Endostatin Overexpression Inhibits Lymphangiogenesis and Lymph Node Metastasis in Mice

Gaëlle Brideau,1 Markus J. Mäkinen,2 Harri Elamaa,1 Hongmin Tu,1 Gunnar Nilsson,3 Kari Alitalo,1 Taina Pihlajaniemi,1 and Ritva Heljasvaara3

1Collagen Research Unit, Biocenter Oulu and Department of Medical Biochemistry and Molecular Biology, and 2Department of Pathology, University of Oulu, Oulu, Finland; 3Department of Medicine, Clinical Immunology and Allergy Unit, Karolinska Institutet, Stockholm, Sweden; and 4Molecular/Cancer Biology Laboratory and Ludwig Institute for Cancer Research, Biomedicum Helsinki, Helsinki, Finland

Abstract

Endostatin, a proteolytic fragment of collagen XVIII, is a potent inhibitor of angiogenesis and tumor growth. We studied the development of carcinogen-induced skin tumors in transgenic J4 mice overexpressing endostatin in their keratinocytes. Unexpectedly, we did not observe any differences in tumor incidence and multiplicity between these and control mice, nor in the rate of conversion of benign papillomas to malignant squamous carcinomas (SCC). We did find, however, that endostatin regulates the terminal differentiation of keratinocytes because the SCCs in the J4 mice were less aggressive and more often well differentiated than those in the control mice. We observed an inhibition of tumor angiogenesis by endostatin at an early stage in skin tumor development, but more strikingly, there was a significant reduction in lymphatic vessels in the papillomas and SCCs in association with elevated endostatin levels and also a significant inhibition of lymph node metastasis in the J4 mice. We showed that tumor-infiltrating mast cells strongly expressed vascular endothelial growth factor-C (VEGF-C), and that the accumulation of these cells was markedly decreased in the tumors of the J4 mice. Moreover, endostatin inhibited the adhesion and migration of murine MC/9 mast cells on fibronectin in vitro. Our data suggest that endostatin can inhibit tumor lymphangiogenesis by decreasing the VEGF-C levels in the tumors, apparently via inhibition of mast cell migration and adhesion, and support the view that the biological effects of endostatin are not restricted to endothelial cells because endostatin also regulates tumor-associated inflammation and differentiation, and the phenotype of epithelial tumors. [Cancer Res 2007;67(24):11528–35]

Introduction

Endostatin, a proteolytic fragment of the vascular and epithelial basement membrane collagen type XVIII, is an efficient antiangiogenic and antitumor molecule (1). Recombinant endostatin inhibits the proliferation and migration of endothelial cells and induces their apoptosis, which leads to reduced tumor vascularization and subsequently suppresses tumor growth in mice (reviewed in 2, 3). Transgenic mice overexpressing endostatin specifically in their endothelial cells show a 1.6-fold increase in circulating endostatin levels, which is sufficient to inhibit tumor vascularization and slow down the growth of transplanted tumors (4).

Diverse molecular mechanisms are associated with endostatin signaling. Among other effects, endostatin can reduce endothelial cell motility by interfering with basic fibroblast growth factor–induced signal transduction (5), induce apoptosis of endothelial cells (6), and block vascular endothelial growth factor (VEGF)-mediated signaling by directly interacting with the receptor VEGFR-2 (7). The most consistent effect of endostatin is the inhibition of growth factor–induced endothelial cell migration, which is thought to occur by binding to integrin α5β1 (8), and subsequent disruption of cell-matrix adhesion via caveolin-1/Src tyrosine kinase/Rho (9, 10) or via FAK/Ras/Raf/extracellular signal-regulated kinase1/p38 signaling pathways (11). Using genome-wide expression profiling, Abdollahi and coworkers (12) showed that endostatin affects the expression of up to 12% of the genes in human endothelial cells, down-regulating the angiogenic stimulators, and up-regulating many antiangiogenic genes. Moreover, they showed that endostatin affects signaling events that are not directly associated with angiogenesis, demonstrating the importance of interpathway communications in a complex signaling network.

We have recently reported on the generation and characterization of transgenic mice overproducing endostatin in the skin and in the eye lens capsule under the keratin 14 promoter (J4 mice; ref. 13). These mice develop cataract of the lens due to loosening of the contact of the epithelial cells with the lens capsule and due to abnormal proliferation and clustering of these cells. They also have significantly broadened epidermal basement membranes, and a similar phenotype has been observed in mice lacking collagen XVIII (14), indicating that endostatin and its precursor have a role in maintaining the structural integrity of the basement membranes (13).

