Fibroblasts in omentum activated by tumor cells promote ovarian cancer growth, adhesion and invasiveness

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Omentum metastasis is a common occurrence in epithelial ovarian cancer (EOC), which is often accompanied by ascites that facilitates the spread of EOC cells. A subpopulation of fibroblasts—the cancer-associated fibroblasts (CAFs) are important promoters of tumor progression. We have shown previously that CAFs exist not only in omentum with EOC metastasis but also in omentum without metastasis. In the present study, using primary human fibroblasts isolated from normal omentum (NFs) and omentum with ovarian cancer metastasis (CAFs), we established in vitro coculture models and a 3D culture model mimicking human omentum structure for investigation of interactions between fibroblasts and cancer cells. We demonstrate that EOC cells activate NFs and promote their proliferation via transforming growth factor-β1 (TGF-β1) signaling, and the activated fibroblasts contribute to the invasion and adhesion of EOC cells. Moreover, EOC cells and NFs coculture led to overexpression of hepatocyte growth factor (HGF) and matrix metalloproteinase-2 (MMP-2) and adhesion and invasion of EOC cells could be partially suppressed by blocking the function of HGF or MMP-2. Additionally, mouse peritoneal dissemination models of EOC confirmed the activation of fibroblasts by cancer cells and the tumor growth and metastasis-promoting effects of activated fibroblasts in vivo. Our findings indicate that activated fibroblasts in omentum form a congenial environment to promote EOC cells implantation. It is an intriguing concept that targeting the activation of omentum fibroblast through the inhibition of TGF-β1 signaling can be used as a new therapeutic strategy against ovarian cancer omentum metastases, which needs further study.

Introduction

Abdominal peritoneum and the omentum are the most common sites of ovarian cancer metastasis (1). With the formation of ascites, the exfoliated tumor cells can spread throughout the peritoneal cavity (2). Surgery is rarely able to render patients free of disease because of its diffuse nature, resulting in a 5 years survival of only 20% in patients with peritoneal microscopic or visible metastatic foci even if postoperative chemotherapy is performed (3). Thus, it is necessary to improve our understanding of the mechanisms involved in the spread of ovarian carcinoma to the omentum to identify novel therapeutic targets.

The omentum is a large fatty structure, which literally hangs off the middle of the colon and drapes over the intestines inside the abdomen. The morphology of the omentum is simple: a single layer of mesothelial cells covers a submesothelial region composed of connective tissue with a few fibroblasts, mast cells, macrophages and blood vessels (4). As the second most numerous cell types in the omentum, fibroblasts represent a dynamic population of cells that show functional and phenotypic diversity. Among the various fibroblastic phenotypes, activated fibroblasts (which are sometimes referred to as myofibroblasts) are the most important and characterized by the expression of α-smooth muscle actin (α-SMA) (5). In peritoneal tissues, activated fibroblasts were initially found in the peritoneal dialysis patients with simple sclerosis (6). Activated fibroblasts that are found in association with cancer cells are known as cancer-associated fibroblasts (CAFs) (5). CAFs play an important role in the malignant progression of cancer such as breast, prostate, pancreatic, esophageal and lung cancer, etc. including the initiation, proliferation and metastasis of cancer cells (7–10). In addition to secreting growth factors including hepatocyte growth factor (HGF), stromal cell-derived factor-1 (SDF-1) and fibroblast growth factor (FGF) that directly affect cell motility (9,11), CAFs are the source of extracellular matrix (ECM)-degrading proteases such as the MMPs. MMPs probably allow cancer cells to cross tissue boundaries and escape the primary tumor site (12).

Recent studies have provided evidence that CAFs of different tumor types probably display functional differences (13). To date, little research has been done to evaluate the role of omentum fibroblasts in cancer cell omentum metastases. In our previous study, we found CAFs also existed in omental tissues of ovarian carcinoma patients, even in some cases of omentum tissue lacking detectable cancer cell infiltration, and the stroma exhibited increased numbers of CAFs as the omental metastasis enlarges, but there was no CAFs detected in omentum tissues from patients with benign ovarian diseases (14).

