). Interestingly, fibroblasts constitute a major stromal compartment, and many of them associated with tumor cells are seen significantly differentiated into so-called “myofibroblasts” (7–9), which are also found in the process of wound healing, possessing more reactive and plastic properties than normal fibroblasts. The trait of myofibroblasts containing both fibroblasts and smooth muscle cell lineages is defined by positive expression of stromal cell type markers such as prolky 1-4-hydroxylase (10, 11), integrin α6β1, vimentin, and CD34, and by positive expression of smooth muscle cell markers such as α-smooth muscle actin, smooth muscle myosin, calponin, and α1-integrin (9). However, the differences between myofibroblasts and fibroblasts involved in the oncogenic ability are quite unknown. It was postulated previously that several tumorigenic features could be enhanced by coexistence of the fibroblasts and malignant epithelial cells. In brief, several in vitro coculture (12–15) and in vivo xenografts studies (16–19), using both cells with a combination of various cell types among normal, initiated, and/or tumorigenic stages, have suggested that the fibroblasts could accelerate progression of malignant epithelial cells because of activated proteolysis (20, 21), increased cancer cell proliferation (14, 15), and attenuation of cancer cell death (18). These consequences might be feasible in assessing the cancer cell progression mechanisms, although the molecular signaling of the fibroblasts-epithelial interaction is also still quite obscure.

We have tried to perform immunostaining of frozen sections from 10 independent endometrial cancer samples using different antibodies, which specifically recognize several key proteins involved in cell growth, cell cycle regulation, sex hormone activation, hypoxia, and angiogenesis. Significant positive staining of various factors in interstitial myofibroblasts has raised an interesting possibility that the myofibroblasts might actually support tumor progression.
PATIENTS AND METHODS

Selection of Patients. Between November 1998 and December 1999, 10 patients of all of the endometrial cancer patients (mean age, 60.3 years; range, 38–79 years) to whom hysterectomy was performed were enrolled in the study under informed consent, according to no complication of chronic diseases. Additionally, any hormonal drugs such as GnRH agonist and progestogen had never been prescribed before the operation.

Antibody and Immunohistochemistry. A small portion of each endometrial cancer mass was cut down and promptly embedded into Tissue-Tek O.C.T. Compound (Sakura Finetech- technical Co., Ltd., Tokyo, Japan). Then serial 4-μm frozen sections were generated and stained with H&E. Immunohistochemistry was performed sequentially as described previously (22). Coverslides were fixed, preblocked in 10% normal goat or rabbit serum, and reacted using each first antibody. The reaction by first antibody was performed in 1:100 for mAb and in 1:400–600 for polyclonal antibody, respectively. Anti-prollyl 4-hydroxylase mAb (5B5; DAKO Corp., Carpinteria, CA), anti-integrin αvβ3 mAb (P1E6; DAKO Corp.), antivimentin mAb (V9; Santa Cruz Biotechnology, Inc.), and anti-cdk6 polyclonal antibody (QBEND10; Immunotech, Marsielle, France) were used as a positive marker of stromal cell lineage. In addition, anti-α-smooth muscle actin mAb (1A4; Sigma Chemical Co.) was used as a positive marker of smooth muscle cells, and anticytokeratin 8, 18, and 19 mAb (NCL-5D3; PROGEN, Heidelberg, Germany) was used as a positive marker of epithelial cells. Additionally, anti-VEGF3 polyclonal antibody (C-1; Santa Cruz Biotechnology, Inc.), anti-IGF-I polyclonal antibody (G-17; Santa Cruz Biotechnology, Inc.), anti-EGFR polyclonal antibody (Chemicon International Inc., Temecula, CA), anti-EGF mAb (Chemicon International Inc., Temecula, CA), anti-IGF-I polyclonal antibody (1005)-G (Santa Cruz Biotechnology, Inc.), anti-Fetal liver kinase-1/kinase Insert Domain-containing receptor/VEGFR-2 mAb (A-3; Santa Cruz Biotechnology, Inc.), and anti-fms-like tyrosine kinase-1/VEGF receptor-1 polyclonal antibody (C-17; Santa Cruz Biotechnology, Inc.) were used as a marker of growth factors and the cognate receptors. As a marker of cell cycle regulators, anticyclin D1 mAb (R-124; Santa Cruz Biotechnology, Inc.), anticyclin D3 mAb (Upstate Biotechnology Inc., Waltham, MA), anticyclin A polyclonal antibody (A-3; Santa Cruz Biotechnology, Inc.), anti-cdk4 polyclonal antibody (C-22);G (Santa Cruz Biotechnology, Inc.), and anti-cdk6 polyclonal antibody (C-21; Santa Cruz Biotechnology, Inc.) were also used. Moreover, anti-PCNA mAb (PC10; Santa Cruz Biotechnology, Inc.) and anti-Ki-67 polyclonal antibody (C-20; Santa Cruz Biotechnology, Inc.) were used as a marker of proliferating cells. In addition, anti-ER α polyclonal antibody (PA1–308; Affinity Bioreagents, Inc., Golden, CO), anti-PR mAb (MA1–410; Affinity Bioreagents, Inc.), and anti-EFP

