Contributions of Stromal Metalloproteinase-9 to Angiogenesis and Growth of Human Ovarian Carcinoma in Mice

Suyun Huang, Melissa Van Arsdall, Sean Tedjarati, Marya McCarty, Wenjuan Wu, Robert Langley, Isaiah J. Fidler

Background: The expression level of several matrix metalloproteinases (MMPs), including MMP-2 and MMP-9, in ovarian cancer cells is directly associated with their invasive and metastatic potentials. MMP-9 is also expressed in stromal cells adjacent to the tumor. To investigate the contribution of MMP-9 expression in stromal cells to ovarian tumor growth, we examined angiogenesis and progressive growth of human ovarian cancer cells implanted into mice with and without the MMP-9 gene. Methods: Human ovarian cancer cells SKOV3.ip1 and HEY-A8 were implanted into the peritoneal cavities of nude mice that lacked the gene for MMP-9 (MMP-9–/–) or were wild type for MMP-9 (MMP-9+/+) (10 mice of each genotype per cell line). Tumor incidence, tumor size, and volume of ascites fluid were recorded for each mouse at 30 and 45 days after HEY-A8 and SKOV3.ip1 cell injections, respectively. Blood vessel density and macrophage infiltration into the lesions were analyzed in excised tumors by immunohistochemistry and double immunofluorescence. Tumor growth was also studied in MMP-9–/– nude mice that had been reconstituted with spleen cells collected from either MMP-9+/+ or MMP-9–/– nude mice. All statistical tests were two-sided. Results: HEY-A8 cells expressed high levels of MMP-9, and SKOV3.ip1 cells expressed low levels. Nevertheless, tumor incidence and growth were statistically significantly lower in MMP-9–/– mice than in MMP-9+/+ mice injected with cells from either line (for tumor size, P = .006 and .042 for HEY-A8 and SKOV3.ip1 cells, respectively). Compared with MMP-9+/+ mice injected with human ovarian cancer cells, MMP-9–/– mice injected with human ovarian cancer cells displayed decreased microvessel density and decreased macrophage infiltration into the lesions. Compared with MMP-9+/+ mice that received spleen cells (a rich source of macrophages) from MMP-9–/– mice, those that received spleen cells from MMP-9+/+ mice before cancer cell injections displayed increased angiogenesis and tumorigenicity of the cancer cells. The growing tumors contained MMP-9-expressing macrophages. Conclusion: Host-derived MMP-9 expression, most likely in tumor-infiltrating macrophages, appears to play a critical role in angiogenesis and progressive growth of human ovarian tumors in mice. [J Natl Cancer Inst 2002;94:1134–42]

Among all the human gynecologic cancers, ovarian cancer is the leading cause of death (1). At the time of diagnosis, ovarian cancer patients usually have locally advanced or disseminated disease that is characterized by diffuse intraperitoneal tumors and, in many cases, malignant ascites (2). Both the progressive growth of ovarian cancer and the formation of ascites fluid are dependent on angiogenesis (3,4), the extent of which inversely correlates with prognosis (5–7).

To produce new vasculature during angiogenesis, endothelial cells must migrate, divide, and form tubes (4). Proteolysis of components of the extracellular matrix (8,9) allows endothelial cells to migrate and releases stored angiogenic signaling molecules from the extracellular matrix (10,11). High levels of matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9, also called type IV collagenases or gelatinases) in tissues have been associated with active neovascularization (8–11). Indeed, inhibitors of MMPs, such as tissue inhibitor of MMP-2 (TIMP-2), have been shown to inhibit in vitro proliferation and tube formation by endothelial cells (12,13). TIMP-2 as well as tissue inhibitor of MMP-1 (TIMP-1) have been shown to suppress in vivo angiogenesis by mouse B16 melanoma cells (14) and Burkitt’s lymphoma cells (15). Studies in mice that were genetically modified to lack MMP-9 expression (10,11,16,17) have shown that MMP-9 contributes to the angiogenic switch that occurs during carcinogenesis (10,11). Furthermore, these studies suggested that MMP-9 is expressed by inflammatory cells and not by neoplastic cells, at least in these animal models.

Human ovarian cancer cells produce both MMP-2 and MMP-9 (18–20), and increased expression of these MMPs in human ovarian cancer cells is associated with their invasive and metastatic potentials (21–25). However, in situ hybridization studies have demonstrated that MMP-9 mRNA expression is detectable not only within neoplastic epithelial areas of the tumors (23) but also in stromal areas, which raises the possibility that the MMP-9 expressed by stromal cells, in addition to that expressed by tumor cells, may contribute to the malignant behavior of ovarian cancers.

