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Effect of Vascular Endothelial Growth Factor Inhibition on Endometrial Implant Development in a Murine Model of Endometriosis

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Abstract

The main factor involved in neovascularization of ectopic endometrial tissue in endometriosis is the vascular endothelial growth factor (VEGF), which is produced both by the endometrial implant and by peritoneal macrophages. On the other hand, bevacizumab is an antiangiogenic agent used in the treatment of different tumors, like colorectal, pulmonary, and recently mammary. We evaluated the effect of the inhibition of VEGF activity with bevacizumab (Avastin) on ectopic endometrial growth in a murine model of endometriosis. Two months old female BALB/c mice had surgery performed to induce endometriotic-like lesions. Treatment with bevacizumab started on post-surgery day 15 and continued during 2 weeks. Then, animals were sacrificed, peritoneal fluid was collected, and endometriotic-like lesions were counted, measured, and removed. Cell proliferation, vascular density, and apoptosis were assessed by immunohistochemistry for proliferating cell nuclear antigen (PCNA), immunohistochemistry for CD34, and Terminal Deoxynucleotidil Transferase-Mediated dUTP Nick End Labeling (TUNEL), respectively. Vascular endothelial growth factor levels were evaluated in the peritoneal fluid by enzyme-linked immunoassay (ELISA). Treatment with bevacizumab significantly inhibited endometriotic lesion development ($P < .05$). Consistently, bevacizumab significantly inhibited cell proliferation in lesions ($P < .01$), reduced vascular density ($P < .001$), as well as increased the apoptotic cell percentage ($P < .001$). In addition, bevacizumab reduced VEGF levels in peritoneal fluid of endometriosis-induced animals ($P < .05$). In conclusion, this study suggests a direct effect of bevacizumab on the reduction of endometrial implant growth and supports further research on VEGF inhibition as a novel therapeutic modality in endometriosis.

Keywords

endometriosis, bevacizumab, BALB/c mice, apoptosis, cell proliferation

Introduction

Endometriosis is one of the most common gynaecological diseases in women of reproductive age and is characterized by the implantation and growth of endometrial tissue within the abdominal cavity.[1] Its prevalence approaches 14% of the general population, but in women with pelvic pain, infertility or both, the frequency reaches 35% to 50%.[2] However, the real prevalence may even be higher, because the disease is often not diagnosed due to its heterogeneous clinical manifestation. Surgery commonly provides temporary relief, although endometriosis is characterized by a recurrence rate of 21.5% at 2 years and 40% to 45% at 5 years.[3]

As the disease is estrogen-dependent, medical therapies are principally aimed at downregulating the ovarian estrogen production using GnRH agonists, progestins, androgenic agents, or oral contraceptives. However, side effects limit their long-term use.[4,5]

As observed in tumor growth, angiogenesis of the endometrial implant appears to be essential for its survival and development in an ectopic location.[6] Therefore, the use of angiostatic agents promises to provide a new therapeutic option for this pathological process.

Angiogenesis is a dynamic process involving several factors. Many studies have demonstrated that peritoneal fluid from patients with endometriosis contains significantly greater amounts of vascular endothelial growth factor (VEGF), one of

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the most potent angiogenic factors.[7] Furthermore, it has been observed that VEGF expression is increased in eutopic and ectopic endometrium from patients with endometriosis compared to control women.[8,9] Moreover, VEGF has been shown to be released by macrophages present in the peritoneal fluid of women with endometriosis.[10]

The relation between VEGF and pathological angiogenesis in endometriosis has been studied in nude mice and several experiments were performed with VEGF ligands[11] and other angiogenesis inhibitors.[12] In all cases, a significant decrease of the number of endometrial implants was reported.

Bevacizumab is a recombinant, humanized, monoclonal antibody targeting human VEGF. The antibody was engineered by assembling VEGF-A-binding residues from the murine-neutralizing antibody (MabA.4.6.1.) into a framework of a human immunoglobulin,[13] retaining similar ligand binding affinity to its mouse counterpart. Anti-VEGF therapy with bevacizumab has demonstrated efficacy in various human malignancies.[14] Bevacizumab was FDA-approved as a first-line treatment for patients with metastatic colorectal cancer.[15]

In clinical practice, women usually attend to the physician when the endometriotic lesions are already established. Hence, treatment options have to be developed targeting the inhibition of the maintenance and growth of established lesions. For this reason, and based on the data reviewed, the aim of the present study was to investigate the effect of an antiangiogenic therapy with bevacizumab on established endometriotic lesions experimentally induced in immunocompetent mice.

For this purpose, we evaluated lesion size, cell proliferation, vascular density, and apoptosis in the endometriotic lesions, as well as the release of VEGF into the peritoneal fluid.

