Anti-tumor effect of endostatin mediated by retroviral gene transfer in mice bearing renal cell carcinoma

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ABSTRACT We investigated whether transfer of the gene encoding the angiogenesis inhibitor endostatin into the NIH/3T3 fibroblast cell line could inhibit renal tumor growth in vivo. NIH/3T3 cells were transduced with retroviral vectors containing the murine endostatin (ES) gene. SCID mice bearing CaKi-1 derived tumors were given a subcutaneous injection of either ES-transduced cells or control cells and were monitored for tumor growth. At the end of the in vivo experiment, the mean tumor volume of treated mice was 51.6 ± 2.4 mm³, while the tumor volume of control was 234.5 ± 14.8 mm³. Microvascular density was significantly decreased on treatment (control 9.79 vs. ES 2.53%, <0.001) accompanied by a 23-fold increase in intratumoral necrotic area and a 2.94-fold increase in the apoptotic index, determined by immunohistochemistry with anti-activated caspase-3. Apoptotic cells were found in foci enriched in infiltrating leukocytes. In conclusion, retroviral endostatin gene transfer led to secretion of functional endostatin that was sufficiently active to inhibit tumor angiogenesis and tumor growth. A second mechanism may also be implied in endostatin-dependent tumor regression, associated with tumor infiltration of leukocytes. Besides its antiangiogenic properties, endostatin may be a promising adjuvant to immunotherapy. —Coutinho, E. L., Nogueira de Sousa Andrade, L., Chammas, R., Morganti, L., Schor, N., Bellini, M. H. Anti-tumor effect of endostatin mediated by retroviral gene transfer in mice bearing renal cell carcinoma. FASEB J. 21, 3153–3161 (2007)

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Clear cell carcinomas represent just ~85% of the newly diagnosed renal cell cancers (RCC), which occur at an estimated rate of 4.4–11.1 cases per 100,000 people per year. RCC incidence is steadily increasing with rates varying from 2.3 to 4.3% each year in countries like United States (1). RCC are highly vascularized tumors, which may explain the 30–40% prevalence of metastatic disease at initial diagnosis, when systemic therapies are then necessary. In this group of patients, 1 yr survival rates are ~25%, illustrating the limited role of both chemotherapy and radiotherapy in the management of advanced stages of RCC (2). RCC secrete the proangiogenic cytokines vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) in vitro and in vivo (3–7), which are responsible for the tumor-associated angiogenesis. Conventional chemotherapy is ineffective since RCC cells overexpress genes that confer resistance to multiple drugs and radiotherapy is only useful for palliation of tumor symptoms (8–9). Systemic immunotherapy protocols using IFN-α, interleukin-2, or a combination of both were designed. However, results of large clinical trials have shown overall poor objective responses (~20% of the patients were somehow responsive) and of short duration, accompanied with severe toxicities (10–13). Altogether, these studies suggested that the vascularization in RCC might be an effective target for novel therapeutic approaches, as it has been proposed for a variety of tumor progression models (14, 15).

The angiogenic switch is tightly controlled. Stimulators of angiogenesis, such as VEGF, are opposed by a variety of endogenous inhibitors, such as thrombospondin, interferons, and protein fragments generated by limited proteolysis. These latter antiangiogenic factors include the plasminogen-derived protein fragment angiotatin, the collagen IV-derived protein fragment tumstatin, and the collagen XVIII-derived protein fragment endostatin (ES). ES was originally isolated from hemangioendothelioma cells and identified as the carboxyl-terminal segment of collagen XVIII (16). Although the mechanism through which ES suppresses angiogenesis in a tumor-specific manner generally remains unclear, it has been shown that ES induced endothelial cell apoptosis and inhibited endothelial migration. Several potential molecular targets of ES have been postulated by previous studies (17–19). Numerous studies (20–22) have shown that recombinant ES significantly inhibited tumor growth as well as...
metastasis when injected subcutaneously into tumor-bearing animals. A potential problem that needs to be overcome in antiangiogenic protocols is the need for sustained levels of the inhibitors for a critical period or treatment window. In this regard, gene transfer strategies that allow for the generation of a genetically modified producing cell that can be implanted into animals have been exploited. These studies, including the implant of ES-producing cells, led to tumor suppression and prolonged animal survival (23–25). In this study, we showed that retroviral endostatin gene transfer may be a therapeutic alternative for RCC. The tissue microenvironmental remodeling characterized by leukocyte infiltration observed in tumors exposed to ES suggests that this approach may be used as an adjuvant strategy for immunotherapy protocols.