We have used the J4 mice to analyze the effects of elevated endostatin levels on chemically induced skin tumors and tumor vascularization. Most in vivo studies reporting the antiangiogenic and antitumor effects of endostatin have been performed using tumors implanted in mice and have involved substantial doses of recombinant endostatin (reviewed in 2). To our knowledge, only one previous study describes the antitumor effects of recombinant endostatin administration on carcinogen-induced mammary tumors in the rat (15). We used a multistage mouse skin carcinogenesis protocol involving 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) treatments to induce tumor formation (16). This approach allowed us to follow the consequences of continuous skin-specific endostatin overexpression on tumor angiogenesis and tumor progression at different stages in skin carcinogenesis. We present here in vivo and
in vitro evidence that endostatin regulates the infiltration of VEGF-C–producing mast cells into the tumor tissue, which leads to decreased lymphangiogenesis and lymph node metastasis, and that it also modulates the terminal differentiation of SCCs in the mouse skin.

Materials and Methods

Mice. Age- and sex-matched J4 transgenic mice overexpressing endostatin in the epidermal basal cells (13) and wild-type control mice, both of a FVB/N background, were used for the skin carcinogenesis studies.

Chemical skin carcinogenesis. Each experimental group consisted of 25 to 30 J4 and control mice aged for 12 weeks. Their dorsal skin was shaved and tumors were induced by a single topical application of 100 μg of DMBA (Sigma-Aldrich) in 100 μL of acetone. After 1 week, tumor formation was promoted by treating the mice weekly for 12 to 20 weeks with 5 μg of TPA (Sigma-Aldrich) in 100 μL of acetone. Tumor growth was monitored as follows: Group 1, DMBA initiation followed by TPA promotion for 12 weeks and sacrifice at 13 weeks; Group 2, DMBA initiation followed by TPA promotion for 20 weeks and sacrifice at 24 weeks; and Group 3, DMBA initiation followed by TPA promotion for 20 weeks and sacrifice at 34 weeks. The number of tumors on each mouse was counted weekly for the duration of the experiment, and tumor size was measured with a gauge. The tumor incidence (percentage of mice with a tumor) and tumor multiplicity (number of papillomas per mouse) were recorded. Mice were sacrificed if moribund or if the tumor load was excessive. All the animal experiments were approved by the Animal Care and Use Committee of the University of Oslo and by the State Provincial Office of Oslo.

Tumor and tissue sample harvesting. Mice were sacrificed, the skin tumors were removed, and a complete autopsy, including lymph node dissection, was performed. The samples were evaluated by a pathologist in a blinded manner on the basis of HE-stained sections, and the skin alterations were classified as representing hyperplasia, dysplasia, papilloma, keratoacanthoma, well-, moderately, or poorly differentiated SCC or spindle cell carcinoma. The autopsy samples were evaluated histopathologically for the presence of metastases.

Histochemical and immunohistochemical analyses. Tissue sections were either treated with trypsin for 30 min at 37°C and stained with a rat antimouse CD-31 antibody (platelet/endothelial cell adhesion molecule 1; BD Biosciences Pharmingen), heated in a microwave oven in EDTA-Tris buffer (pH 9.0) and stained with an antirabbit VEGF-C antibody (Santa Cruz Biotechnology, Inc.), or treated with 10 mmol/L citrate buffer (pH 3.0) for 30 min at 37°C and stained with a rat antimouse monoclonal F8/80 antibody against macrophages (Serotec). The tyramide signal amplification kit (TSA; Perkin-Elmer Life Sciences) was used according the manufacturer’s instructions to intensify the color reactions. A biotinylated antinestin or antirabbit antibody (Vector Laboratories) was used as a secondary antibody. For Ly-1 and VEGFR-3 immunostainings, sections were heated in a microwave oven in 10 mmol/L citrate buffer (pH 6.0) and incubated with an antirat Ly-1 antibody or with an antimouse VEGFR-3 antibody (17), using the TSA signal amplification protocol. A biotinylated antirabbit or antirabbit IgG antibody (Vector Laboratories) was used for detection. Mast cells were detected by Leder’s method of chloroacetate esterase histochemistry (18). The number of mast cells within the tumor and peritumoral stroma was counted from ten random fields of 0.1 mm², covering 1.0 mm² area in total.