To examine the contribution of mouse stromal MMP-9 to the progressive growth of human ovarian cancer cells, we generated a strain of nude mice that have a homozygous null mutation in the MMP-9 gene (i.e., MMP-9–/– nude mice). We implanted human ovarian cancer cells SKOV3.ip1 and HEY-A8 into the peritoneal cavities of MMP-9–/– nude mice and into those of nude mice with intact MMP-9 genes (MMP-9+/+ nude mice) and...
measured tumor incidence, angiogenesis, and progressive growth.

**Materials and Methods**

### Human Ovarian Cancer Cells

The SKOV3 cell line was originally obtained from the American Type Culture Collection (Manassas, VA). The SKOV3.ip1 variant cell line was isolated from ascites fluid that had accumulated in a nude mouse that was injected intraperitoneally with SKOV3 cells (26). The HEY-A8 cell line was obtained from Dr. Gordon B. Mills (The University of Texas M. D. Anderson Cancer Center). Human ovarian cancer cells were maintained in culture (5% CO₂ and 95% air at 37 °C) in minimal essential medium (MEM; Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and vitamin solution (Life Technologies).

### Mice

Athymic BALB/c nude mice with wild-type MMP-9 genes (MMP-9+/+ nude mice) were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). Mice that lacked an intact MMP-9 gene were originally developed *via* homologous recombination in mice with a 129/CD1 genetic background (17). We generated nude mice that lacked an intact MMP-9 gene (MMP-9–/– nude mice) in our animal facility by interbreeding MMP-9+/– 129/CD1 mice with MMP-9+/+ mice for eight generations. The genotypes of the resulting mice were determined by using polymerase chain reaction analysis (17). The nude mice were housed in laminar flow cabinets under pathogen-free conditions and were used for all studies when they were 8 weeks old. Animals were maintained according to institutional regulations in facilities approved by the American Association for Accreditation of Laboratory Animal Care, in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and the National Institutes of Health.

### Collagenase Activity Assay

SKOV3.ip1 cells, HEY-A8 cells, and macrophages (2 × 10⁵) were seeded in six-well plates and incubated overnight at 37 °C. The cells were washed twice with Hank’s balanced salt solution (HBSS) and cultured for an additional 24 hours in serum-free medium. Macrophages were treated with lipopolysaccharide (at 0 µg/mL, 0.1 µg/mL, or 1.0 µg/mL; Sigma Chemical Co., St. Louis, MO) for an additional 30 minutes. Culture supernatants were collected for assays of collagenase activity; cells attached to the plate were stained for viability with trypan blue, and the viable cells were counted. Culture supernatants (40 µL) were resolved on a 7.5% sodium dodecyl sulfate polyacrylamide gel that contained 1 mg/mL gelatin (Sigma Chemical Co., St. Louis, MO). The gel was washed for 30 minutes at room temperature in wash buffer (50.0 mM Tris–HCl [pH 7.5], 15.0 mM CaCl₂, 1.0 mM ZnCl₂, 2.5% Triton X-100) and then incubated for 24 hours at 37 °C in the same buffer that contained Triton X-100 at a final concentration of 1%. The gel was then stained with 0.1% Coomassie Brilliant Blue R-250; clear zones against the blue background indicated the presence of gelatinolytic (i.e., collagenase) activity.

### Collection of Mouse Peritoneal Exudate Macrophages

We collected peritoneal exudate macrophages from MMP-9+/+ and MMP-9–/– nude mice by peritoneal lavage with HBSS 4 days after the mice were given an intraperitoneal injection with 2 mL of thioglycollate broth (Baltimore Biological Laboratories, Cockeysville, MD) (27). The macrophages were concentrated by centrifugation for 5 minutes at 895g and resuspended in MEM supplemented with 5% fetal bovine serum, and 3 × 10⁶ cells were plated into each tissue culture flask and incubated at 37 °C for 2 hours. The flasks were washed with MEM to remove nonadherent cells. The adherent cells were greater than 98% pure macrophages.

### Matrigel Invasion Assay

The *in vitro* invasion assay was performed as previously described (28) with minor modifications. Invasion chambers containing polycarbonate filters (8-µm pore size; BD Biosciences, Franklin Lakes, NJ) were coated with growth-factor-reduced Matrigel matrix (50 µg/filter; BD Biosciences, Bedford, MA). Macrophages were harvested from cultures by tapping the flask sharply to dislodge cells, counted, and seeded (2 × 10⁵ cells per chamber) in the upper compartment of each invasion chamber in MEM containing 0.1% bovine serum albumin (BSA) for 24 hours at 37 °C. Either conditioned medium from 1 × 10⁶ SKOV3.ip1 or HEY-A8 cells cultured for 48 hours or MEM was placed in the lower compartment and served as a chemoattractant. After 24 hours at 37 °C, cells were harvested from the lower compartment and from the undersurface of the filter. Each assay was performed on duplicate filters, and the experiments were repeated twice. The harvested cells were counted, and the mean number of cells per chamber was calculated and recorded.