Table 1 Summarized results of semi-quantitive immunohistochemical staining of tumor-associated stroma in 10 endometrial cancers

<table>
<thead>
<tr>
<th>Sample</th>
<th>Patients</th>
<th>Age</th>
<th>Grade</th>
<th>Steroid hormone</th>
<th>Growth factors</th>
<th>Cell cycle regulators</th>
<th>Antigenics</th>
<th>Mesoenchymal cell markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Y.U.</td>
<td>55</td>
<td>G1</td>
<td>ER/PR</td>
<td>TGF</td>
<td>Flk-1/Flt-1</td>
<td>HIF1</td>
<td>αSM, βSM, αvβ3, αvβ5, αvβ8, αvβ6</td>
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<tr>
<td>2</td>
<td>N.Y.</td>
<td>39</td>
<td>G2</td>
<td>ER/PR</td>
<td>TGF</td>
<td>Flk-1/Flt-1</td>
<td>HIF1</td>
<td>αSM, βSM, αvβ3, αvβ5, αvβ8, αvβ6</td>
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<tr>
<td>3</td>
<td>S.T.</td>
<td>79</td>
<td>G3</td>
<td>ER/PR</td>
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<td>Flk-1/Flt-1</td>
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<td>N.Y.</td>
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<td>ER/PR</td>
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<td>S.T.</td>
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<tr>
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<td>Flk-1/Flt-1</td>
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*The abbreviations used are: VEGF, vascular endothelial growth factor; IGF-I, insulin-like growth factor I; mAb, monoclonal antibody; EGF, epidermal growth factor; TGF, transforming growth factor; EGF-R, epidermal growth factor receptor; cdk, cyclin-dependent kinase; ER α, estrogen receptor α; PR, progesterone receptor; EFP, estrogen-responsive protein; PCNA, proliferating cell nuclear antigen; Flt-1, fms-like tyrosine kinase-1; HIF1 α, hypoxia-inducible factor 1 α.
Polyclonal antibody (23) were used as detection of sex hormone receptors and downstream target genes. Lastly, anti-HIF-1α mAb (clone H1α67; Novus Biologicals, Inc., Littleton, CO) was used as a marker of hypoxia. After biotinylated antirabbit IgG antibody, antimouse IgG antibody, or antigoat IgG antibody was reacted selectively, horseradish peroxidase-streptavidin and substrate solution were added. Then these sections were slightly stained by hematoxylin.

RESULTS

Immunohistochemical Identification of Myofibroblasts in Endometrial Cancer. Serial frozen sections from 10 endometrial cancer samples were stained by H&E to classify them histologically into three pathological grades (G1–G3). We found that there were 3 cases with G1 grade, 4 cases with G2 grade, and 3 cases with G3 grade, respectively. Actually, 8 samples (from 3 to 10 in Table 1) showed significant stroma cell invasion in the interstitium, whereas 2 samples (1 and 2) showed little stroma cell invasion (data not shown). Herein, a view of the sample 6 (Y.U.) was representatively shown. Morphologically there were a number of fibroblasts with spindle-like appearance in the stroma invasion on the specimen stained by H&E (Fig. 1, panel 1). To confirm them immunohistochromically, immunostaining was done using positive markers of stroma cell lineage including fibroblasts as prolyl 4-hydroxylase (Fig. 1, panel 2), integrin α2β1 (Fig. 1, panel 3), vimentin (Fig. 1, panel 4), and CD34 (data not shown), and using a strict positive marker of smooth muscle cell lineage as α-smooth muscle actin (Fig. 1, panel 5), which never reacts with fibroblasts. In the markers of stroma cell lineage, the anti-prolyl 4-hydroxylase antibody was reported as more specific to the fibroblasts than others showing no cross-reactivity, at least with other mesenchymal cell lineages such as lymphocytes, monocytes, dendritic cells, granulocytes (10, 11), and smooth muscle cells (data not shown). It was found that a number of cells in the interstitium were positive with respect to each marker on serial sections from the sample 6 (Fig. 1, panels 2–5), suggesting that the majority of invasive stroma appears to be composed of a great many myofibroblasts, because positive signals of both markers of fibroblastic and smooth muscle cell lineage were colocalized. In contrast, anticytokeratin 8, 18, and 19 antibody, strictly specific to the epithelial cells, stained only the epithelial cells strongly but no stroma in the interstitium as was expected (Fig. 1, panel 6).