MATERIALS AND METHODS

Cell lines and cell culture conditions

NIH/3T3 and the human renal carcinoma cell line Caki-1 were purchased from ATCC (HTB-16 and CRL-1658, respectively), cultured in DMEM containing high glucose (4.5 g/l) and 25 mM HEPES (Life Technologies, Grand Island, NY, USA; ref. 26–27). Human umbilical vein endothelial cells (HUVECs) CRL-1730 were cultured in medium 199 supplemented with Earle’s salts (Cultilab, Campinas, Brazil) and 10% FBS (Life Technologies) buffered with 25 mM HEPES (Life Technologies) and supplemented with fresh 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell lines were maintained in a humid chamber at 37°C in an atmosphere containing 5% CO₂.

Construction of retroviral vector and production of replication-defective retrovirus

The endostatin-expressing replication-deficient retrovirus vector was constructed by subcloning the murine ES cDNA fragment from the pSecend vector (constructed in our laboratory) into the retroviral vector pLXSN, generating the vector LendSN, using appropriate restriction enzymes and T4 ligase (Promega Bioscience, San Luis Obispo, CA, USA). GP+E-86 ecotropic retroviral packaging cells were then transfected with retroviral vector (LendSN or LXSN) separately by CaCl₂ precipitation method. Subsequently, GP+E-86 medium was used to infect amphotropic GP+envAm12 packaging cell line, and individual G418-resistant clones were selected as described (28). Both packaging cell lines were kindly donated by Dr. A. Bank (Columbia University, Genetix Pharmaceuticals, Tarrytown, NY, USA). For each isolated clone, the viral titer was determined using the supernatants from both LXSN and LendSN-transfected packaging cell lines. After 48 h, the medium was switched and supplemented with 0.5 mg/ml G418. These conditions were maintained for 2 wk, when individual resistant clones were isolated (30).

Western blot analysis

Western blot analysis was performed using conditioned media obtained from NIH/3T3-LendSN cells and NIH/3T3-LXSN, used herein as a control of transduction. Forty microliters from each condition were electrophoretically separated on 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane and immunodetection performed using polyclonal antibody endostatin Res.16 (kindly donated by Dr. T. Pihlajaniemi (Collagen Research Unit, University of Oulu, Oulu, Finland) as primary antibody and goat anti-mouse biotinylated antibody as secondary antibody. Detection of bands was performed using the enhanced NBT/BCIP system (Bio-Rad, São Paulo, Brazil) following the manufacturer’s instructions.

Recombinant endostatin determination by ELISA

Endostatin in the culture media and animal sera was measured with a murine endostatin enzyme immunoassay kit (Chemicon International, Temecula, CA, USA), following the manufacturer’s protocols. All samples were measured in duplicates. ELISA plates were read using the Multiskan EX Microplate Reader (Labsystems, Milford, MA, USA).

Cell viability

HUVEC viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, 5 × 10⁴ HUVEC were plated in 96-well tissue culture plates in MEM containing 2% FBS in a final volume of 0.2 ml. After 24 h, they were treated with culture medium from transduced NIH/3T3-LendSN cells and 3 ng/ml ECGF (Sigma) were then added for additional 72 h. Then, cell proliferation was assessed by directly adding 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 7 dye (5 ng/l) to the medium for another 4 h. Formazan crystals were solubilized by adding 10% SDS in 0.01 M HCl (100 μl/well) at room temperature for 10 min before reading the absorbance at 590 nm using a Multiskan EX Microplate Reader (Labsystems, Milford, MA, USA).

