Abstract. The term vasculogenic mimicry (VM) indicates the process by which aggressive tumor cells are able to generate in vitro non-endothelial cell-lined channels delimited by extracellular matrix. Although VM has been described in several human malignancies, the molecular basis of this phenomenon is not entirely understood. In the present study, we examined VM in two ovarian cancer cell lines with different invasion capability (CABA I, low invasion activity; SKOV3, high invasion activity). Specifically, we focused on the potential role played by CD147/extracellular MMP inducer, a membrane spanning molecule highly expressed in tumor cells, in VM. Previous studies have shown that CD147 may be involved in the progression of malignancies by regulating the expression of metalloproteinases in peritumoral stromal cells. In this study, we found significant correlations between expression of CD147 in ovarian cancer cell lines and tumor invasiveness, the activity of the proteases and the ability to form vascular channels. The treatment of SKOV3 cells with small interfering RNA against CD147 suppressed the ability of these cells to generate non-endothelial cell-lined channels. In contrast, transfection of CD147 cDNA into the CABA I cell line resulted in an increased tumor invasiveness and enabled the formation of vascular channels. Altogether, our data suggest that CD147 may play a critical role in VM of CABA I and SKOV3, human ovarian cancer cell lines.

Introduction

Tumors require a blood supply for growth and hematogenous dissemination (1,2). The term angiogenesis describes the formation of new vessels independent from pre-existing vessels. Several authors have recently reported a process termed vasculogenic mimicry (VM), which consists of the formation of patterned, tubular networks by aggressive tumor cells in three-dimensional cultures in vitro, that mimics endothelial-formed vasculogenic networks (1,4-5). VM has been described in several types of aggressive tumors, including cutaneous, uveal and mucous membrane melanoma, inflammatory and ductal carcinoma of the breast, ovarian and prostatic carcinoma, synovial sarcoma, rhabdomyosarcoma, osteosarcoma and pheochromocytoma. There is evidence to suggest that the matrix metalloproteinase 2 (MMP-2) and its activator, the membrane-type 1 matrix metalloproteinase (MT1-MMP), may play a role in VM (14,15). CD147, also named EMMPRIN (Extracellular Matrix MetalloPRoteinase Inducer) or basigin, is a plasma membrane glycoprotein enriched on the surface of many malignant tumor cells and functions as an upstream modulator of metalloproteinase production in the local tumor environment (16,17). CD147-positive tumor cells stimulate neighboring fibroblast cells to express MMPs and therefore facilitate tumor invasion and metastasis. Recent investigations have provided evidence that CD147 plays a crucial role in cancer progression not only by inducing MMP production (18-25) but also by stimulating tumor angiogenesis via induction of vascular endothelial growth factor (VEGF) expression (26).

Data on the pathophysiologic role of CD147 in VM are scarce. In this context, the aim of our study was to investigate the potential role played by CD147 in VM. We examined our hypothesis in two ovarian cancer cell lines with different invasion capability (CABA I, low invasion activity; SKOV3, high invasion activity).

Materials and methods

Cell culture. The CABA I cell line was established from the ascitic fluid of an ovarian carcinoma patient not undergoing drug treatment (27). CD147-transfected CABA I cells were maintained as monolayers in RPMI-1640 (Euroclone, Devon, UK).
UK) containing 5% FCS and 400 μg/ml G418. Human ovarian carcinoma cell lines SKOV3 cells were cultured in RPMI medium containing 10% FCS.

Small interfering RNA and transfection. Transfection of CD147/green fluorescent protein cDNA (a kind gift of S. Zucker) into human CABA I ovarian cancer cells with low CD147 expression was performed by the method of Zucker et al (28). Cells were stably transfected using the FuGene (Roche, Basel, Switzerland) according to the manufacturer's protocol. G418-resistant clones were microscopically screened for green fluorescent protein fluorescence and positive clones were pooled to minimize clonal variations. Control transfectants carrying the pcDNA3 vector alone were generated.

Identical results were obtained with non-transfected CABA I cells or CABA I cells transfected with pcDNA3 (data not shown). We silenced CD147 in SKOV3 cells by using CD147 small interfering RNA (siRNA) h-sc-35298, scrambled oligos sc-37007 as control, and sc-29528 siRNA transfection reagent (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's protocol. The same results were obtained with non-silenced SKOV3 cells or SKOV3 cells transfected with scrambled oligos.