### Macrophage Chemotaxis Assay

Macrophage chemotaxis was examined by using 24-well transwell migration chambers (8-µm pore size; BD Biosciences) as described previously (29). Macrophages (1 × 10⁶) in MEM containing 0.1% BSA were seeded in the upper compartment of each chamber. MEM containing recombinant mouse monocyte chemoattractant peptide-1 (JE/MCP-1) at 1–100 ng/mL (R&D Systems, Minneapolis, MN) was added to the lower compartment. The cells were incubated at 37 °C and then harvested after 3 hours or 24 hours by scraping from the lower compartment and the undersurface of the filter and counted. All assays were performed in triplicate, and the experiments were repeated twice. The mean number of cells per chamber was calculated and recorded.

### Tumor Growth In Vivo

Cultured human tumor cells were harvested by a brief treatment with 0.25% trypsin and 0.02% EDTA. Single-cell suspensions of 1 × 10⁶ cells that had a viability of greater than 95% by trypan blue dye exclusion were injected into the peritoneal cavities of female MMP-9+/+ and MMP-9–/– nude mice (10 mice in each group) (21,30). The mice were monitored daily for evidence of disease (e.g., abdominal swelling, hunched posture, listlessness) and killed when they became moribund or at day 30 (HEY-A8 cells) or day 45 (SKOV3.ip1 cells) after the intraperitoneal injection, whichever came first. All of the mice were necropsied, and the size and volume of ascites fluid for each were recorded.

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Immunohistochemistry and Quantitation of Microvessel Density

Peritoneal tumors of similar size were harvested at autopsy and processed for immunostaining as previously described (30) using the rat polyclonal antibody F4/80, which recognizes macrophage-specific antigen F4/80 (Serotec, Inc., Raleigh, NC) (27), anti-MMP-9 monoclonal antibody (human specific; Oncogene Research Products, Cambridge, MA), anti-vascular endothelial growth factor (VEGF) polyclonal antibody (mouse and human cross-reactive; Santa Cruz Biotechnology, Santa Cruz, CA), anti-basic fibroblast growth factor (anti-bFGF) monoclonal antibody (mouse and human cross-reactive; Sigma Chemical Co.), and anti-CD31/PECAM-1 monoclonal antibody, which recognizes platelet–endothelial cell adhesion molecule-1 (PECAM-1) on endothelial cells (mouse specific; BD PharMingen, San Diego, CA), and the appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Negative controls were stained with nonspecific immunoglobulin G (IgG) and the appropriate horseradish peroxidase-conjugated secondary antibody. All sections were counterstained with Gill’s hematoxylin. The immunostained tumor sections were examined by bright field microscopy, and the macropathies and blood vessels in 10 random 0.159-mm² fields of each sample were counted (21,30). Microvessel density was defined as the mean number of blood vessels per field of tumor at ×100 magnification (1 field = 0.159 mm²). Images were digitized by using a Sony 3CD color video camera (Sony Corp., Tokyo, Japan) and a personal computer equipped with Optimas image analysis software (Optimas Corp., Bothell, WA).

Immunofluorescence Double Staining for F4/80 and MMP-9

Frozen peritoneal tumors were cut into 8-μm sections and fixed in cold acetone. Sections were incubated with 4% fish gelatin in phosphate-buffered saline for 10 minutes to block nonspecific binding and then incubated with a 1:50 dilution of a goat monoclonal anti-mouse MMP-9 antibody (R&D Systems) for 18 hours at 4 °C. Bound antibody was detected by using biotinylated mouse anti-goat IgG (1:200 dilution; Biocare Medical, Walnut Creek, CA) and streptavidin-conjugated Alexa 594 (1:400 dilution; Molecular Probes, Eugene, OR). The sections were incubated for 10 minutes with 4% fish gelatin to block nonspecific binding and then overnight with F4/80 (1:10 dilution). Bound F4/80 was detected by using anti-rat Alexa 488 (1:200 dilution; Molecular Probes). Sections were mounted in medium containing 0.1 M propyl gallate to minimize photo-bleaching (Sigma Chemical Co.). Immunofluorescence microscopy was conducted on a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Images were captured with a cooled C5810 camera (Hamamatsu Photonics KK, Bridgewater, NJ) using Optimas software (Media Cybernetics, Silver Spring, MD) run on a Dell personal computer. MMP-9 staining was identified by red fluorescence, and F4/80 staining was detected by green fluorescence. Colocalization of MMP-9 and F4/80 was detected by yellow fluorescence.