Determination of vessel density. The immunostained tumor sections were analyzed at ×100 magnification to identify the areas of high vascularization, either in the tumor itself (blood vessels only) or in the surrounding area (blood vessels and lymphatic vessels). Vessels were counted in 10 fields (carcinomas) or 5 fields (papillomas) representing the areas of highest vascular density at ×200 magnification. Branching structures were considered as a single vessel, and the average of the microvessel counts was calculated.

Apoptosis assay. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling (TUNEL) assay was performed using an in situ cell death detection kit (Roche Diagnostics) according to the manufacturer’s instructions with some modifications. The paraformaldehyde-fixed sections were incubated in 3% H₂O₂/methanol for 10 min at room temperature, permeabilized with proteinase K for 10 min at 37°C, and incubated for 30 min at 37°C in the TUNEL reaction buffer containing the label solution and the enzyme solution, both diluted 1:1 with TUNEL-diluting buffer. The following steps were carried out as suggested except that the duration of the incubation in 3,3′-diaminobenzidine was reduced to 15 s. Five random fields at ×200 magnification were counted to determine the numbers of apoptotic cells.

Proliferation assay. Antigen retrieval was performed by heating the sections for 10 min in a microwave oven in 10 mmol/L citrate buffer (pH 6.0). The sections were incubated overnight at 4°C with a monoclonal rat antimonoo Ki67 antibody (DAKO/Cytomation), and a Cy3-conjugated goat antirat antibody (Jackson Immunoresearch Laboratories, Inc.) was used as a secondary antibody. Five random fields at ×200 magnification were counted to determine the numbers of proliferating cells in the sections.

Cell culture. The murine mast cell line MC/9 (a gift from Dr. Gunnar Nilsson, Karolinska Institutet, Stockholm, Sweden) was maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 10 ng/mL of recombinant mouse interleukin-3 (Sigma-Aldrich), 0.1 mmol/L norsesential amino acids, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4 mmol/L L-glutamine, and 50 μmol/L L-mercaptoethanol.

Expression and purification of recombinant endostatin. The cloning and the purification of recombinant human endostatin have been described earlier (8).

Adhesion assay. 96-well plates were coated overnight at 4°C with 25 μg/mL of fibronectin (BD Biosciences) in PBS or with 3% bovine serum albumin (BSA) in PBS to determine the spontaneous adhesion (data not shown). The remaining binding sites were blocked with 5% BSA in PBS for 2 h at 37°C. 5 × 10^5 MC/9 mast cells in complete culture medium were preincubated with different concentrations of recombinant endostatin (or with BSA as a control) for 1 h at 37°C and added to the substrate-coated wells. To induce the cell adhesion, stem cell factor (SCF; Sigma-Aldrich), at 35 μg/mL, was added to the wells immediately after cell loading (19). Cells were allowed to attach on substrate for 80 min at 37°C in 5% CO₂ after which the wells were washed twice with PBS to remove nonadherent cells. Trypsin-EDTA was added to the wells to recover adherent cells. The numbers of the recovered adherent and nonadherent living cells were determined using a hemocytometer. Each well was done at least in duplicate, and the experiment was repeated several times. To test the specificity of endostatin, 5 μg/mL of recombinant endostatin was preincubated with 1 μg/mL of antihuman endostatin antibody (HES6; ref. 20), or with a nonrelevant polyclonal IgG antibody (Santa Cruz, Inc.), for 1 h at room temperature before adding to the cells.

Migration assay. The migration assay was performed by using 8.0-μm pore size Transwell inserts (Greiner Bio One). Micropore filters were coated overnight at 4°C with 40 μg/mL of fibronectin in PBS, and the remaining binding sites were blocked with 5% BSA in PBS for 1 h at 37°C. 5 × 10^5 MC/9 cells in complete culture medium were preincubated with different concentrations of recombinant endostatin (or with BSA as a control) for 1 h at 37°C, and added to the upper chamber of the Transwell insert. Complete medium supplemented with the chemotactrant SCF (35 μg/mL) was added in the lower chamber to induce the cell migration (19). Controls without SCF were performed to determine the passive diffusion (always <7%^ data not shown). Cells were allowed to migrate for 8 h at 37°C in 5% CO₂. The numbers of the living cells in the upper and lower chamber were counted separately using a hemocytometer. Each sample was assayed in duplicate, and the experiment was repeated twice. To test the specificity of endostatin action, 5 μg/mL of recombinant endostatin was preincubated with 1 μg/mL of antihuman endostatin antibody, or with a nonrelevant polyclonal IgG antibody (Santa Cruz, Inc.), for 1 h at room temperature before adding to the cells.