Materials and Methods

Animals

In this study, 2-months-old female BALB/c mice were used. All procedures were performed according to NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Ethics and Research Committee from IBYME, Buenos Aires, Argentina.

Surgical Induction of Endometriosis

The induction of experimental endometriosis was carried out at random phases of the estrous cycle. Endometriotic-like lesions were induced through transplantation of one of the uterine horns to the bowel mesentery as previously described.[16] Briefly, animals were deeply anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Afterward, mice underwent laparotomy by midventral incision to expose the uterus and intestine. The right uterine horn was removed and placed in a Petri dish containing warmed DMEM-F12 (Gibco, Paisley, United Kingdom) supplemented with 100 IU/mL of penicillin and 100 µg/mL of streptomycin (Gibco). The uterine horn was opened longitudinally and then cut into square pieces measuring approximately 4 mm^2. Three equal pieces of tissue were then sutured onto serosal layer with a single 6-0 nylon suture (Supralon, Ethicon, Somerville, NJ). Endometrial tissue was sutured facing the serosa. The abdomen was then closed with a 5-0 nylon suture.

Anti-VEGF Treatment

Treatment was initiated 2 weeks after surgery and continued for 2 weeks. After surgery, animals were randomly assigned to 2 experimental groups: Bevacizumab ($n = 12$) and Control ($n = 12$). The first group received 5 mg/kg of bevacizumab (Avastin-Roche, Argentina) in saline intraperitoneally on post-surgery days 15, 18, 21, 24, and 27.[17] Control mice received an equal volume of saline solution intraperitoneally. Mice were monitored daily and no evidence of toxicity was noted based on body weight, food consumption, grooming behavior, or activity levels compared with controls.

Endometriotic Lesion Evaluation

After 2 weeks of treatment, animals were sacrificed by cervical dislocation. The abdomen was opened by ventral midline incision. Implantation sites were localized by the presence of a lesion or by a suture alone. Lesions were counted and measured in 2 perpendicular diameters ($d < D$) using a caliper. Lesion volumes were determined using the formula: $V = (4/3)\ \pi r^2 R$ (r and R are the radiuses, $r < R$).[18] Then lesions were excised and fixed in 10% formaldehyde for histological analysis. Formalin-fixed specimens were paraffin-embedded and cut into 5 µm serial sections. Several sections from each specimen were stained with hematoxylin-eosin and examined microscopically for the presence of histological hallmarks of endometriosis.

Immunohistochemistry

Serial sections of the endometriotic lesions were subjected to standard immunohistochemistry.[19] Tissue sections were incubated overnight with the primary antibody (rabbit anti-mouse Proliferating Cell Nuclear Antigen [PCNA] polyclonal, 1:300, FL-261, Santa Cruz Biotechnology, Santa Cruz, California; or rat anti-mouse CD34 monoclonal, 1:50, ab8158 Abcam, Cambridge, Massachusetts) at 4°C. After that, sections were treated for 60 min with the proper secondary biotinylated antibody (anti-rabbit IgG, 1:200, B7389 Sigma-Aldrich, Saint Louis, Missouri; or goat anti-rat IgG 1:500, B7139 Sigma-Aldrich) followed by incubation with streptavidin-peroxidase (LSAB+ System, Dako, Carpinteria, California). Binding was visualized by incubating sections with diaminobenzidine and lightly counterstaining with hematoxylin. As a negative control, immunoglobulin of the same immunoglobulin class and concentration as the primary antibodies was used.

Proliferating cell nuclear antigen−positive cells were identified by the presence of brown nuclear reactivity. A total of 300 epithelial cells were counted by 2 independent observers in 10 representative fields, considering all lesions, and the

percentage of PCNA-positive cells was established per mouse. All percentages were used to get the mean value per group. Any nuclear staining was regarded as positive.

Staining for CD34, a useful endothelial cell marker,[20] was assessed to study vascular density identifying blood vessels. The percentage of total area expressing immunoreactivity for CD34 was established by analyzing 10 representative fields from each slide containing all lesions with the Image J 1.33u software (NIH, USA). The CD34-positive area was calculated per field and then averaged per mouse. All percentages were used to get the mean value per group.

Apoptosis Detection System

For apoptosis quantification, sections were processed for in situ immunolocalization of nuclei exhibiting DNA fragmentation, by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP digoxygenin nick-end labeling (TUNEL) technique, using the apoptosis detection kit Apoptag Plus (Chemicon International, Temecula, California). Sections were treated according to the manufacturer's instructions as previously described.[19] The positive control was provided by the supplier of the kit. As a negative control, a number of tissue samples were subjected to treatment without TdT. A total of 300 epithelial cells were counted by 2 independent observers in 10 representative fields, considering all lesions, and the percentage of TUNEL-positive cells was established per mouse. All percentages were used to get the mean value per group.