In ovarian cancer, Ena Wang et al. found that there were differences between the transcriptional repertoires of peritoneal tissues lacking detectable cancer in patients with epithelial ovarian cancer (EOC) and benign gynecologic disease, and several of the transcripts identified suggest the presence of gene products that may foster cancer growth and metastasis (15). This research raises a hypothesis that peritoneal structures may be affected by the presence of EOC before tumor implantation. In other cancer tissues such as breast and lung cancers, researchers found settlement of metastasizing tumor cells is facilitated by the establishment of special niches in premetastatic organs through stimulation of bone marrow-derived hematopoietic progenitor cells and local resident fibroblasts (16). So, we speculated that changes of fibroblastic phenotypes occurring within omentum tissues also created a ‘soil’ conductive for ovarian tumor implantation.

In the present study, we found that EOC cells can contribute to fibroblast activation and hyperplasia through transforming growth factor-β1 (TGF-β1). We used a novel 3D culture model and an in vivo assay to investigate the role of fibroblasts in adhesion and invasion of ovarian cancer cells to the omentum. Evidently, fibroblasts isolated from the normal omentum tissues of ovarian cyst patients (normal fibroblasts, NFs) cannot promote the EOC cells invasion, whereas EOC cells can activate the fibroblasts and foster the ability of fibroblasts to promote the invasion of EOC cells. A83-01 which can block signaling of type I receptors for cytokines of the TGF-β superfamily (known as activin receptor-like kinases; ALKs) (17) can reverse both fibroblasts activation and the followed metastases of EOC cells promotion.

Materials and methods

Isolation and culture of primary human mesothelial and fibroblasts

Primary human mesothelial cells and normal fibroblasts (NFs) were isolated from omental tissues of patients undergoing surgery for benign ovarian cysts and CAFs were obtained from omentum with EOC metastasis as described (18,19). Briefly, after washings with sterile phosphate-buffered saline (PBS), several 2–3 cm² pieces of omentum were incubated with 0.125% trypsin/
0.01% ethylenediaminetetraacetic acid at 37°C for 20 min. To isolate mesothelial cells, the solution containing cells in suspension was centrifuged at 1500 r.p.m. for 5 min, and the pellet was plated with Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA). To isolate fibroblasts, the tissue was further digested for two 40 min periods. Cells obtained in the second period were resuspended in 5 ml DMEM/F12 supplemented with 20% FBS. Mesothelial cells were verified by both vimentin and cytokeratin 8-positive staining (20), whereas fibroblasts were verified by vimentin positive but cytokeratin 8-negative staining (21). CAFs were further characterized by expression of α-SMA, whereas NFs were α-SMA-negative. Although ovarian epithelial cells also often show vimentin-positive staining, they could be distinguished from fibroblasts by positive expression of cytokeratin 8 (22). To quantify the purity of primary cells, the percentage of marker-expressing cells to the 100 cells randomly counted was calculated. The primary cultures were incubated at 37°C and the medium was exchanged after 24 h for the first time and every third day thereafter. All primary fibroblasts used for this study were between passages 2 and 5, and mesothelial cells were only used in passage 2.

**EOC cell lines and culture conditions**

Human EOC cell lines SKOV3, CAOV3, OVCAR3 and ES2 were purchased from China Center for Type Culture Collection. SKOV3, CAOV3 and ES2 were cultured in DMEM supplemented with 10% FBS. OVCAR3 was cultured in RPMI-1640 supplemented with 10% FBS and 0.01 mg/ml bovine insulin. All of these cell lines were grown in a humidified 5% CO2 environment. For TGF-β1 stimulation studies, SKOV3 cells were cultured overnight with RPMI-1640 containing 10% FBS and then the supernatants were replaced and cultured with fresh culture medium RPMI-1640 containing 1% FBS with recombinant human TGF-β1 of different concentrations (R&D system, Minneapolis, MN) for 2–48 h.

**Direct and indirect coculture**

For direct coculture, fibroblasts labeled with a tracking dye (CFDA, V12883; Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The sequences of primers used were as follows: α-SMA, 5′-CTGTTCCACCATCCTGCTCATC-3′ (sense) and 5′-CCGATTCTTCTGTGACT-3′ (antisense); β-actin, 5′-GCCAACACGAGTCTGGTGGC-3′ (sense) and 5′-GCTAGAGGGAGGAAATGATCCTTG-3′ (antisense). Polymerase chain reaction was performed as described by the manufacturer using the SYBR Green PCR Master Mix (Toyobo, Japan). Amplification protocols were followed: 95°C for 3 min; 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. After polymerase chain reaction, a melting curve was constructed by increasing the temperature from 65 to 95°C with a temperature transition rate of 0.2°C/s. The transcript level of each specific gene was normalized to the β-actin amplification and was calculated using the comparative threshold cycle (Ct) method (2−ΔΔCt) (23).