Real-time PCR. Total RNA was extracted from the tumors using Tri-pure reagent (Sigma-Aldrich) according to the manufacturer’s instructions. 0.5 μg of total RNA was reverse transcribed using 100 ng of random hexamers and Superscript II reverse transcriptase (Invitrogen). The PCR primers for VEGF-A, VEGF-C, VEGF-D, VEGFR-2, and VEGFR-3 were obtained from a published study (21). Specific primers for 18S RNA were

www.aacrjournals.org 11529

Downloaded from cancerres.aacrjournals.org on April 30, 2017. © 2007 American Association for Cancer Research.
Results

Endostatin modulates skin carcinogenesis. The multistage mouse skin carcinogenesis protocol leads to the outgrowth of highly differentiated benign papillomas that can progress to malignant SCCs and often also to metastatic dissemination into the lymph nodes (16). We applied this carcinogenesis model to transgenic J4 mice, which show a clear increase in monomeric endostatin in the skin and the lens capsule (13), and measured the concentrations of circulating endostatin. These were ~7-fold higher (189 ± 55 ng/mL) than in the control FVB/N mice (27 ± 6.9 ng/mL) and increased further in both strains upon cancer induction, although relatively less so in the control mice, so that levels of 495 ± 61 ng/mL (2.6-fold) were reached in the tumor-bearing J4 mice versus 35.5 ± 10.8 ng/mL (1.3-fold) in the tumor-bearing wild-type mice.

A comparison of carcinogen-induced skin tumorigenesis between the J4 mice and the wild-type control mice in three experimental groups (13, 24, and 34 weeks) is presented in Table 1 and Fig. 1. In view of the well-documented antitumor effects of endostatin (2), we expected to find a lower tumor incidence and/or multiplicity and/or smaller tumor size in the J4 mice, but in practice, the tumors began to emerge after a similar latency period (7–8 weeks) in both strains, and no differences in incidence were detected, as the same proportion of both the J4 mice and the wild-type mice (95%) developed papillomas upon chemical treatment (Fig. 1A). Tumor multiplicity showed no changes in the J4 mice either; the transgenic mice yielding an average of 9.0 papillomas per mouse compared with 9.7 in the control group, but a sharper decrease in the number of papillomas was observed in the J4 mice after 30 weeks (not significant, t test; Fig. 1B). However, from the 20-week time point onwards, the papillomas in the J4 mice were smaller in size than those in the wild-type mice, and this difference was statistically significant (P < 0.05 at most time points and P < 0.001 in several time points, t test; Fig. 1C). In addition, histopathologic evaluation of the skin lesions at 13 weeks time point showed that there was a delay in the development of the papillomas in the J4 mice at an early stage in skin carcinogenesis, 36% having papillomas at week 13 compared with 64.7% of the wild-type mice (P < 0.05, t test; Table 1).

The mice were evaluated for the development of skin SCC, but no significant differences in the time of appearance of carcinomas were found between the J4 and wild-type mice, or in the rate of malignant conversion at the end of the experiment (70% and 68%, respectively; Table 1; data not shown). Nevertheless, the histopathologic analysis of the SCCs revealed clear differences in keratinocyte differentiation between the controls and the J4 mice (Table 1 and Fig. 1D); those in the J4 mice being more often well-differentiated at week 34, whereas moderately or poorly differentiated SCCs were more common in the control mice (P = 0.001, χ² test; Table 1 and Fig. 1D). The depth of local invasion of the SCCs seemed to be higher in the control group, in which the cancers more often extended into the s.c. tissues and muscular layer (75% versus 55% in the J4 mice), but this difference was not statistically significant (P = 0.210, χ² test; data not shown). Moreover, histopathologic analysis of the autopsy samples collected from most of the mice bearing SCCs indicated that lymph node

Table 1. Classification of carcinogen-induced skin tumors in endostatin-overexpressing J4 and FVB/N control (wild-type) mice (n = number of tumors; % = number of tumors / total number of tumors)