Zymography. Zymography was performed using SDS-polyacrylamide gels copolymerized with 1 mg/ml gelatin type B (Sigma). Five-fold concentrated cell-conditioned medium were diluted in SDS-PAGE sample buffer under non-reducing conditions without heating. After electrophoresis, gels were washed twice for 30 min in 2.5% Triton X-100 at room temperature and incubated overnight in medium were diluted in SDS-PAGE sample buffer under non-reducing and non-reducing conditions without heating. After electrophoresis, gels were washed twice for 30 min in 2.5% Triton X-100 at room temperature and incubated overnight in reducing reagent (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's protocol. The same results were obtained with non-silenced SKOV3 cells or SKOV3 cells transfected with scrambled oligos.

Western blot analysis of CD147. Cells were lysed in buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, and 1% NP40. Protein concentration of cell lysate and microvesicles containing 50 mM Tris (pH 7.8), 150 mM NaCl, and 1% NP40. Protein concentration of cell lysate and microvesicles was determined as described above. Cell lysates (40 μg) were resolved by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing and reducing conditions and then transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Non-specific binding sites were blocked by incubation with 10% non-fat dry milk in TBST for 1 h at room temperature. The blots were incubated with a monoclonal antibody raised against human CD147 (8D6 mouse monoclonal IgG, 1:500 dilution; Santa Cruz Biotechnology) for 1 h, followed by peroxidase-conjugated secondary antibody in blocking buffer. After washing, reactive bands were visualized using a chemiluminescence detection kit (ECL, Amersham-Pharmacia Biotech, Piscataway, NJ).

Expression levels were normalized to the expression levels of identical results were obtained with non-transfected CABA I cells or CABA I cells transfected with pcDNA3 (data not shown). We silenced CD147 in SKOV3 cells by using CD147 small interfering RNA (siRNA) h-sc-35298, scrambled oligos sc-37007 as control, and sc-29528 siRNA transfection reagent (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's protocol. The same results were obtained with non-silenced SKOV3 cells or SKOV3 cells transfected with scrambled oligos.

Statistical analysis. All data shown are from at least three independent experiments and are expressed as mean ± SD. Statistical significance was determined by using the Mann-Whitney U test. Calculations were performed using GraphPad Prism 4 software (GraphPad, San Diego, CA). Statistical significance was set at P<0.05.

Results

Vasculogenic mimicry of ovarian cancer cells. To verify whether ovarian cancer cell lines are capable of generating patterned vascular channels through vasculogenic mimicry, CABA I and SKOV3 cells were seeded onto a 3D matrix coating Matrigel.
In complete medium containing 10% serum, SKOV3 cells were able to form vascular channels after 2 and 24 h (Fig. 1A), whereas this was not the case for CABA I cells (Fig. 1C). PAS staining was performed on SKOV3 cells plated on Matrigel to identify the extracellular matrix-associated vascular channels formed by cells (Fig. 1B).

**Correlation between CD147 expression levels and proteolytic and invasion capability of ovarian cancer cell lines.** The expression of CD147 in ovarian cancer cell lines was assessed by Western blot analysis. A high level of expression was found in the SKOV3 cells, whereas CABA I cells did not express CD147 (Fig. 1D). The invasive capability of SKOV3 cells was higher than that of CABA I as assessed by an in vitro invasion assay using Matrigel (Fig. 1E). Gelatin zymography was performed to examine the presence of matrix-degrading proteases in cell conditioned media (Fig. 1F). The conditioned medium of SKOV3 cells showed higher proMMP-2 and proMMP-9 activities as compared with the conditioned medium of CABA I cells.

**CD147 regulates MMPs expression and plays a role in VM of ovarian cancer cell lines.** To investigate the role played by CD147 in tumor progression, we compared CD147-transfected CABA I cells with vector-transfected CABA I cells. Expression of the CD147 fusion protein was confirmed by Western blot analysis (Fig. 2A). The invasive capacity and MMP activity was higher in CD147-transfected CABA I compared with vector-transfected cells (Fig. 2B and C). Real-time PCR was performed to quantify MT1-MMP, MMP-2 and MMP-9 gene expression in CABA I CD147-transfected cells relative to the parental CABA I population. CD147-transfected cells showed a higher expression of MT1-MMP, MMP-2 and MMP-9 (Fig. 2D). To investigate the vasculogenic potential of CABA I CD147-transfected cells, we seeded these cells onto Matrigel-coated wells in complete medium containing 10% serum. After 24 h, CABA I CD147-transfected cells were found to form vascular channels (Fig. 2E), whereas this was not the case for vector-transfected CABA I cells (Fig. 2F). To evaluate that the activity of MMPs induced by CD147 causes for VM, CABA I-CD147 cells...
were treated with 500 mM CTT. The inhibition of vascular channel formation in the presence of MMP inhibitor is shown in Fig. 2G.