**Immunofluorescence**

For immunofluorescence, the CFDA-labeled fibroblasts mixed with EOC cell lines were plated on sterilized coverslips in six-well plates. After washing with PBS, the cells in coverslips were fixed in 4% paraformaldehyde for 30 min and washed in PBS followed by permeabilizing with ice-cold acetone for 10 min and blocked in 10% bovine serum albumin. The cells were then incubated with antibodies against α-SMA for 30 min at 37°C. Anti-mouse IgG-CY3 (Sigma, Aldrich, Missouri) was used as secondary antibodies (1:100 in a blocking medium). Following further washing, cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1:1000; Invitrogen, Carlsbad, CA) and examined under the fluorescence microscope. Total fibroblasts number was obtained by counting the CFDA-stained cells in five fields at ×200 magnification. The percentage of CFDA labeled α-SMA-expressing cells to the total number of the fibroblasts was calculated.

**Enzyme-linked immunosorbent assay**

EOC cell lines (0.2 × 10⁵ cells per well) were cultured in six-well plates overnight with DMEM containing 10% FBS. Supernatants of these cells were removed and replaced by fresh culture medium DMEM containing 2% FBS. Cells were cultured for another 24 and 48 h. TGF-β1 in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). The assays were performed according to the manufacturer’s instructions.

**Western blotting**

Equal amount (70 µg) of cell extracts were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter membrane followed by incubating with primary mouse monoclonal antibody against human α-SMA; rabbit monoclonal antibodies against human HGF, SDF-1 (1:200 dilution; R&D Systems), smad2, phospho-smad2 (Ser465/467) (1:500 dilution; Cell Signaling Technology, Beverly, MA), MMP-2 and MMP-9 (1:500 dilution; Epitomics, Burlingame, CA). Horseradish peroxidase-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA) was used for detection. Proteins were visualized by enhanced chemiluminescence detection reagents (Pierce, Rockford, IL) and bands were quantitated by Quantity One Software (Bio-Rad, Hercules, CA).

**Cell proliferation analysis**

The thymidine nucleotide analog 5-ethyl-2′-deoxyuridine (EdU) was used for the in vitro labeling of the nuclei of dividing fibroblasts and EOC cells. After incubation for 1–5 days, respectively, EdU incorporation assays were conducted. Briefly, for indirect coculture, half of the media was replaced with fresh media containing 20 µM EdU and incubated for another 12 h, the cells in lower compartment were fixed with formaldehyde followed by 0.5% Triton X-100 permeabilization and then stained by incubating for 30 min with reaction cocktail which was prepared according to the manufacturer’s instructions. Cells were counterstained with Hoechst 33342 and imaged by fluorescence microscopy. For direct coculture, the fibroblasts were prelabeled with CFDA. The percentage of EdU-positive cells was used to evaluate the cell proliferation activity.

**3D omental culture**

To investigate the role of fibroblasts on the adhesion and invasion ability of ovarian cancer cells, we established a 3D culture model mimicking human omentum conducted according to the method of Lengyl et al. (24). Briefly, a 24-well plate was coated with 2 mg neutralized isoeionic collagen I mixed with 1 × 10⁶ CAFs or NFs. After solidification, the mesothelial cells (5 × 10⁴) in 100 µl of growth media were added to the above and incubated at 37°C for 24 h until a confluent layer of mesothelial cells formed and then 5 × 10⁶ EOC cells in 100 µl of growth media were plated on top. After incubation for 1, 24, 48 h at 37°C, the 3D omental culture gel was fixed in 10% formalin. Gels were parafin embedded, sliced at 4 µm and standard hematoxylin and eosin staining was performed.