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Stage/grade</th>
<th>13 wk</th>
<th>24 wk</th>
<th>34 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>J4</td>
<td>Wild-type</td>
<td>J4</td>
<td>Wild-type</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Dysplasia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 (48.0)</td>
<td>8 (23.5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>4 (16.0)</td>
<td>4 (11.8)</td>
<td>2 (4.0)</td>
<td>0</td>
</tr>
<tr>
<td>Keratoacanthoma</td>
<td>0</td>
<td>0</td>
<td>3 (6.8)</td>
<td>0</td>
</tr>
<tr>
<td>Papilloma</td>
<td>Papilloma</td>
<td>16 (64)</td>
<td>15 (44.1)</td>
<td>16 (32.0)</td>
</tr>
<tr>
<td>Papilloma with dysplasia</td>
<td>5 (20.0)</td>
<td>7 (20.6)</td>
<td>32 (64.0)</td>
<td>30 (68.2)</td>
</tr>
<tr>
<td>Total*</td>
<td>25 (100)</td>
<td>34 (100)</td>
<td>50 (100)</td>
<td>44 (100)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-differentiated</td>
<td>0</td>
<td>0</td>
<td>10 (100)</td>
<td>8 (80.0)</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>0</td>
<td>0</td>
<td>2 (20.0)</td>
<td>7 (35.0)</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (5.0)</td>
</tr>
<tr>
<td>Total†</td>
<td>0</td>
<td>0</td>
<td>10 (100)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Spindle cell carcinoma</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Total number of tumors (including: dysplasia, hyperplasia, keratoacanthoma, and papillomas) histopathologically evaluated.
† Total number of SCCs histopathologically evaluated.
metastases at 34 weeks were statistically more frequent in the wild-type mice (40% in the control mice versus 7.7% in the J4 mice; \( P < 0.05 \), \( \chi^2 \) test; Fig. 1D).

**Effects of endostatin on tumor angiogenesis and lymphangiogenesis.** A statistically significant difference in the number of blood vessels between the two mouse strains was found only in the tumors collected at 13 weeks, where the capillary densities in the central tumor area were markedly lower in the J4 mice, as also were those in the area surrounding the tumor. The average number of blood vessels in the tumor area was 9.09 ± 2.18 per field in the J4 mice, which was markedly lower than in the wild-type mice, 14.1 ± 3.97 per field (\( P = 0.009 \), t test), whereas the difference in vessel densities was smaller in the peritumoral area (11.4 ± 2.15 per field in the J4 mice versus 15.2 ± 3.7 in the control mice; \( P = 0.011 \), t test; Fig. 2A). No significant differences in blood vessel densities were observed between the two mouse strains in the other experimental groups; that is, at 24 and 34 weeks after DMBA treatment, neither in terms of papillomas nor of SCCs (Fig. 2A; data not shown).

The difference in lymph node metastases between the J4 and control mice prompted us to study the possible effects of endostatin overexpression on lymphangiogenesis. Because the tumor area itself was found to contain very few lymphatic vessels, their numbers were calculated only in the area surrounding the tumor. A decrease in the numbers of Lyve-1–positive vessels was detected in this area within the SCC samples collected from the J4 mice at 34 weeks (\( P = 0.008 \), t test) and in the benign papillomas at the same point in time (\( P = 0.007 \), t test; Fig. 2B–D). A statistical difference in the numbers of lymphatic vessels within skin tumors was also noted at other time points (\( P = 0.007 \) and \( P = 0.004 \) in the papillomas at 13 weeks and 24 weeks, respectively, and \( P = 0.013 \) in the SCCs at 24 weeks). Furthermore, the J4 mouse skin outside the carcinogen-treated area contained significantly less Lyve-1–positive vessels (\( P = 0.009 \), t test), suggesting that endostatin overexpression affects the development of the cutaneous lymphatic system in these mice (Fig. 2D).

**Effects of endostatin on cell proliferation and apoptosis in skin tumors.** We evaluated the effects of endostatin overexpression on apoptosis by calculating the TUNEL-positive cells in tumor sections. An increase in the incidence of apoptotic keratinocytes was detected both in papillomas and SCCs in the transgenic J4 mice relative to the control mice (Fig. 3A), a difference that was already evident at an early stage in tumor development (at 13 weeks; \( P = 0.01 \), t test) and remained significant throughout the process of malignancy. Cell death was even higher in the late-stage benign papillomas of the J4 mice (at 34 weeks; \( P = 0.0066 \) between the mouse strains, t test) than in the samples collected at earlier time points. The SCC samples collected from the J4 mice also showed increased numbers of apoptotic keratinocytes at the two later time points, again with a statistical difference between them (\( P < 0.05 \) at 24 weeks and 34 weeks, t test). Elevated endostatin expression had an inhibitory effect on the proliferation of keratinocytes. At an early stage in tumor development (13 weeks), the number of Ki67-positive cells was significantly higher in the tumor samples derived from wild-type mice than in those derived from the J4 mice (\( P = 0.0004 \), t test; Fig. 3B), whereas at later time points, the numbers of proliferating cells did not show significant differences between the two mouse strains.