Spleen Cell Reconstitution Experiment

Spleens were harvested from 4-week-old MMP-9+/+ and MMP-9–/– nude mice, placed in MEM, and forced through a wire mesh to disaggregate spleen cells. The spleen cells were washed with HBSS, treated for 60 seconds with ammonium chloride (140 meq/L), then washed again with HBSS. We determined spleen cell viability by trypan blue exclusion; 1 × 10⁷ viable spleen cells were injected intravenously into each mouse. Spleen cells were injected on days 1, 3, and 5. Tumor cells (1 × 10⁶) were injected intraperitoneally on day 6. Mice with SKOV3.ip1-derived tumors were killed on day 50. Mice with HEY-A8-derived tumors were killed on day 35. Tumors were excised and weighed, and the volume of ascites fluid for mice injected with SKOV3.ip1 cells was measured.

Statistical Analysis

The statistical significance of the in vitro results was determined by using Student’s t test (two-tailed). The in vivo data were analyzed by the Mann–Whitney U test. All statistical tests were two-sided.

RESULTS

Human Ovarian Cancer Cell Growth and Ascites Fluid Formation in Mice That Lack an Intact MMP-9 Gene

In the first set of experiments, we injected SKOV3.ip1 and HEY-A8 cells into the peritoneal cavities of MMP-9+/+ and MMP-9–/– nude mice (10 mice of each genotype for each cell line). All of the MMP-9+/+ nude mice injected with either SKOV3.ip1 or HEY-A8 cells developed peritoneal tumors. By contrast, only four of the MMP-9–/– mice injected with HEY-A8 cells and six of the MMP-9–/– mice injected with SKOV3.ip1 cells developed peritoneal tumors. Moreover, the peritoneal tumors in the MMP-9–/– nude mice were statistically significantly smaller (P = .006 and .042 for HEY-A8 and SKOV3.ip1 cells, respectively) and produced statistically significantly less ascites fluid (P = .005 for SKOV3.ip1 cells) than those that developed in the MMP-9+/+ nude mice (Table 1). For example, the median weight of tumors in MMP-9+/+ nude mice injected with HEY-A8 and SKOV3.ip1 cells was 3.8 g (inter-quartile [IQ] range = 1.7–4.4 g) and 2.3 g (IQ range = 1.1–2.9 g), respectively, whereas in MMP-9–/– nude mice, it was 0 g (IQ range = 0–1.7 g) and 0.9 g (IQ range = 0–1.3 g), respectively. Thus, disruption of the MMP-9 gene in the recipient mice decreased tumorigenicity and progressive growth of human ovarian cancer cells in those animals.

Effect of Tumor Cell-Derived MMP-9 in MMP-9–/– Mice

Because both the human ovarian cancer cells and cells from the MMP-9+/+ mice were potential sources of MMP-9, we next determined whether the difference in tumor incidence between the MMP-9+/+ and MMP-9–/– mice was dependent on MMP-9 expressed by mouse cells or on MMP-9 expressed by the human cancer cells. Using gelatin zymographic analysis to detect collagenase activity, we found that SKOV3.ip1 cells expressed a low level of MMP-9 activity, whereas HEY-A8 cells expressed a high level of MMP-9 activity (data not shown). The two cell lines expressed similar levels of MMP-2 activity in vitro (data not shown). We also determined the in vivo expression level of MMP-9 in tumor cells growing in mice by immunohistochemistry with an anti-human MMP-9 antibody. As shown in Fig. 1, the level of MMP-9 in SKOV3.ip1 and HEY-A8 tumor cells in MMP-9–/– nude mice was similar to that in tumor cells growing in MMP-9+/+ nude mice. These data suggested that expression of
MMP-9 by human ovarian cancer cells was not sufficient to promote tumorigenicity in this mouse model.

**Macrophage Infiltration Into Ovarian Tumors Growing in MMP-9+/+ and MMP-9−/− Nude Mice**

MMP-9 is expressed in macrophages, which are a major component of the lymphoreticular cells that infiltrate ovarian tumors that grow in humans as well as in nude mice (21,31). We used immunohistochemistry to characterize the tumor-infiltrating macrophages in SKOV3.ip1 cell- and HEY-A8 cell-derived tumors of similar size. Specific staining for macrophages with the F4/80 antibody (27) revealed the presence of macrophages throughout the HEY-A8- and SKOV3.ip1-derived tumors in MMP-9+/+ nude mice (the mean number was 269 macrophages [95% confidence interval (CI) = 231 to 307 macrophages] and 144 macrophages [95% CI = 129 to 159 macrophages], respectively) (Fig. 1 and Table 2). By contrast, in MMP-9−/− mice, tumors derived from HEY-A8 and SKOV3.ip1 cells contained fewer macrophages (the mean number was 104 macrophages [95% CI = 87 to 121 macrophages] and 51 macrophages [95% CI = 42 to 60 macrophages], respectively) (Table 2).