We also examined the invasive potential of SKOV3-CD147 silenced cells compared with parental SKOV3 cells. We silenced CD147 in SKOV3 cells by using CD147 small interfering RNA (siRNA). Silencing was confirmed by Western blot analysis (Fig. 3A). SKOV3 cells with silenced CD147 showed lower invasive potential and a marked inhibition in the proMMP-2 and a weak inhibition of proMMP-9 activity as compared to parental SKOV3 cells (Fig. 3B and C). Real-time PCR was performed to quantify MMP gene expression in cells transfected with siRNA relative to the parental SKOV3 population. Cells with silenced CD147 showed a marked inhibition in the MT1-MMP, MMP-2, and MMP-9 gene expression (Fig. 3D). Differently from parental SKOV3 cells, SKOV3 cells with silenced CD147 showed a reduced expression, and were not able to form vascular channels when seeded in a three-dimensional matrix (Fig. 3E and F). When SKOV3 cells, plated with a cellular growth medium containing 10%, FCS were treated with 500 mM CTT, their capability to form vascular channel was inhibited (Fig. 3G).

Discussion

The growth of cancers is dependent upon vascularization, assuring supplying oxygen and nutrients and removing waste products. The best-known mechanism driving tumor vascularization is angiogenesis, consisting in the formation of new blood vessels from the endothelium of the existing vasculature. However, several studies have now been published that suggest that not only angiogenesis but also vasculogenesis may be involved in response to pathological conditions such as tumor formation. Vasculogenesis is the process by which the primordial microcirculation is formed during embryogenesis. The term ‘vasculogenic mimicry’ has been used to describe the manner in which highly aggressive tumor cells are able to form vasculogenic-like networks similar to embryonic vasculogenesis (32).
The microcirculation characteristic of vasculogenic mimicry was described for the first time in uveal (intra-ocular) melanoma (1). Specifically, vasculogenic mimicry refers to abnormal vessel development in which cancer cells directly line vessels or blood channels within tumors.

Previous studies have shown that aggressive melanoma cells are capable of expressing endothelium-associated genes and to form vasculogenic-like networks in three-dimensional cell cultures (1,4,5-12). There are three key factors that govern the formation of a microcirculation by cells other than endothelial cells in vascular mimicry, namely the plasticity of highly malignant and invasive cells, extracellular matrix remodeling, and the connection of the host vessels via vascular mimicry to acquire a blood supply (32).

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We thus hypothesized that the same phenomenon could occur in aggressive ovarian cancer cells. In particular, in this study we focused on the potential role played by CD147 in vascular mimicry of ovarian cancer cells. CD147 expressed on tumor cells plays a key role in triggering the production of matrix metalloproteinases by surrounding stromal cells and tumor cells themselves. In the present study, we examined vascular mimicry in two ovarian cancer cell lines with different invasion capability (CABA I, low invasion activity; SKOV3, high invasion activity). Our results showed that only the highly aggressive SKOV3 cells were able to generate patterned vascular channels when seeded in a three-dimensional collagenous matrix (Matrigel). Interestingly, a positive correlation was seen between the cell expression of CD147 and ovarian cancer cell invasive ability and protease activity. In turn, transfection of CD147 cDNA into the CABA I cell line resulted in increased invasion, increased expression of MMP-2 and MMP-9 in both active and proenzymatic form. We believe that ovarian cancer cells can secret and activate MMP-2 and MMP-9, and that remodelling the extracellular matrix can assist the formation of VM. Notably,
CD147-transfected CABA I cells were able to form vascular channels when seeded in a three-dimensional matrix. In contrast, the treatment of SKOV3 cells with siRNA against CD147 resulted in a suppression of their invasive potential and matrix metalloproteinase activity. As expected, SKOV3 cells with silenced CD147 expression were unable to form capillary-like structures when tested in the Matrigel assay.

Altogether, our results provide strong in vitro evidence that CD147 plays an important role in the formation of tumor-lined vessels by ovarian cancer cells. It is intriguing to speculate that the use of anti-CD147 therapeutic targeting may provide a novel opportunity to circumvent the ability of CD147-expressing tumors, such as ovarian cancers, to grow and metastasize.

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References