**Endostatin overexpression decreases the mRNA levels of lymphangiogenesis-related VEGF signaling components.** To determine whether the difference observed in the numbers of
tumor lymphatic vessels between the endostatin-overproducing mice and the controls might involve changes in the expression of VEGF family ligands or receptors, a real-time PCR analysis was carried out using total RNA extracted from papillomas collected at week 34. mRNA expression of VEGF-A, VEGF-D, and VEGFR-2 showed no differences between J4 and control mice, but the expression of VEGF-C decreased almost 7-fold, and mRNA for its receptor VEGFR-3 decreased 15-fold in tumors of the J4 mice (Fig. 4A).

**Endostatin overexpression reduces the number of VEGF-C–producing mast cells.** We next asked which cells contribute to the expression of VEGF-C and its receptor VEGFR-3 in skin tumors. VEGF-C is produced by tumor cells in many cancers (22) but also by tumor-infiltrating inflammatory cells, in particular macrophages and mast cells (23–25), and the numbers of these cells have been shown to correlate with blood and lymphatic vessel densities (24–26) and with the frequency of lymph node metastasis (24, 25). Immunohistochemical staining of VEGF-C in J4 and control papillomas at 34 weeks revealed that it is strongly expressed by inflammatory mast cells in the peritumoral area, as identified by Leder’s method (Fig. 4B), but also to some extent by keratinocytes in the SCCs (data not shown). Interestingly, there was a significant reduction in the numbers of mast cells in tumors of the same differentiation grade in the endostatin-overexpressing J4 mice at weeks 24 and 34 by comparison with the control mice, with P values of 0.0035 and 0.042, respectively (t test; Fig. 5A and B). Macrophages were abundant in the peritumoral area and in the surrounding stroma of both mouse strains at all stages of carcinoma development (data not shown). We could not detect any differences in their numbers between the J4 and control mice nor could we confirm the production of VEGF-C by these cells. Staining of serial papilloma sections with the antibodies for VEGFR-3 and Lyve-1 indicated that VEGFR-3 was expressed by the Lyve-1–positive lymphatic endothelial cells (Fig. 4C).

**Endostatin inhibits the adhesion and migration of mast cells on fibronectin.** The tissue distribution of mast cells is regulated by the interactions between their cell surface receptors and the extracellular matrix (27). We hypothesized that endostatin might directly control the trafficking of mast cells into the tumor tissue, and tested this by studying the effects of recombinant endostatin on the adhesion and migration of murine MC/9 mast cells on fibronectin-coated surfaces. As Fig. 5C shows, endostatin inhibited the SCF-induced adhesion of MC/9 cells to fibronectin in a concentration-dependent manner. Of the untreated control cells, up to 72% adhered to fibronectin in the presence of SCF, whereas treatment with 10 μg/mL of endostatin decreased the cell adhesion to 37% (P < 0.001, t test; Fig. 5C). The antiendostatin antibody HES.6 was able to block the inhibitory effect of endostatin and restored the MC/9 cell adhesion to fibronectin to the level of the control cells, whereas the addition of an unspecific polyclonal antibody could not reverse the effect of endostatin on mast cells. This showed that the reduction in the mast cell adhesion to fibronectin was endostatin specific (Fig. 5C).

Similarly, we examined the potential effects of endostatin on the mast cell migration using a Transwell assay. Endostatin also inhibited the MC/9 cell migration in a concentration-dependent manner (Fig. 5D). Without endostatin treatment, 56% of the mast cells migrated through the fibronectin-coated filters in response to chemoattractant SCF, whereas in presence of 5 μg/mL of endostatin, the migration decreased to 25% (P < 0.001, t test; Fig. 5D). As in the cell adhesion assays, the antiendostatin antibody, but not a nonrelevant IgG fraction, reversed the inhibitory effect of endostatin on MC/9 cell migration, confirming that the effect was specifically due to endostatin administration (Fig. 5D).

**Discussion**

We set out here to analyze the roles of endostatin in carcinogen-induced skin tumorigenesis by using transgenic J4 mice, which overexpressed endostatin in their keratinocytes (13). We found that elevated endostatin levels inhibited angiogenesis at an early stage of tumor development, but this did not significantly alter tumor incidence or multiplicity. Endostatin overproduction significantly reduced the number of tumor lymphatics and also prevented tumor cell dissemination into the lymph nodes, which, at least in part, seems to be due to the ability of endostatin to inhibit the distribution of VEGF-C–producing mast cells in the tumor matrix as shown by the in vitro mast cell adhesion and migration assays. The elevated endostatin level also had a clear effect on the differentiation of the skin tumors because the SCCs in the J4 mice...
were more often well differentiated, whereas those in the control mice were frequently moderately or poorly differentiated.