**Invasion of Matrix-Coated Filters by Peritoneal Macrophages**

To determine the role of MMP-9 in macrophage function, we collected and analyzed macrophages from peritoneal exudates from 10 MMP-9+/+ and 10 MMP-9−/− nude mice that had not been injected with either human tumor cell line. The mean number of peritoneal macrophages recovered from the MMP-9+/+ mice was 30.1 × 10^6 macrophages per mouse (95% CI = 21.6 × 10^6 to 38.5 × 10^6 macrophages), which was statistically significantly more than the mean number of peritoneal macrophages recovered from the MMP-9−/− mice (95% CI = 14.7 × 10^6 to 26.0 × 10^6 macrophages).

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**Table 1. Growth of human ovarian carcinomas in MMP-9+/+ and MMP-9−/− nude mice**

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>Nude mice</th>
<th>Incidence</th>
<th>Median tumor weight, g (IQ range)</th>
<th>P†</th>
<th>Incidence</th>
<th>Median volume, mL (IQ range)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEY-A8</td>
<td>MMP-9+/+</td>
<td>10/10</td>
<td>3.8 (1.7–4.4)</td>
<td></td>
<td>0/10</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>HEY-A8</td>
<td>MMP-9−/−</td>
<td>4/10</td>
<td>0 (0–1.7)</td>
<td>.006</td>
<td>0/10</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>SKOV3.ip1</td>
<td>MMP-9+/+</td>
<td>10/10</td>
<td>2.3 (1.1–2.9)</td>
<td>.042</td>
<td>10/10</td>
<td>2.7 (1.1–3.5)</td>
<td>.005</td>
</tr>
<tr>
<td>SKOV3.ip1</td>
<td>MMP-9−/−</td>
<td>6/10</td>
<td>0.9 (0–1.3)</td>
<td></td>
<td>6/10</td>
<td>0.4 (0–1.6)</td>
<td></td>
</tr>
</tbody>
</table>

*Tumor cells (1 × 10^6) were injected intraperitoneally into 10 nude mice. The mice were killed on day 30 (HEY-A8 cells) or day 45 (SKOV3.ip1 cells). The volume of ascites fluid for mice injected with SKOV3.ip1 cells was measured, and tumor lesions in the peritoneal cavity were excised and weighed. CI = confidence interval; IQ = interquartile range; — = not applicable.

†The P values were calculated by comparing the MMP-9+/+ and MMP-9−/− mice injected with each cell line.
exudate macrophages recovered from the MMP-9+/+ mice (16.9 × 10^6 macrophages per mouse, 95% CI = 9.8 × 10^6 to 23.4 × 10^6 macrophages) (P = .035, Mann–Whitney U test). Gelatin zymographic analysis revealed that peritoneal exudate macrophages from MMP-9−/− nude mice had no detectable MMP-9 activity, whereas those from MMP-9+/+ nude mice had substantial activity (Fig. 2, A). Moreover, the MMP-9 activity in peritoneal exudate macrophages from MMP-9+/+ nude mice could be induced by lipopolysaccharide in a dose-dependent manner (Fig. 2, A). In MMP-9+/+ nude mice, human ovarian tumors derived from SKOV3.ip1 and HEY-A8 cells were highly vascularized and had a microvessel density of 70 vessels/field (95% CI = 54 to 88 vessels/field) and 118 vessels/field (95% CI = 93 to 144 vessels/field), respectively, whereas in MMP-9−/− nude mice, the mean vessel densities were 32 vessels/field (95% CI = 21 to 44 vessels/field) and 44 vessels/field (95% CI = 31 to 57 vessels/field), respectively (P = .003).

We also used immunohistochemistry to evaluate the expression levels of the proangiogenic molecules VEGF and bFGF in the peritoneal tumors. VEGF was expressed at higher levels in both SKOV3.ip1 and HEY-A8 tumors growing in MMP-9+/+ nude mice than in tumors growing in MMP-9−/− nude mice (Fig. 1). In MMP-9+/+ nude mice, human ovarian tumors derived from SKOV3.ip1 and HEY-A8 cells were highly vascularized and had a microvessel density of 70 vessels/field (95% CI = 54 to 88 vessels/field) and 118 vessels/field (95% CI = 93 to 144 vessels/field), respectively, whereas in MMP-9−/− nude mice, the mean vessel densities were 32 vessels/field (95% CI = 21 to 44 vessels/field) and 44 vessels/field (95% CI = 31 to 57 vessels/field), respectively (P = .003).