Immunohistochemical Staining of Several Growth Factors and Cell Cycle Regulators in Invasive Carcinoma-associated Myofibroblasts. First, we hypothesized that growth factors would be more feasible as the factor that mediates the myofibroblastic-epithelial interaction. A number of sections from 10 endometrial cancer samples were used for immunostaining. In Fig. 2A, the representative views of invasive myofibroblasts in interstitial stroma (Fig. 2A, panels 2, 4, 6, and
Fig. 2  Immunohistochemical staining of several growth factors and cell cycle regulators in invasive carcinoma-associated myofibroblasts. A, representative views of 2 samples (1 and 6, shown in Table 1) of 10 endometrial cancer samples are shown. Panels 1, 3, 5, and 7 are from sample 1, and panels 2, 4, 6, 8, 9, and 10 are from sample 6. The benign regions (panels 9 and 10) from sample 6 are also shown. Each serial frozen section was immunostained using the following antibodies: panels 1, 2, and 9, anti-VEGF polyclonal antibody; panels 3, 4, and 10, anti-IGF-I polyclonal antibody; panels 5 and 6, anti-EGF mAb; and panels 7 and 8, anti-EGFR polyclonal antibody. EP, epithelial cells; CAF, carcinoma-associated fibroblast; BE, benign region. Scale bar, 100 μm. B, representative views of two samples (1 and 6) are shown as in (A). Similarly, here are views from sample 1 (panels 1, 3, 5, 7, and 9) and those from sample 6 (panels 2, 4, 6, 8, and 10). Panels 1 and 2, immunostaining with anticyclin D1 mAb; panels 3 and 4, anticyclin D3 mAb; panels 5 and 6, anticyclin A polyclonal antibody; panels 7 and 8, anti-cdk4 polyclonal antibody; and panels 9 and 10, anti-cdk6 polyclonal antibody. Positive signals are shown in brown, and incidental nuclear staining of whole cells is shown as blue or violet by hematoxylin. Abbreviations and scale bars are the same as in A.
8), the vicinal tumor epithelial cells (Fig. 2A, panels 2, 4, 6, and 8), and the portion of benign epithelial cells (Fig. 2A, panels 9 and 10) were presented from the serial sections of the sample 6 that had also been shown in Fig. 1, whereas malignant epithelial cells with few invasive myofibroblasts from sample 1 were also shown in Fig. 2A, panels 1, 3, 5, and 7.

Indeed, the myofibroblasts (Fig. 2A, panels 2, 4, and 6) showed immunohistochemically significant positive staining of VEGF, IGF-I, and EGF, whereas vicinal malignant epithelial cells (Fig. 2A, panels 2, 4, and 6) were quite less stained. In contrast, the malignant epithelial cells without invasive myofibroblasts (Fig. 2A, panels 1, 3, and 5) showed strong staining of VEGF, IGF-I, and EGF, and the stain of VEGF (Fig. 2A, panel 9) and IGF-I (Fig. 2A, panel 10) in the benign epithelium was also shown strongly, indicating the down-regulation of these factors in malignant epithelial cells adjacent to invasive myofibroblasts, although there were differences among respective samples (data not shown). In addition, EGFR staining was
strongly positive in invasive myofibroblasts (Fig. 2A, panel 8), vicinal tumor epithelial cells (Fig. 2A, panel 8), and tumor epithelial cells with few invasive myofibroblasts (Fig. 2A, panel 7).