**Adhesion and invasion assay**

All of the EOC cells used in the adhesion and invasion assay were fluorescently labeled with CFDA. For adhesion assay, cells were plated in 96-well plates (5 × 10⁴ cells per well) which had been precoated with collagen I (5 µg/well), mesothelial cells (5 × 10⁶ cells per well), fibroblasts (1 × 10⁰ cells per well) including CAFs, NFs, NFs cocultured with SKOV3 cells for 4 days and NFs treated by TGF-β1 for 2 day. After incubation at 37°C for 0.5 h, cells were washed three times with PBS and then fixed in 10% formalin. For invasion assay, 24-well transwell plates (corning) with 8 µm pore size were coated with 15 µg collagen I or collagen I mixed with 2 × 10⁵ fibroblasts and a confluent layer of 1 × 10⁵ mesothelial cells. 1 × 10⁶ fluorescently labeled EOC cells in 100 µl serum-free media were plated on top. The lower chamber was filled with 600 µl full growth media containing 10% FBS. After incubation for 48 h at 37°C, cells on the top of the membrane were scraped off. The membranes were fixed in 10% formalin. The fixed cells in adhesion and invasion assays were visualized by fluorescent microscopy at ×200 magnifications, selected five random regions by two independent observers. The number of cells per field was quantified by measuring the fluorescence intensity. All assays were run in triplicate.

To determine the effect of activation of fibroblasts in EOC cells adhesion and invasion, A83-01 was added to the pretreating step (fibroblasts culture before they were added into collagen) or the 3D culture system directly (in the collagen gel and media). To investigate the role of HGF and MMP-2 in EOC cell adhesion and invasion in 3D culture, the neutralization HGF antibody (1 µg/ml) (R&D...
systems. NFs were directly cocultured with EOC cell lines SKOV3, OVCAR3, CAOV3 and ES2, respectively, for 4 days and then the \( \alpha \)-SMA expression was assayed by immunofluorescence staining (Figure 2A). NFs cultured alone were used as control. On average 37.6–70.0% of cocultured NFs expressed \( \alpha \)-SMA, whereas only 1.72% of the control NFs expressed this CAF marker (Figure 2B). In addition, increased \( \alpha \)-SMA expression in NFs indirectly cocultured with EOC cell lines was detected by polymerase chain reaction and western blot (Figure 2C,D). Among the four EOC cell lines studied, SKOV3 showed the strongest ability to activate fibroblasts in direct as well as indirect coculture models. Thus, SKOV3 was chosen for subsequent experiments in \textit{vitro} and \textit{in vivo}. Using western blot, the \( \alpha \)-SMA levels in NFs physically separated from SKOV3 cells (indirectly cocultured) for up to 5 days were determined. We found that the \( \alpha \)-SMA expression increased time dependently, and the difference was significant from day 4 \((P < 0.01, \text{Figure 2E})\). These results indicate that fibroblasts can be activated by EOC cells even if a direct cancer cell fibroblast contact is not present. Besides, the original phenotype of primary fibroblasts remained after culturing was confirmed by the fact that the CAFs were \( \alpha \)-SMA positive and NFs were \( \alpha \)-SMA negative in the control group. However, 3 days after the end of coculture, the expression of \( \alpha \)-SMA in NFs reduced to the level in control NFs (Figure 2F), suggesting stable \( \alpha \)-SMA expression in fibroblasts needs long-term stimulation by cancer cells.

In addition, the effect of EOC cells on proliferation of NFs was evaluated by EdU incorporation assay. It was showed that hyperproliferation of NFs began to be evident after indirect coculture with SKOV3 cells for 96 h \((P = 0.0004)\) (Figure 2G, H, J). Similar pro-proliferation effect of SKOV3 cells on NFs was observed in direct coculture assay \((P = 0.039)\) (Figure 2I and J). But neither CAFs nor NFs could promote the proliferation of SKOV3 cells (data not shown).