Angiogenesis in Human Ovarian Tumors in MMP-9+/+ and MMP-9−/− Nude Mice

Because tumor-infiltrating macrophages have been shown to augment neoplastic angiogenesis (33), we examined whether macrophage infiltration into the human ovarian tumors was associated with the formation of blood vessels. Tumors produced by SKOV3.ip1 or HEY-A8 cells in MMP-9+/+ and MMP-9−/− nude mice were resected, and tumor sections (not necessarily adjacent) were processed for immunohistochemistry. The blood vessels in the tumors were identified in different sections by staining the sections with an anti-CD31 antibody, and macrophages were identified by using an anti-F4/80 antibody (Fig. 1). In MMP-9+/+ nude mice, human ovarian tumors derived from SKOV3.ip1 and HEY-A8 cells were highly vascularized and had a microvessel density of 70 vessels/field (95% CI = 54 to 88 vessels/field) and 118 vessels/field (95% CI = 93 to 144 vessels/field), respectively, whereas in MMP-9−/− nude mice, the mean vessel densities were 32 vessels/field (95% CI = 21 to 44 vessels/field) and 44 vessels/field (95% CI = 31 to 57 vessels/field), respectively (P = .003).

We used immunohistochemistry to evaluate the expression levels of the proangiogenic molecules VEGF and bFGF in the peritoneal tumors. VEGF was expressed at higher levels in both SKOV3.ip1 and HEY-A8 tumors growing in MMP-9+/+ nude mice than in tumors growing in MMP-9−/− nude mice (Fig. 1). No substantial differences were found in the levels of bFGF among the four groups of mice (data not shown).

Effect on Tumorigenicity of Reconstitution of MMP-9−/− Nude Mice With Spleen Cells From MMP-9+/+ Nude Mice

The spleens of young mice are a rich source of monocytes and macrophages (34). We tested whether injection of nucleated spleen cells from young MMP-9+/+ nude mice into MMP-9−/− nude mice would stimulate the growth of SKOV3.ip1 and HEY-A8 cells injected into the peritonea of the MMP-9−/− nude mice.

Table 2. Effect of injected spleen cells from MMP-9+/+ and MMP-9−/− nude mice on the growth of human ovarian cells in MMP-9−/− nude mice*

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>Host</th>
<th>Spleen cells</th>
<th>Incidence</th>
<th>Median tumor weight, g (IQR range)</th>
<th>P §</th>
<th>Incidence</th>
<th>Median volume, mL (IQR range)</th>
<th>P §</th>
<th>Mean No. of cells (95% CI)</th>
<th>P §</th>
<th>Mean vessels/field (95% CI)</th>
<th>P §</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKOV3.ip1</td>
<td>+/+</td>
<td>8/8</td>
<td>2.3 (1.0–3.5)</td>
<td>.023</td>
<td></td>
<td>8/8</td>
<td>1.9 (1.8–3.8)</td>
<td>.002</td>
<td>144 (129 to 159)</td>
<td>.001</td>
<td>64 (51 to 77)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−/−</td>
<td>4/7</td>
<td>0.6 (0–1.1)</td>
<td>—</td>
<td></td>
<td>4/7</td>
<td>0.1 (0–1.0)</td>
<td>—</td>
<td>51 (42 to 60)</td>
<td>&lt;.001</td>
<td>38 (32 to 44)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td>−/+</td>
<td>7/8</td>
<td>1.7 (1.2–2.4)</td>
<td>—</td>
<td></td>
<td>6/8</td>
<td>0.9 (0.3–1.5)</td>
<td>—</td>
<td>102 (91 to 133)</td>
<td>.035</td>
<td>52 (43 to 61)</td>
<td></td>
</tr>
<tr>
<td>HEY-A8</td>
<td>+/+</td>
<td>5/8</td>
<td>0.7 (0–1.3)</td>
<td>.016</td>
<td></td>
<td>5/8</td>
<td>0.3 (0–1.0)</td>
<td>.011</td>
<td>42 (35 to 49)</td>
<td>&lt;.001</td>
<td>33 (23 to 43)</td>
<td>.003</td>
</tr>
<tr>
<td></td>
<td>−/−</td>
<td>2/5</td>
<td>0 (0–2.5)</td>
<td>0.041</td>
<td></td>
<td>2/5</td>
<td>0</td>
<td>—</td>
<td>269 (231 to 307)</td>
<td>&lt;.001</td>
<td>105 (80 to 130)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td>+/+</td>
<td>5/8</td>
<td>2.2 (1.5–4.0)</td>
<td>0.05</td>
<td></td>
<td>0/5</td>
<td>—</td>
<td>—</td>
<td>112 (96 to 128)</td>
<td>&lt;.001</td>
<td>48 (28 to 68)</td>
<td>.002</td>
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<td>—</td>
<td>112 (96 to 128)</td>
<td>&lt;.001</td>
<td>48 (28 to 68)</td>
<td>.002</td>
</tr>
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</table>