In vitro apoptosis detection

For detection of apoptosis, cells were stained using the DNA dye Hoechst 33342 and the number of cells with condensed chromatin was determined using a fluorescence microscope. Briefly, 1 × 10⁶ HUVEC were cultured as described above. After cell adhesion, the medium was replaced by either the supernatant of NIH/3T3-LendSN or control medium for additional 72 h. After incubation, cells were stained with 5 mg/ml Hoechst 33342 (Boehringer Mannheim, Indianapolis, IN, USA) for 30 min in the dark. The supernatant was collected and the cells were removed from the dish with trypsin and centrifuged for 5 min, and the pellet was resuspended in 50 μl of PBS. Assays were done in triplicates, and two samples of each replica were analyzed independently. To count nuclei with condensed chromatin, cells were observed and photographed under a Nikon fluorescence microscope. The percentage of dying cells, i.e., the percentage of cells with condensed chromatin, was then determined.
Animals and tumor induction

Six-week-old male SCID mice were obtained from the animal housing facility (CEDEME) at UNIFESP-EPM and housed in laminar airflow cabinets under pathogen-free conditions with a 12 h light/12 h dark schedule and fed autoclaved standard chow and water ad libitum. Six mice were injected subcutaneously in the left hind flank with 4.6 × 10⁶ Caki-1 cells/mouse in a volume of 0.1 ml of sterile phosphate buffered saline (PBS). Tumor dimensions were measured with an electronic caliper, and volumes were calculated as follows: Tumor volume = length × width² × 0.52. Animals were divided in two groups. The tumors were allowed to grow until 100 mm³, when treatments started by injecting NIH/3T3 LendSN cells (3.6 × 10⁶ cells/mouse) in the right hind flank of the mouse. All experiments were performed in accordance with institutional guidelines for animal care.

Histological analysis

At the end of the experiments, animals were euthanized following the guidelines for euthanasia of the American Veterinary Medical Association. Tumors were excised, washed in PBS, fixed in PBS-buffered 3.7% formalin for 24 h, and then processed routinely for paraffin-embedding. Histological analysis was performed in 4 μm sections stained with hematoxylin and eosin. The evaluation of necrotic areas was done by morphometry by determining the relative area of necrosis within the tumors. For immunohistochemical detection of activated caspase 3, tissue sections were routinely processed and subjected to an antigen retrieval method. Heat-induced epitope retrieval was performed on all slides by immersion in 1 mM EDTA (pH 8) and incubation at 120°C at 25 psi for 3 min using an electric pressure cooker (BioCare Medical, Walnut Creek, CA, USA). Caspase 3 labeling was performed using a monoclonal antibody (Upstate, CA, USA), at a 1:100 dilution for 28 min at 42°C, followed by incubation with an appropriate secondary antibody (Dako, Carpinteria, CA, USA) and developed using a diaminobenzidine based detection system (Dako). Microvascular density was measured using the animal lectin galectin-3, which binds to glycoconjugates expressed by endothelial cells (31). The probe used was recently developed and consisted of human galectin-3 fused to bacterial alkaline phosphatase, Gal-3/AP (Melo et al., unpublished observations). Tissue sections were incubated with 16 μg/ml of Gal-3/AP at 4°C for 22 h, thoroughly rinsed with Tris-buffered saline and incubated with the alkaline phosphatase substrate Fast Red (Dako), and lightly counterstained with hematoxylin. Micovascular density was quantified as follows: grids were projected on random fields of tumor sections at ×10 magnification, and the number of grid intersections that overlaid stained vascular structures was counted. Twenty fields were counted per tumor. Data were expressed as the relative area of blood vessels within tumor parenchyma.

For all cases, the images were collected (Digital camera DXM1200F, Nikon) and were analyzed using the software Eclipse Net for NIKON cameras version 1.16.3.

RESULTS

Recombinant endostatin production

The mouse endostatin cDNA containing the sequences for murine Ig k-chain V-J2-C signal peptide was inserted into LXSN plasmid resulting in the retroviral vector designated LendSN. These vectors were used to transfect packaging cell lines, the supernatants of which were then used to infect NIH/3T3 cells. The supernatants of one clone infected with the LXSN retrovirus (negative control, Fig. 1, lane 1) and of three clones derived from the cells infected with LendSN retrovirus (NIH/3T3-LendSN-3; NIH/3T3-LendSN-2 and NIH/3T3-LendSN-1, Fig. 1, lanes 2–4, respectively) were collected and subjected to Western blot analysis using the anti-mouse endostatin antibody. As shown in Fig. 1, the bands of recombinant endostatin produced by the engineered cells displayed the expected apparent molecular weight, compatible with mouse endostatin (Fig. 1, lane 5). The amount of endostatin secreted by each clone was quantified using an ELISA assay (Table 1), which confirmed the notion that NIH/3T3-LendSN-clone 3 was the best endostatin producer, yielding ~7–8 times more endostatin than the other two clones. This clone, rated as a good producer as compared to