\textit{Paracrine TGF-\( \beta \)1 from cancer cells is responsible for NFs activation}

Having demonstrated the activating effect of EOC cells on fibroblasts, we were interested in the underlying molecular mechanisms. ELISA showed notably increased secretion of TGF-\( \beta \)1 in routinely cultured EOC cell lines at 48 h \((P < 0.05, \text{Figure 3A})\), and during the coculture with SKOV3 cells, in NFs, the phosphorylation level of SMAD2, which is a cytoplasmic signaling molecule for TGF-\( \beta \)1 began to be elevated at 2 days (Figure 3B). It implies that the EOC cells-induced expression of \( \alpha \)-SMA may be mediated by TGF-\( \beta \)1. To verify this
Fig. 2. EOC cells increase expression of α-SMA in NFs. (A) Immunofluorescent staining for α-SMA expression (red) are observed in CAFs, NFs directly cocultured with EOC cells SKOV3, OVCAR3, CAOV3 or ES2, but not in NFs cultured alone. Fibroblasts are fluorescently labeled (green), nuclei are labeled with 4′,6-diamidino-2-phenylindole (blue). (B) Quantified results of immunofluorescence. The percentage of α-SMA-positive cells to the total number of the fibroblasts is increased in 4 days cocultured NFs compared with control. (C and D) Result of real-time polymerase chain reaction and western blot analysis for α-SMA expression in CAFs, NFs cultured alone and NFs indirect cocultured with EOC cell lines SKOV3, OVCAR3, CAOV3, ES2 for 4 days. Graphs show relative expression levels, normalized to β-actin. (E) Western blot analysis for α-SMA expression in NFs cocultured with SKOV3 cells for indicated time periods. The CAFs and NFs cultured alone were used as control. Quantification of western blot is shown below. (F) Real-time polymerase chain reaction show decreased α-SMA expression in NFs separated from coculture system for 1–4 days. (G) Growth rates of NFs were determined by EdU incorporation assay each day for 5 days. The growth rates of SKOV3 treated NFs were slightly increased on days 4 and 5 compared with control NFs. (H) Representative results of EdU assay of NFs in direct coculture models. NFs are labeled for EdU (red). All of the NFs are counterstained with Hoechst33342 (blue). (I) Representative result of EdU assay of cells in direct coculture models. NFs are labeled for EdU (red). All of the NFs are counterstained with Hoechst33342 (blue).
hypothesis, we examined α-SMA expression in NFs treated with different concentrations of TGF-β1 (0.1, 0.5, 1, 5, 10 ng/ml) for 2 days using quantitative real-time polymerase chain reaction as well as western blotting. As shown in Figures 3C and 3D, the expression level of α-SMA and phospho-SMAD2 in NFs were significantly increased by treatment with TGF-β1 of 1 ng/ml or higher concentrations (P < 0.01). To find out the chronological order of the changes in expression of α-SMA and phospho-SMAD2, we assessed their expression levels at five time points (0, 2, 12, 24 and 48 h) during treatment with 1 ng/ml TGF-β1 using western blotting. The expression of α-SMA as well as phospho-SMAD2 in NFs increased time dependently and significant increase of phospho-SMAD2 occurred at
2 h ($P < 0.001$) while $\alpha$-SMA expression started to rise until after TGF-$\beta 1$ stimulation for 48 h ($P < 0.001$, Figure 3E), confirming the role of TGF-$\beta 1$ signaling pathway in activation of fibroblasts from another angle. In addition, similar to coculture with EOC cells, exogenous TGF-$\beta 1$ stimulation could also promote proliferation of fibroblasts ($P = 0.002$, Figure 3F).

A specific inhibitor of TGF-$\beta 1$ (A83-01) was used to further verify the role of TGF-$\beta 1$ in fibroblast activation. The $\alpha$-SMA expression and SMAD2 activation in NFs induced by SKOV3 cells or exogenous TGF-$\beta 1$ could be attenuated by administration of 5 $\mu$mol/l A83-01 (Figure 3G). Besides, we found TGF-$\beta 1$ inhibition also reduced the proproliferation effect of TGF-$\beta 1$ ($P = 0.003$) and SKOV3 cells ($P < 0.001$) on NFs (Figure 3H). We therefore conclude that TGF-$\beta 1$ is a key factor mediating fibroblasts activating effect of ovarian cancer cells.

The activated fibroblasts play key roles in ovarian cancer cell adhesion and invasion

To examine the role of fibroblasts in omental metastasis of EOC, we established a 3D culture model consisted of primary human omentum fibroblasts in a collagen matrix and a confluent layer of mesothelial cells. The primary mesothelial cells were isolated from normal human omentum. During the initial growth phase in culture, the subconfluent mesothelial cells showed multipolar morphology, and a classic cobbledstone appearance was present after cell confluence. In addition to morphology, they were further identified by immunohistochemical expression of the cell type-specific markers cytokeratin 8 and vimentin (Figure 4A). The mesothelial character of these cells was clear and the purity of second generation mesothelial cells was ~95%.