Lymph node metastases were observed in only one of the 13 transgenic J4 mice bearing SCCs (7.7%), by comparison with 40% of the corresponding control mice (6 of 15). Cancer cell metastasis to distant organs occurs via the vascular and lymphatic systems, the lymphatic system being more important in the case of SCCs (28, 29). Moreover, several reports have shown that lymphangiogenesis correlates with lymph node metastases (30, 31). Our finding of significantly less Lyve-1–positive vessels in the J4 mice than in the control mice, both in papillomas and in SCCs, is consistent with these observations and suggests that the reduction in lymph node metastases may be due to the suppression of tumor lymphangiogenesis induced by endostatin.

There are, to our knowledge, only two papers that describe endostatin's effects on lymphangiogenesis: Shao et al. (32) showed that recombinant endostatin inhibits the proliferation and migration of lymphatic endothelial cells in vitro, and Fukumoto et al. (33) showed that endostatin inhibits lymphangiogenesis and lymph expansion by down-regulating VEGF-C expression in cultured SCC cells. We also showed down-regulation of VEGF-C mRNA in papillomas in the presence of endostatin overproduction (Fig. 4A). This may partly be due to decreased expression of VEGF-C by the tumor cells, as the present study and others (19) showed an expression of this endothelial growth factor by SCCs. We also showed a strong expression of VEGF-C by tumor-associated inflammatory mast cells and observed a notably reduced number of mast cells in the skin tumors of the J4 mice. Furthermore, we showed that endostatin inhibited the adhesion and the migration of murine MC/9 mast cells on fibronectin in vitro. These data suggest that elevated endostatin levels regulate the amount of lymphangiogenesis by reducing the number of VEGF-C–producing inflammatory mast cells in the tumor tissue, which subsequently affects the amount of tumor metastasis.

Interestingly, the α5β1 and αvβ3 integrins, which mediate the effects of endostatin on endothelial cells (8, 9), have been implicated in the adhesion of human cutaneous mast cells to fibronectin and vitronectin, respectively (34, 35). It is thus possible that the binding of endostatin to these receptors inhibits mast cell adhesion and migration on the tumor matrix, although this remains to be shown. Furthermore, Coussens and coworkers (26) showed that mast cells activate angiogenesis and neoplastic progression during skin carcinogenesis in the mouse, and that the angiogenic switch is dependent on the activation of pro–matrix metalloproteinase (MMP)-9 matrix metalloproteinase by the mast cell serine protease. We have earlier reported that endostatin inhibits activation of pro–MMP-9 in vitro (36), and thus, the overproduction of endostatin may further decrease the amount of active MMP-9 in tumors of J4 mice and thereby suppress angiogenesis, lymphangiogenesis, and tumor progression.

We also showed a pronounced reduction in the mRNA expression of VEGFR-3 within the papillomas of the J4 mice.

Figure 3. Cell proliferation and apoptosis in skin tumors of endostatin-overexpressing J4 and control (WT) mice. Papillomas and SCCs collected at different time points during skin carcinogenesis were analyzed for apoptotic and proliferating keratinocytes. A, TUNEL labeling of apoptotic cells. B, Ki67 immunofluorescence staining for proliferating cells. Columns, mean; bars, SD. pap, papilloma. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 4. Expression of VEGF signaling components by mouse skin tumors. A, real-time PCR analysis was carried out using total RNA isolated from papillomas that were collected from endostatin-overexpressing J4 and control mice (WT) at 34 wk. B, expression of VEGF-C by mast cells. Serial sections of papillomas at 34 wk were stained for mast cells (left) and VEGF-C (right; original magnification, ×200). *, a Lyve-1–negative blood vessel.
Endostatin down-regulated the transcription of VEGFR-2 in endothelial cells (12), and thus, it is plausible that VEGFR-3 production could also be affected by elevated endostatin. However, we think that the weak VEGFR-3 mRNA expression observed in the J4 papillomas most likely reflects the loss of lymphatic vessels in the tumors of the J4 mice.