Figure 1. Endostatin produced by engineered NIH/3T3 cells is secreted to medium. Conditioned media from NIH/3T3 cells either infected with a control retrovirus-coding retrovirus were analyzed regarding presence of endostatin using Western blots. Protein secreted by NIH/3T3 LendSN clones 1, 2, and 3 displayed same apparent molecular mass of murine endostatin used as standard, suggesting the molecule produced by different clones was not further processed or modified. Molecular weight markers were separated in first lane.
other retrovirus-infected cells (32), was then used for the in vivo experiments of this study.

ES produced by NIH/3T3 LendSN clones inhibited human endothelial cell but not NIH/3T3 cell proliferation

Whereas the proliferation of the distinct NIH/3T3 clones was not altered by the presence of secreted endostatin in the conditioned media, as observed by the growth curves of all four lines generated in this study (Fig. 2A), addition of endostatin-containing media to HUVEC led to a dose-dependent growth inhibition, as determined using the MTT assay (Fig. 2B). Conditioned media of NIH/3T3 LendSN-clone 3 were more potent than the conditioned media of the other two clones, as indicated in Fig. 2B. Inhibitory rates were from 40–75% and could be due to either increased cell death or arrested endothelial cell growth in the presence of exogenous endostatin. These results demonstrated that ES produced by the NIH/3T3-LendSN clones specifically inhibited the proliferation of endothelial cells.

ES induces HUVEC death

To determine if the inhibitory ES effect on HUVEC proliferation was due to increased cell death, we incubated the endothelial cells with increasing concentrations of ES for 72 h and determined the fraction of cells with condensed chromatin using Hoechst 33342 staining. After this time period, up to 25% of the cells presented nuclear alterations compatible with apoptotic death (Fig. 3). As apoptotic cell death is asynchronous, we concluded that most of the effects observed using the MTT assay was likely due to increased cell death of ES-treated endothelial cells.

In vivo evaluation of tumor engraftment and ES treatment

SCID mice were injected subcutaneously with human Caki-cells. Tumors reached 100 mm³ after 9 days of xenografting. Serum levels of endogenous endostatin were measured every 4 days. In the first 9 days of the experiment, the presence of the tumor cells led to a 3-fold increase on endostatin levels. Regardless the endogenous production of endostatin, Caki tumors formed and developed to a clinically detectable level. At the ninth day, SCID mice were divided in two groups, part of them received a subcutaneous inoculum of $3.6 \times 10^6$ NIH/3T3-LXSN cells, while the others were injected with equal amount of NIH/3T3-LendSN clone 3 cells. As shown in Fig. 4, tumors from the former group grew steadily, increasing their volume 124.4% in 2 wk, while tumors in the later group displayed a significant regression (decrease of 59.2% in volume). Tumor regression was accompanied by a

### Table 1. Quantitative analysis of ES secretion by 3 selected NIH/3T3 LendSN clones

<table>
<thead>
<tr>
<th>CLONES</th>
<th>Endostatin levels (µg/ ml)</th>
</tr>
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<tbody>
<tr>
<td>NIH/3T3 LendSN –Clone 1</td>
<td>0.181</td>
</tr>
<tr>
<td>NIH/3T3 LendSN –Clone 2</td>
<td>0.252</td>
</tr>
<tr>
<td>NIH/3T3 LendSN –Clone 3</td>
<td>1.353</td>
</tr>
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ES concentration in conditioned media of 3 indicated clones was determined using a commercial ELISA assay.

Figure 2. ES produced by NIH/3T3 cells was an effective inhibitor of human endothelial, but not murine, fibroblast cell proliferation. A) Growth curves of all 4 retrovirally engineered NIH/3T3 cells produced in this study showed that secreted endostatin did not interfere with murine fibroblast proliferation, since all curves were not significantly different. On the other hand, on evaluating proliferation of HUVEC in the presence of supernatant media from NIH/3T3 LendSN cells using MTT assay (B), it was possible to show an effective impairment of endothelial cell proliferation in endostosing-containing medium. Condition medium of NIH/3T3 LendSN clone 3, which contained 7–8 times higher amount of endostatin inhibited cell proliferation to a greater extent than conditioned media of other 2 clones. Assay was performed in 96-well plate with $5.0 \times 10^5$ HUVEC/well (*$P<0.0001$).
sustained increase in serum endostatin levels, which reached a plateau 2 wk after inoculation of the engineered NIH/3T3 cells. In control animals, endostatin serum levels fluctuated around 100–150 ng/ml in the time course of the experiment.