In the omental 3D culture model with CAFs, SKOV3 cells efficiently attached to the surface of the artificial omentum at 1 h and formed a dense and adherent layer of SKOV3 cells coated the mesothelial cells at 24 h and then they began to invade into the collagen at 48 h. But when we replaced CAFs with NFs, the adhesion and invasion of SKOV3 cells were significantly delayed (Figure 4B).

To further determine whether the activity level of fibroblasts is associated with adhesion and invasion of EOC cells, we performed adhesion and invasion assays for SKOV3 cells in omental 3D culture models with CAFs, NFs and activated NFs, respectively. Model without fibroblasts was used as blank control. The presence of CAFs, NFs cocultured with SKOV3 cells and NFs exposed to TGF-$\beta 1$ increased the number of adhered SKOV3 cells 4.77 ($P = 0.007$), 3.08 ($P = 0.022$) and 3.14-fold ($P = 0.018$), respectively, and augmented the number of invaded SKOV3 cells 6.81 ($P = 0.0001$), 3.80 ($P = 0.03$) and 6.06-fold ($P = 0.0005$), respectively, compared with the control. However, there was no significant difference between naive NFs 3D model and blank control, indicating unactivated NFs are insufficient to induce the adhesion and invasion of SKOV3 cells to the omentum. The adhesion- and invasion-promoting effects of NFs stimulated by SKOV3 cells and exogenous TGF-$\beta 1$ could be partially (44.9 and 61.6% in adhesion, 58.5 and 54.8% in invasion, respectively) attenuated by addition of A83-01 in the culture period of NFs before they were added into collage, i.e. pretreating step while A83-01 directly added into the gel of 3D culture system did not influence SKOV3 cell adhesion (Figure 4C and D). These results suggest that TGF-$\beta 1$ promotes adhesion and invasion of EOC cells through regulating activity of fibroblasts, and A83-01 can inhibit the activation of NFs mediated by TGF-$\beta 1$ but can neither restrain SKOV3 cells adhesion or invasion nor influence activity of CAFs per se.

Activated fibroblasts increase the adhesion and invasion of EOC cells through regulating HGF expression

To explore the molecular mechanisms underlying promotion of EOC metastasis by activated fibroblasts, we examined two important growth factors HGF and SDF-1 in the tumor microenvironment, which were previously identified in melanoma and oral cancers as fibroblast-secreted factors that promote tumor progression (26,27). The NFs activated by SKOV3 cells or exogenous TGF-$\beta 1$ expressed significantly higher levels of HGF compared with primary NFs (3.6-fold, $P = 0.008$; 4.3-fold, $P = 0.002$). Both of these increases could be attenuated by adding A83-01 to the coculture systems (78.8 and 66.9%, $P = 0.005$) (Figure 5A). To determine whether HGF participates in adhesion and invasion of EOC cells, we used inhibitory antibody to block its function in the 3D coculture system. Figures 5B and 5C demonstrates that inactivation of HGF significantly reduced the adhesion and invasion of SKOV3 cells promoted by NFs cocultured with SKOV3 (56.9 and 30.7%, respectively) as well as NFs treated with TGF-$\beta 1$ (57.9 and 27.5%, respectively) but did not decrease to the level produced by SKOV3 cells cultured alone, suggesting that promoting effect of activated NFs was only partly mediated by HGF. No increase of SDF-1 was detected in activated NFs compared with control (data not shown).

Inhibition of MMP-2 reduces adhesion and invasion of SKOV3 cells mediated by activated fibroblasts