Next, we attempted immunostaining using various antibodies specific to cell cycle regulators such as cyclin D1, D3, cyclin A, cdk4, and cdk6. These regulators were significantly positive in invasive myofibroblasts even more than vicinal tumor epithelial cells (Fig. 2B, panels 2, 4, 6, 8, and 10), whereas tumor epithelial cells with few invasive myofibroblasts also showed strong positive signals (Fig. 2B, panels 1, 3, 5, and 7) of all of these factors. However, cdk6 signals appeared predominant in stroma (Fig. 2B, panel 9), indicating that other interstitial cells also produce cdk6 in this sample. Then we performed immunostaining using each antibody that recognizes PCNA and Ki-67 to assess whether these cells are proliferating. There were positive PCNA and Ki-67 signals in both tumor epithelial cells and invasive myofibroblasts (Fig. 3, panels 2 and 4) and also in tumor epithelial cells with few invasive myofibroblasts (Fig. 3, panels 1 and 3). These results show that both the myofibroblasts and tumor epithelial cells are actually proliferating.

**Immunohistochemical Analysis of Sex Hormone Receptors and a Few Factors Implicated in Hypoxia and Angiogenesis in Cancer-associated Myofibroblasts.** Next, immunostaining of ER α was examined because of the feature of estrogen-dependent growth of endometrial cancer cells. The expression of EFP (22, 23) and PR (24), which are downstream target genes of ER α in the uterus, was also examined.

Invasive myofibroblasts showed significantly overlapped staining of ER α (Fig. 4, panel 2), EFP (Fig. 4, panel 4), and PR (Fig. 4, panel 6), whereas respective signals of vicinal tumor epithelial cells were moderately positive in ER α (Fig. 4, panel 2) and PR (Fig. 4, panel 6) and almost negative in EFP (Fig. 4, panel 4), suggesting the possible regulation by sex hormones even in the invasive myofibroblasts. In contrast, tumor epithelial cells with few invasive myofibroblasts also showed strong staining in ER α (Fig. 4, panel 1), EFP (Fig. 4, panel 3), and PR (Fig. 4, panel 5). Lastly, we have attempted immunostaining of HIF1 α, which is a marker protein of hypoxia and also that of Flk-1 and Flt-1, which are markers for initiation of angiogenesis as the receptors of VEGF. In Fig. 5, the immunoreactivity of HIF1 α (Fig. 5, panel 1), Flk-1 (Fig. 5, panel 2), and Flt-1 (Fig. 5, panel 3) was significantly positive in invasive myofibroblasts, whereas vicinal tumor epithelial cells were also moderately positive in HIF1 α and Flk-1 but negligible in Flt-1. The significance of positive staining in invasive myofibroblasts is discussed later.

**DISCUSSION**

The supportive role of stroma surrounding cancer cells, which is composed of majority of myofibroblasts, to tumor is originally demonstrated (4, 5). Interestingly, new reports are showing currently that myofibroblasts have oncogenic ability to promote not only tumor initiation but also the progression in the prostate cancer (19, 25). However, the molecular mechanism of a sort of tumorigenic ability in the myofibroblasts is still unknown.

Actually, it was reported previously that 83% of the whole curetteting samples from 204 endometrial cancers with G1–G3 grades showed significant stromal invasion adjacent to cancer epithelial cells (26). Therefore, we have evaluated the immunoreactivity of several factors regarding cancer promotion in stroma surrounding the endometrial cancer cells.

This time, we have demonstrated that almost all of the cells of the invasive stroma in 4 (samples 3, 6, 7, and 8, shown in Table 1) of 8 endometrial cancer samples (from 3 to 10) are likely to be real myofibroblasts according to highly positive signals of prolyl 4-hydroxylase and α-smooth muscle actin, whereas the interstitium of the remaining 4 samples (4, 5, 9, and 10) probably consists of smooth muscle cells and other mesen-
chymal cells containing fewer myofibroblasts, because the cell population is less positive of prolyl 4-hydroxylase compared with more cell population positive of α-smooth muscle actin, integrin αβ1 and vimentin (Table 1). Consistently, it was reported previously that myofibroblasts were composed of >80% of breast cancer stroma cells (27). In future, double immunostaining using anti-prolyl 4-hydroxylase antibody and α-smooth muscle actin antibody may be required to actually evaluate the correct ratio of myofibroblasts in the whole stroma.

The role of invasive myofibroblasts during tumorigenesis has been examined by several immunohistochemical studies and coculture systems with malignant cells showing elevated production of extracellular matrix proteins such as tenascin (28) and fibronectin (29) and altered secretion of metalloproteinases such as stromelysin-3 (20, 21) and MMP-2 (30), which are proteolytic enzymes. These findings suggested that the myofibroblasts might support cancer-invasive properties through facilitation of the initial attachment and also through infiltrative movement of cancer epithelial cells. In addition, regarding cell proliferation, previous reports have shown altered expression of many growth factors such as hepatic growth factor (15), IGF-I and II (12, 21), TGF-β1 (31), connective tissue growth factor (31), and platelet-derived growth factors receptor (32) in myofibroblasts cocultured with malignant cells, supporting the idea that these growth factors might help accelerate cell growth of malignant epithelial cells.