* Spleen cells (1 × 10^7) were injected intravenously on days 1, 3, and 5. Tumor cells (1 × 10^6) were injected intraperitoneally on day 6. Mice with SKOV3.ip1-derived tumors were killed on day 50. Mice with HEY-A8-derived tumors were killed on day 35. Tumors were excised and weighed, and the volume of ascites for mice injected with SKOV3.ip1 cells was measured. CI = confidence interval; IQR = interquartile range; +/+ = MMP-9+/+ nude mice; −/− = MMP-9−/− nude mice; — = not applicable.

† The number of macrophages was determined by immunohistochemistry with F4/80 antibodies.

‡ The number of blood vessels was determined by immunohistochemistry with anti-CD31 antibodies.

§ Values were calculated from the comparisons between MMP-9+/+ and MMP-9−/− nude mice (for mice that did not undergo spleen cell reconstitution) using the Mann–Whitney U test. One field = 0.159 mm² at ×100 magnification.
The presence of MMP-9-expressing macrophages in the peritonea of MMP-9−/− nude mice was confirmed by MMP-9 collagenase activity assays (data not shown). The tumorigenicity of SKOV3.ip1 cells was determined at 6 weeks after tumor cell injection. As shown in Table 2, reconstitution of MMP-9+/+ nude mice with splenocytes from MMP-9+/+ nude mice was associated with a statistically significant enhancement of the growth of peritoneal tumors derived from SKOV3.ip1 cells (P = .016) and the formation of ascites fluid (P = .011). Moreover, the microvesel density of peritoneal tumors was statistically significantly higher in MMP-9+/+ mice that were injected with splenocytes from MMP-9+/+ mice than that in MMP-9−/− mice injected with splenocytes from MMP-9−/− mice (P = .003), a finding that was consistent with relative levels of macrophage infiltration into the tumors (Table 2). The growth of HEY-A8 cells in the peritoneal cavities of MMP-9+/+ nude mice was statistically significantly lower than that in MMP-9−/− nude mice (the median tumor weights were 0 g and 3.4 g, respectively, P = .041).

Reconstitution of MMP-9−/− nude mice with spleen cells from MMP-9+/+ nude mice (but not with spleen cells from MMP-9−/− nude mice) was associated with a statistically significant increase in the growth of these tumors (the median tumor weights were 2.2 g and 0 g, respectively, P = .043) (Table 2).

MMP Production by Tumor-Infiltrating Macrophages

In the final set of experiments, we examined whether MMP-9 expression in the tumor-infiltrating macrophages was associated with progressive growth of human ovarian cancers in nude mice. Tumors produced by the injection of SKOV3.ip1 or HEY-A8 cells into MMP-9+/+ or MMP-9−/− nude mice were resected and processed for immunohistochemistry. MMP-9-expressing mouse cells were detected with an anti-MMP-9 antibody that was specific for murine MMP-9 and were visualized by red fluorescent signals. Tumor-infiltrating macrophages were detected with an antibody against the macrophage-specific marker F4/80 and were visualized by green fluorescent signals. Colocalization of the two antibodies yielded a yellow fluorescent signal. Fig. 3 shows that human ovarian tumors in MMP-9+/+ nude mice contained F4/80-positive macrophages that expressed MMP-9, whereas tumors in MMP-9−/− nude mice contained F4/80-positive macrophages that did not express MMP-9. In fact, the majority of the tumor-infiltrating mouse cells in human ovarian tumors from MMP-9+/+ mice were positive for both MMP-9 and F4/80.

We then examined whether MMP-9-expressing macrophages were also present in human ovarian tumors from MMP-9−/− nude mice that had received spleen cells from MMP-9+/+ nude mice. Tumors produced by the injection of SKOV3.ip1 or HEY-A8 cells into MMP-9−/− nude mice were resected and processed for immunohistochemistry, and MMP-9-expressing macrophages in the tumors were detected by double staining with anti-MMP-9 and anti-F4/80 antibodies. Human ovarian tumors from MMP-9−/− nude mice that had received spleen cells from MMP-9+/+ nude mice contained macrophages that expressed MMP-9, whereas tumors from MMP-9−/− nude mice that had received spleen cells from MMP-9−/− nude mice contained macrophages that did not express MMP-9 (Fig. 3). These data confirm that the increased growth of human ovarian tumors in MMP-9−/− nude mice reconstituted with spleen cells from MMP-9+/+ mice compared with that of tumors in MMP-9−/− nude mice reconstituted with spleen cells from MMP-9−/− mice was associated with increasing infiltration of MMP-9-expressing macrophages into the former tumors.