Both epithelial cells and stromal fibroblasts can produce enzymes that degrade the mesenchymal ECM, such as matrix metalloproteinases (MMPs) (28). Generally, MMP-2 and MMP-9 are involved in tumor metastasis. Therefore, we were interested in whether if they regulate adhesion and invasion of EOC cells mediated by activated fibroblasts. Using western blot, the MMP-2 and MMP-9 expression in fibroblasts and SKOV3 cells in different culture models were determined. When cultured alone, NFs expressed small amount of MMP-2, and its expression in SKOV3 cells was even fainter. Enhanced expression of MMP-2 was found after 3 days of direct coculture ($P < 0.001$), whereas neither NFs nor SKOV3 cells expressed increased MMP-2 up to 5 days in indirect coculture system (Figure 5D), suggesting that cell–cell contact between EOC cells and fibroblasts is essential for the enhancement of MMP-2 expression. This speculation was further confirmed by the fact that exogenous TGF-$\beta 1$ could not induce the MMP-2 expression in NFs either. We next used specific inhibitor to demonstrate the function of MMP-2. The MMP-2 inhibitor decreased the adhesion and invasion ability of SKOV3 cells promoted by activated fibroblasts in a dose-dependent fashion in the 3D culture models (Figure 5E and F). These results suggest that MMP-2 is involved in omental metastasis of EOC cells mediated by fibroblasts, although MMP-2 expression is not regulated by TGF-$\beta 1$. However, no change in MMP-9 expression was detected in different coculture systems described above (data not shown).

Mouse model of ovarian cancer metastasis

To determine the interactions between fibroblasts and EOC cells in vivo, SKOV3 cells were injected intraperitoneally into BALB/c nude mice either alone or admixed with NFs with/without subsequent A83-01 administration, respectively ($n = 6$ for each group). SKOV3 cells coinjected with NFs produced many tumor nodules in the peritoneal cavity covering and invading the body wall, mesentry and diaphragm in the mice. The SKOV3/NFs tumor weight was 4.12-fold higher than xenografts. Besides, upregulation of MMP-2 was found in SKOV3/NFs tumors and SKOV3/NFs tumors (data not shown), it may be because HGF showed abundant CAFs in SKOV3/NFs tumors and also a few activated fibroblasts present in SKOV3 tumors, which mainly intermingled with cancer cells at margin. They may be recruited from host by tumor cells (Figure 6C) and their inadequate numbers is likely to be the major cause leading to ineffectiveness of A83-01 on SKOV3 tumors. Besides, upregulation of MMP-2 was found in SKOV3/NFs xenografts (Figure 6D). A83-01 administration could reduce the fibrin, $\alpha$-SMA expression and MMP-2 expression in SKOV3/NFs tumors. There was no difference of HGF expression between SKOV3 tumors and SKOV3/NFs tumors (data not shown), it may be because HGF is expressed mainly in fibroblasts, which were very few in xenografts.
Discussion

The surrounding tumor microenvironment seems to be an important determinant in the metastases of cancer (12, 29). Our previously research indicated that CAFs exist not only in omentum with EOC metastasis but also in omentum of patients without metastasis (14), prompting us to explore the mechanisms underlying activation of fibroblasts in omentum and the role of fibroblasts in cancer metastasis. In the present study, we showed the bidirectional interactions between fibroblasts and EOC cells and explored the key factors contributing to metastasis.
fibroblasts activation and fibroblasts mediated metastasis of cancer cells.

It has been shown that CAFs might originally derive from several types of cells, such as pre-existing normal fibroblasts, nearby epithelial cells through an epithelial-to-mesenchymal transition, bone marrow-derived mesenchymal stem cells and endothelial cells (30). One of the best-known factors responsible for the phenotypic emerge of CAFs is TGF-β (30). Many studies have identified that tumor-cell-autonomous TGF-β expression is often increased in human carcinoma including EOC (31), which was confirmed by our results that all of the four EOC cell lines studied secreted high level of TGF-β1. However, EOC cells frequently escape the influence of TGF-β through the acquisition of missense mutations in TGF-β receptor I (32).

In the present study, we showed that TGF-β1 play important roles in induction of the activated phenotype of fibroblasts through stimulating SMAD signaling and promoting the proliferation of NFs in vitro and in vivo. However, the activation of NFs induced by EOC cells was not perpetual in vitro. The activated phenotype was vanished 3 days after the end of coculture. This result suggests that EOC cells induce activation of NFs in vitro but not to fully differentiated CAFs which remain perpetually activated even when the initial insult has regressed (5,11).

