Endostatin Overexpression Inhibits Lymphangiogenesis and Lymph Node Metastasis in Mice

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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/Bad/extracellular signalregulated 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 μL 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 Oulu and by the State Provincial Office of Oulu.

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 H&E-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 antismooth muscle actin (Becton Dickinson), or with a nonrelevant polyclonal IgG antibody (Santa Cruz, Inc.), for 1 h at room temperature before adding to the cells. Trypsin-EDTA was added to the wells to recover adherent cells. The remaining binding sites were blocked with 5% 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 × 10^4 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 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 anti-human endostatin antibody (HES; ref. 20), or with a nonrelevant polyclonal IgG antibody (Santa Cruz, Inc.), for 1 h at room temperature before adding to the cells.

Methylation 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 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 × 10^4 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 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 anti-human endostatin antibody (HES; 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 chemottractant 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, as described above for the adhesion assay.

Real-time PCR. Total RNA was extracted from the tumors using TriPure 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
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 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 × 100/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>12 (48.0)</td>
<td>8 (23.5)</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>4 (16.0)</td>
<td>15 (44.1)</td>
<td>16 (32.0)</td>
<td>11 (25.0)</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>Well-differentiated</td>
<td>0</td>
<td>0</td>
<td>10 (100)</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated</td>
<td>0</td>
<td>0</td>
<td>2 (20.0)</td>
</tr>
<tr>
<td></td>
<td>Poorly differentiated</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total †</td>
<td>0</td>
<td>0</td>
<td>10 (100)</td>
<td>10 (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.
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 tumor. A decrease in the numbers of Lyve-1–positive vessels 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 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.
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 ~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

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**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, ×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 antienodstatin 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 antienodstatin 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 antitumorigenic 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


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


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