DISCUSSION

Our results demonstrate that mouse-derived MMP-9 plays an important role in angiogenesis, tumor growth, and the formation of ascites fluid by human ovarian cancer cells implanted into the peritoneal cavities of nude mice. In mice that expressed MMP-9, intraperitoneally injected SKOV3.ip1 and HEY-A8 cells produced highly vascularized and rapidly growing tumors, whereas in mice that did not express MMP-9, the human cells produced fewer tumors with lower microvesel densities. The decrease in angiogenesis in mice that lacked MMP-9 expression was associated with a decrease in macrophage infiltration into the ovarian tumors which, in mice that had received spleen cells from mice that expressed or lacked expression of MMP-9, was associated with the expression of MMP-9 in macrophages, suggesting that tumor-infiltrating macrophages played a major role in the an-
giogenesis and growth of the human ovarian tumors in the animal model.

MMP-9 promotes the migration and invasion of cancer cells into and out of blood vessels by mediating the proteolytic degradation of type IV collagen in the basement membrane. MMP-9-deficient mice were also reconstituted with spleen cells from either MMP-9+/+(MMP-9+/+SP(MMP-9+/+)) or MMP-9−/−(MMP-9−/−SP(MMP-9−/−)) nude mice prior to cancer cell injection. After 35 days (for HEY-A8 cell injections) or 50 days (for SKOV3.ip1 cell injections), peritoneal tumors of similar size were harvested and processed for immunofluorescence analysis. Macrophages were detected by using macrophage marker F4/80 antibodies (green), and host MMP-9 was detected by using anti-mouse-specific MMP-9 antibodies (red). Double immunofluorescent staining for F4/80 and mouse MMP-9 (yellow) demonstrated that macrophages expressed MMP-9. Bar = 50 μm.

The extent of angiogenesis is determined by the balance between positive and negative regulatory molecules that are produced by tumor cells as well as by stromal (i.e., nontumor) cells (3,4). Activated macrophages influence the angiogenic process by secreting enzymes that can break down the extracellular matrix and by secreting angiogenic molecules and growth factors, such as bFGF, transforming growth factor-alpha and -beta (TGF-α and -β), insulin-like growth factor-I, platelet-derived growth factor, and VEGF/vascular permeable factor (VEGF/VPF) (38–40). These factors induce endothelial cells to migrate...
and proliferate. The number of macrophages that infiltrate hu-
man ovarian cancers has been shown to directly correlate with
microvesseldensity(31),andmacrophagesisolatedfromascitic
fluid aspirated from women with advanced ovarian cancer has
been shown to produce angiogenic effects in vitro and in vivo
(41). In clinical samples of human ovarian tumors, MMP-9 is
expressed in both epithelial and stromal cells (18).

Our finding—that decreased angiogenesis of ovarian tumors in
MMP-9+/–nude mice was associated with a decrease in mac-
rophage infiltration into the tumors—supports the conclusion
that macrophages positively influence the vascularization of hu-
man ovarian tumors. Moreover, we also observed that tumors
from MMP-9+/– nude mice had lower levels of VEGF than did
tumors from MMP-9+/+ nude mice, and that this decrease was
associated with a decrease in macrophage infiltration. Thus, our
data suggest that one mechanism by which macrophages could
promote angiogenesis is through the proangiogenic molecule,
VEGF.

To infiltrate a tissue, macrophages must penetrate the extra-
cellular matrix. Our data provide direct evidence that MMP-9 is
involved in this process. Consistent with the decrease in mac-
rophage infiltration into tumors growing in MMP-9+/– nude
mice, peritoneal exudate macrophages from MMP-9+/– nude
mice were less able to penetrate a reconstituted extracellular
matrix than were those from MMP-9+/+ nude mice, whether
these data are applicable to other infiltrating cells, e.g., neutro-
phils, is controversial (42,43).

In summary, we have demonstrated that host-derived MMP-9
contributes to the angiogenesis, growth, and formation of ascites
fluid by human ovarian cancers in nude mice. In this tumor
model, a major source of MMP-9 is the macrophage. Our data do
not exclude the possibility that other host cells, such as endo-
thelial cells, mast cells, and neutrophils, could have contributed
MMP-9 (10,44–46). In any event, we found that deficiency of
MMP-9 in host cells (but not in tumor cells) inhibited neoplastic
angiogenesis and, hence, the carcinomatosis of two human ovar-
ian cancer cell lines in this animal model. Targeting expression
of MMP-9 in tumor cells, and more so in nontumor cells, may
therefore be an effective approach to control angiogenesis and
carcinomatosis of human ovarian tumors.

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