Elevated endostatin had only minor effects on the induction and progression of skin tumors and tumor angiogenesis. We showed a decrease in blood vessels at early stage of tumor development in the J4 mice, but at later time points, the vessel numbers were comparable between the mouse strains. At 24 weeks, tumor angiogenesis seemed to be even higher in the transgenic mice, but this difference was not statistically significant ($P = 0.169$ and 0.275 in tumor area and surrounding area, respectively, $t$ test; Fig. 2A). Effective therapeutic levels of circulating endostatin are up to 80 to 450 ng/mL, whereas too low and too high concentrations are ineffective (2). In the J4 mice, the circulating endostatin levels were $\sim$190 ng/mL and further increased in the SCC-bearing J4 mice up to 500 ng/mL. As the exogenous endostatin is expressed by the basal keratinocytes, it is possible that the local endostatin concentration in the skin tumors is too high for adequate angiogenesis suppression. Furthermore, angiogenesis was shown to be an early event in the DMBA-TPA–induced skin tumors and play a major role in the development of the papillomas but not in the premalignant progression (37). Consistently, we did not observe differences in the conversion of the papillomas to SCCs between the J4 and control mice, but the papillomas seemed smaller in the endostatin-overexpressing mice (Fig. 1C).

We found an increase in proliferating keratinocytes in the wild-type mice at the early stage of skin tumor development, but proliferation rates during tumor progression were comparable between the J4 and wild-type mice. Furthermore, we observed an increase in cell death in the J4 mice during progression to malignancy to reach a very high level of apoptotic keratinocytes by...

Figure 5. Effects of endostatin on mast cells. A, numbers of mast cells in papillomas collected at different time points during skin carcinogenesis. Columns, mean; bars, SD. B, leder’s coloration for mast cells in papillomas of the wild-type (left) and J4 mice (right) at 34 wk (original magnification, $\times$50). C, SCF-induced murine MC/9 mast cell adhesion to fibronectin in the presence of different concentrations of endostatin. Pretreatment of recombinant endostatin (5 µg/mL) with an antiendostatin antibody (1 µg/mL) or with nonspecific IgG fraction (right). D, Transwell assay. SCF-induced MC/9 cell migration in the presence of different concentrations of endostatin. Pretreatment of recombinant endostatin (5 µg/mL) with an antiendostatin antibody (1 µg/mL) or with a nonspecific IgG fraction (right). For control, instead of endostatin, the MC/9 cells were treated with BSA. Columns, mean; bars, SD. $^* P < 0.05$; $^{***} P < 0.001$. 

the end of the experiment (Fig. 3). Taken together, these data suggest that endostatin delays tumor formation at an early stage by inhibiting keratinocyte cell proliferation. Moreover, it induces apoptosis of these cells, which counterbalances the effect of the high tumor cell proliferation seen in this model, especially at the late stage in tumor progression. These findings, together with the moderately decreased angiogenesis, may explain the delay in papilloma development in the J4 mice observed in the 13-week experimental group (Table 1) and the smaller size of the papillomas from 20 weeks onward (Fig. 1C). The effects of endostatin on endothelial cell proliferation, migration, and apoptosis are acknowledged, but its role in epithelial cell behavior has not been studied in detail. Our results regarding its inhibitory effects on keratinocyte proliferation and inductive effects on keratinocyte apoptosis in vivo support previous in vitro findings with respect to its efficacy against epithelial cells as well (36, 38).

In summary, using an approach involving transgenic mice and a chemical-induced skin cancer model, we have shown that in addition to its antiangiogenic and accompanying antitumorogenic effects, endostatin can reduce lymphangiogenesis and, subsequently, lymph node metastasis in mice, and modulate the differentiation of epithelial tumor cells and the inflammatory reactions associated with cancer. Using in vitro cell culture assays, we have showed that the inhibitory effect of endostatin on lymphangiogenesis is, at least in part, due to its ability to regulate the adhesion and migration of VEGF-C–producing mast cells on extracellular tumor matrix. Our findings thus support the concept of the ability of endostatin to control a broad spectrum of signaling pathways in a coordinated fashion, by different mechanisms and at different levels, to regulate tumor cell differentiation and to restrict angiogenesis and lymphangiogenesis, and subsequently, tumor progression and metastasis.

Acknowledgments


The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Janna Träskelin, Sirkka Vileni, Aila White, and Riitta Vuento for their excellent technical assistance; Dr. Pirko Huhhtala for her advice in cell adhesion and migration assays; and Dr. Carlos López Otín for his valuable comments on the manuscript.

References


Endostatin Overexpression Inhibits Lymphangiogenesis and Lymph Node Metastasis in Mice

Gaëlle Brideau, Markus J. Mäkinen, Harri Elamaa, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/24/11528

Cited articles
This article cites 37 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/24/11528.full.html#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/67/24/11528.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.