**Histological analysis**

Analysis of the Caki-cell-induced tumors at the end of the experiments was performed to evaluate the efficacy of ES treatment on angiogenesis and to explore the possible causes of tumor regression. Microvascular density was measured using the animal lectin galectin-3 fused with bacterial alkaline phosphatase. Conventional markers for angiogenesis, such as anti-CD34 antibodies, were not useful to stain Caki-cells, due to high background (data not shown). As indicated in Fig. 5A–C, the microvascular density decreased ~3-fold in the tumors grown in NIH/3T3 LendSN clone 3-injected animals. Such a decrease in microvascular density was accompanied by an extremely significant increase in the overall necrotic areas found in Caki tumors (Fig. 5D–F), as evaluated by HE staining of the tumors. Dense tumor infiltrates were also observed in HE staining, as shown in Fig. 5E. Analysis of apoptotic cells in situ, using antiactivated caspase-3 antibodies (Fig. 5G–I), indicated a significant increase in caspase-3 activated cells within tumors of the NIH/3T3-LendSN treated animals. Quantitative analysis of these sections showed 9.84% cells expressing activated caspase-3 in the NIH/3T3-LendSN treated groups as compared with 3.37% in the control group. Remarkably, not all areas stained homogeneously for activated caspase-3. Indeed, as illustrated in Fig. 5H, staining for caspase-3 was only observed in areas of leukocyte infiltration. No specific staining for activated caspase-3 was observed within necrotic areas. The immunohistochemical analysis suggests that besides necrosis, usually related to insufficient vascularization or angiogenesis, the alterations within the tumor microenvironme, marked by dense leukocyte infiltration, are followed by apoptosis of the tumor mass. Altogether, these changes may be related to tumor regression.

**DISCUSSION**

Despite advances in multimodality therapy, the 5 yr survival rate for advanced renal cancer is <10%, mainly owing to the failure of systemic therapy to control disseminated disease (33). Antiangiogenic therapies are attractive as potential treatments for renal cancer because previous studies have shown a relationship between tumor angiogenesis and tumor aggressiveness (34).

Endostatin, a 20 kDa C terminal fragment of collagen XVIII is an endogenous inhibitor of angiogenesis. (35–37). This protein fragment has been characterized originally as a product of hemangioendothelioma cells and has been shown to potently inhibit endothelial cell proliferation in vitro and tumor growth in vivo (38). Tumor-bearing patients may have high levels of circulating endostatin, as it is the case for patients with advanced stages of clear cell renal cancer (39). Production of endostatin is dependent on the action of tumor infiltrating macrophages that secrete elastases, which in turn act on collagen XVIII, leading to the release of a bioactive protein fragment (40). Cytokines, such as the proinflammatory TNF-α, interfere with the yield of endostatin production (41). It is still not clear whether all circulating endogenous endostatin is functional. On the other hand, it has been shown that recombinant, and thus exogenous, endostatin can be successfully used to control the growth of renal cell carcinoma in vivo (36). Given its mechanisms of action, it is thought though that a large quantity of endostatin would be necessary for cancer therapy. Gene therapy would seem to solve this problem. Recently, many researchers have investigated the potential of transduction of cDNA
encoding endostatin as a novel therapeutic strategy for several kinds of tumors, including hepatic, lung, breast cancer, and kidney (42–46). Intratumoral administration of a naked DNA plasmid encoding mouse endostatin was efficient in the control of renal carcinoma growth (47). To evaluate the potential of endostatin gene therapy for renal cancer, we have chosen a human renal cancer cell line, Caki-1, which generates palpable tumors within 10 days after implanted subcutaneously in SCID mice. In this model, circulating endostatin levels rose up to 100–150 ng/ml when tumors develop. Endogenous endostatin did not control tumor growth. On the other hand, we have engineered NIH/3T3 cells to produce the endostatin fragment independently of proteolytic activation (recombinant endostatin). A clone that yielded very good expression levels (\(10^6\) cells in 24 h) of endostatin in vitro was implanted in tumor-bearing animals, inducing a sustained increase in circulating levels of endostatin reaching \(>350\) ng/ml. A rapid regression in implanted tumors exposed to exogenous endostatin was observed just 4 days after inoculation of LendSN-cells, and at the end of the assay it was observed a tumor volume reduction of \(~50\%\) (Fig. 4). In keeping with the apoptogenic effects on HUVECs, recombinant endostatin displayed antiangiogenic properties, as shown by the significant decrease in the microvascular density within the tumor. Although it is possible that the endogenous endostatin was not simply enough to control tumor growth, it is conceivable that endostatin produced in a different tissue context, such as the noninflammatory microenvironment of the implanted NIH/3T3 cells, would be more active than the endogenously produced endostatin. This would explain the relative inefficiency of the high levels of endostatin observed in sera of patients with advanced stage of renal carcinomas (48).