Ernst Lengyel et al. showed that normal omental mesothelial cells inhibit, whereas normal omental fibroblasts enhance the attachment and invasion of ovarian cancer cells to the omentum (24). In our study, CAFs could induce EOC cells metastases but the primary NFs could not. The activated NFs obtained the ability to promote the adhesion and invasion of EOC cells in vitro and in vivo, which could be abrogated by inhibition of fibroblast activation. Using an organotypic culture model, recent studies showed that employment of fetal esophageal fibroblasts within the esophageal cancer ECM resulted in a more aggressive invasion, whereas fetal skin fibroblasts which are less activated produce little evidence of invasion of tumor cells (9,12). These data combined with our studies provide compelling evidence that the activation level of fibroblasts is critical in fostering the extent of tumor invasion.

The ascites of ovarian cancer contained different kinds of cells and protein including TGF-β (33). We imagine that during the course of tumor progression, the normal omentum fibroblasts activation is initially triggered by TGF-β released in significant quantities by carcinoma cells and from ascites and then the activated fibroblasts formed a ‘pre-metastatic niche’ favor EOC cells implantation which promote more fibroblasts activation and hyperproliferation then induce more EOC cells implant to the omentum.

HGF is expressed ubiquitously and plays an important role in the tumor stromal microenvironment (34). Secreted by mesenchymal cells, HGF acts on epithelial and endothelial cells. Many types of cancer cells secrete molecules that enhance HGF production in fibroblasts while fibroblast-derived HGF, in turn, is a potent stimulator of cancer cells. HGF was found to stimulate the migration of the ovarian carcinoma cell line SKOV3 (35). In ovarian cancer, HGF was found to stimulate the migration of the ovarian carcinoma cell line SKOV3 (35). Here, we show that activated fibroblasts expressed higher HGF than primary NFs. The neutralization HGF antibody attenuates SKOV3 invasion and adhesion induced by activated NFs provided the fact that HGF is a significant factor responsible for cancer cell metastasis mediated by tumor-stromal interactions. However, this does not preclude the likely involvement of other growth factors and/or cytokines such as fibroblast growth factor and epidermal growth factor, which contribute to tumor growth and metastases (26,37).

The MMP-2 can degrade many ECM components and often associated with cancer cell metastasis (38). The submesothelial ECM of both the peritoneum and omentum is essentially composed of collagen type I, vitronectin and fibronectin (39), whereas the first two components were previously reported as MMP-2 substrates (40). In this paper, we show that cell–cell interaction is a major factor of enhanced MMP-2 expression in vitro, and stable upregulation of MMP-2 was found in SKOV3 cells and NFs inoculation xenografts.

Fig. 5. Activated NFs promote the invasion and adhesion of SKOV3 cells through elevating expression of HGF and MMP-2. (A) Western blot analysis show enhanced HGF expression in NFs stimulated by either exogenous TGF-β1 or EOC cells. A5-3-1 can block the increases. Quantification of western blot is shown below. (B) and (C) Adhesion and invasion analysis show HGF antibodies significantly inhibit the adhesion and invasion ability of SKOV3 cells promoted by SKOV3 cells and TGF-β1 treated NFs but did not influence SKOV3 cells per se. (D) Western blot analysis show MMP-2 expression in NFs and SKOV3 cells and cells cultured in different models. Quantification of western blot is shown below. (E) and (F) Adhesion and invasion experiments. The bars show a dose-dependent effect of MMP-2 inhibition on the adhesion and invasion ability of SKOV3 cells cocultured with control NFs and NFs activated by TGF-β1 or SKOV3 cells. Error bars show mean ± standard deviation (of experiments performed in triplicate). *P < 0.05 **P < 0.01 ***P < 0.001.
Attenuate MMP-2 activity suppressed EOC cells attached and invaded to the 3D culture, which underscored a direct role for MMP-2 activation in EOC metastases. So, we hypothesized that cancer cells break off the surface of the ovarian mass adhere to and disaggregate on omentum, subsequently forming invasive foci (41). In these foci, hyperproliferated and activated stroma fibroblasts interacted with cancer cells resulted in hyper-expression of MMP-2 which could degrade the ECM, mediate metastasis of neoplastic cells (42,43).

In summary, our results partly explain why ovarian cancer cells have a clear predilection for the omentum. Since the high metastases rate of ovarian cancer, the role of CAFs in tumor cell invasion and adhesion makes targeting the omentum fibroblast activation through the inhibition of TGF-β1 signaling an appealing therapeutic option.

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References

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