In this study, we have succeeded to immunohistochemically detect predominant positive signals in invasive myofibroblasts associated with malignant epithelial cells in actual endo-

![Fig. 4 Immunohistochemical analysis of sex hormone receptors in carcinoma-associated myofibroblasts.](image)

![Fig. 5 Immunohistochemical analysis of a few factors implicated in hypoxia and angiogenesis in carcinoma-associated myofibroblasts.](image)
metrial cancer samples using respective antibodies of several factors involved in cell growth, cell cycle regulation, sex hormone activation, hypoxia, and angiogenesis. Interestingly, the invasive myofibroblasts showed significant positive signals of growth factors, such as VEGF, IGF-I, and EGF, and those of the cognate receptors, such as Flk-1 and Flt-1, which are receptors of VEGF, and EGFR, which was positively detected also in the vicinal epithelial cells. Therefore, it is likely that the invasive myofibroblasts take a share in stimulation of malignant epithelial cells through growth factor receptor signals in a paracrine manner and also stimulate themselves in an autocrine manner. Moreover, the invasive myofibroblasts were actually proliferating as exemplified by the significant positivity of cyclin D1, D3, cyclin A, cdk4, and cdk6, and also of PCNA and Ki-67.

From these observations, we strongly suggest the possibility that these growth factors would be important molecules, which mediate the myofibroblastic-epithelial interaction in carcinogenesis. Summarized results in Table 1 with predefined arbitrary scale show that the high expression of these factors in the invasive myofibroblasts is a common feature among most endometrial cancer samples (8 of 10). Notably, the more these factors are expressed, the more the cells appeared to infiltrate as myofibroblasts (see samples 3, 6, 7, and 8).

The present study is the first to actually demonstrate predominant existence of various growth factors, cell cycle regulators, and cell proliferating markers in the invasive myofibroblasts of endometrial cancer. Incidentally, it was reported previously that most myofibroblasts in nodular palmar fibromatosis were positive for Ki-67 and PCNA (33). Also reported are several data indicating expression and existence of a few growth factors such as IGF-II (21), TGF-β1 (31), and Connective Tissue Growth Factor (31) in invasive myofibroblasts of breast cancer, although their positive signals are not so dramatic.

On the other hand, nearly one-half of endometrial carcinoma has the feature of estrogen-dependent growth and, hence, estrogen is well known as one of the risk factors that stimulates the endometrial cancer progression (34, 35). As shown in Fig. 4, the positive staining of ER α colocalized with EFP (22, 23) and PR (24), which are known as downstream genes of ER α, in the myofibroblasts is remarkable and suggests that ER α might be ligand-independently activated even in some specimens from postmenopausal women.

Next, the existence of a few angiogenic factors in myofibroblasts was examined, referring to previous reports, which had indicated the possible role of myofibroblasts implicated in angiogenesis (36, 37). It is currently believed that enhanced HIF1 α expression correlates with tumor growth and angiogenesis (38, 39), of which the mechanism is likely to be through up-regulation of VEGF expression (40). Then the significant staining of HIF1 α and VEGF in the invasive myofibroblasts in this study lets us imagine the possibility of increased initiation of angiogenesis through activation of VEGF-Flk-1 and VEGF-Flt-1 signals in the interstitium.

Finally, it is recapitulated that our immunohistochemical data supported the cooperative role of the myofibroblasts involved in endometrial cancer cell progression because of accelerated cell proliferation and initiation of angiogenesis in vivo. Significantly, a previous clinical report about endometrial cancer has indicated the importance of stromal invasion in predicting prognosis, because many atypical hyperplasias with significant stromal invasion were more frequently accompanied by more invasive residual carcinomas compared with the one with less stromal invasion (26). Hence, the evaluation of the pathological grades of endometrial cancers might be done seriously based on the insight into the existence of stromal invasion as well as malignant epithelial cells. In future, correlation among the extent of stromal invasion, existence of several growth factors, and the prognosis should be more positively evaluated, and it would also be worthy of examining carefully whether drug intervention into the paracrine factors and other signals of the stromal-epithelial interaction is applicable to therapeutic approach of cancer progression.

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