In this study, attempts to use classical angiogenesis markers, such as CD34, failed due to increased cross-reactivity of the anti-CD34 mAb with the tumor cells themselves. Alternatively, we made use of the animal lectin galectin-3 as a marker of angiogenesis. It is well established that extracellular galectin-3 induces angiogenesis (49–50). As a lectin, galectin-3 exerts its function through binding to glycans expressed on the surface of the endothelial cells. Although the galectin-3 ligands involved in angiogenesis are still largely unknown, galectin-3 proved to be a useful marker of vasculature structures within tumors and allowed us to determine the microvascular density within the tumor microenvironment. The functional consequence of the decrease in microvascular density was a massive necrosis found in the whole tumor tissue. Necrosis was at least in part responsible for the endostatin-mediated tumor regression. Besides necrosis, tumors of the treated group also displayed foci of apoptotic cells, which were associated with leukocyte infiltration. Although previous studies showed that endostatin can selectively induce apoptosis of tumor endothelial cells in vitro and in animals, depriving the tumor of oxygen and nutrients, resulting in tumor cell death (51–54), the histological analysis suggest an alternative scenario for this finding that may be relevant to the process of tumor regression.

It is not possible to ascertain if endothelial cells are the ones, and the only ones, that bear activated caspase-3 within the tumor microenvironment of endostatin-treated animals. Indeed the association of apoptosis with leukocyte infiltration suggests that either leukocytes or tumor cells are undergoing apoptosis. Using a comprehensive approach, van Wijngaarden et al. (55) identified genes that are differentially expressed in renal cell carcinomas after the treatment with endostatin. Granulocyte-specific genes were among the genes that had greater variation on endostatin treatment, suggesting that alterations in the activation status of granulocytes accompanied the microenvironmental changes observed (55). More recently, it has been shown that endostatin may interfere with
endothelial cell anergy, leading to increased leukocyte-endothelium interactions that promote leukocyte infiltration within tumors (56). As we have used an immunodeficient model system, tumor regression was likely dependent on elements of the innate immune system. Studies on immunocompetent mice are now underway. Regardless, our results indicate that besides the classical antiangiogenic effect of endostatin, there may be a second endostatin-dependent pathway that interferes with the recruitment of leukocytes to renal tumors. Both effects combined led to tumor regression. The notion that antiangiogenic therapy with endostatin may also serve as a potent adjuvant to immunotherapy is promising and deserves further studies.

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Figure 5. ES left to marked changes within tumor microenvironment. Histological analyses of parameters of tumor microenvironment, such as microvascular density (A–C), necrosis (D–F) and presence of apoptotic cells, evaluated by activation of caspase-3 (H–I) were determined using lectin histochemistry with a chimeric galectin-3 (A, B), HE staining (D, E), and immunohistochemistry with appropriate antibodies (G, H). Quantitative analysis of these parameters comparing tumors of control group (A, D, and G) with tumors of NIH/3T3 LendSN treated animals (B, E, and H) was done by morphometry. Bars in C, F, and I represent mean and se of each parameter. Both a significant decrease in microvascular density and significant increase in necrotic area and in foci of apoptotic cells were observed in animals that received inoculum of endostatin producing NIH/3T3 cells. *P < 0.001, P < 0.005, Student’s t test (scale bar =×40 magnification).

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