Quantification of VEGF

After 2 weeks of treatment, animals were sacrificed by cervical dislocation. Peritoneal fluid was collected by rinsing the abdominal cavity with 1.5 mL of saline and centrifuged at 4°C during 10 minutes at 1500 rpm. Supernatants were stored at −20°C until assayed for VEGF using a commercial enzyme-linked immunoassay (ELISA) kit according to the manufacturer's instructions (MMV00, R&D Systems Inc., Minneapolis). The sensitivity level for the VEGF ELISA was 3 pg/mL. The intra-assay variability for VEGF was ±4.3%, while the inter-assay variability was ±5.7%. All samples were assessed in triplicate.

Statistics

Statistical analyses were performed using GraphPad PRISM Software V4.0, (GraphPad Software Inc., San Diego, California). Statistical comparisons between 2 groups were performed using Student *t*-test, with Welch correction in groups with significantly different variances. Apoptotic and PCNA-positive cells were counted blindly by 2 independent observers. Results were expressed as mean ± SEM. In all cases, statistical significance was considered to be $P < .05$.

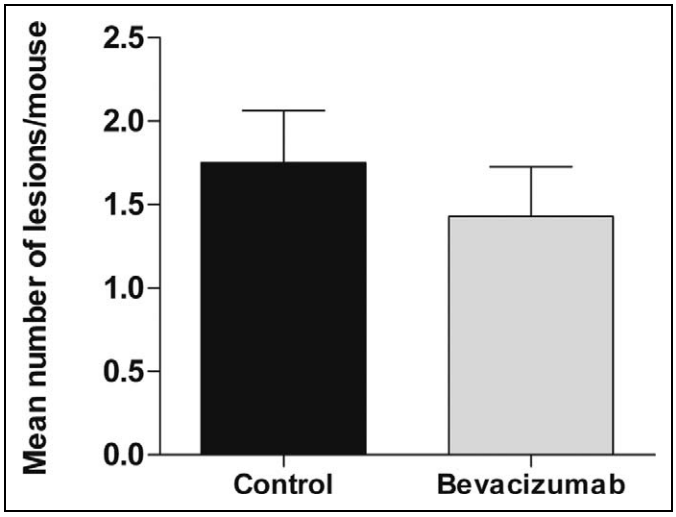

Figure 1. Developed endometriotic lesions. Mice with surgical induced endometriosis were treated with saline (control, n = 12) or bevacizumab (n = 12) starting on post-surgery day 15 and following the next 2 weeks. The number of established lesions (> 0 mm^3) per mouse was calculated for each treatment group (mean ± S.E.M).

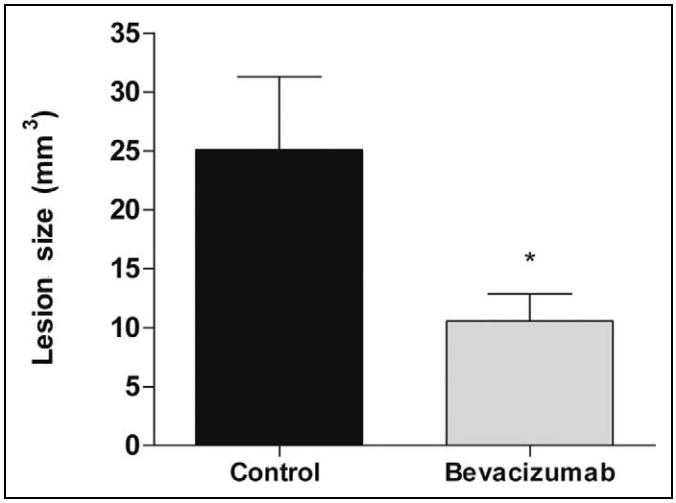

Figure 2. Endometriotic lesion size. Mice with surgical induced endometriosis were treated with saline (control, n = 12) or bevacizumab (n = 12) starting on post-surgery day 15. After 2 weeks of treatment, mice were sacrificed and endometriotic lesions were measured with a caliper. Each volume was calculated with the formula $V = (4/3).\pi.r^2.R$. Volume values of all developed lesions per mouse were averaged and mean ± S.E.M. was calculated for each treatment group. * $p < 0.05$ vs. Control.

Results

Effect of Bevacizumab on Endometriotic Lesion Growth

Treatment with either bevacizumab or saline via intraperitoneal injection was initiated 2 weeks after surgery. Lesions were counted and measured after another 2 weeks of treatment at necropsy. The number of established lesions observed per mouse was not affected by bevacizumab treatment ($P > .05$; Figure 1), since we started the treatment once lesions were

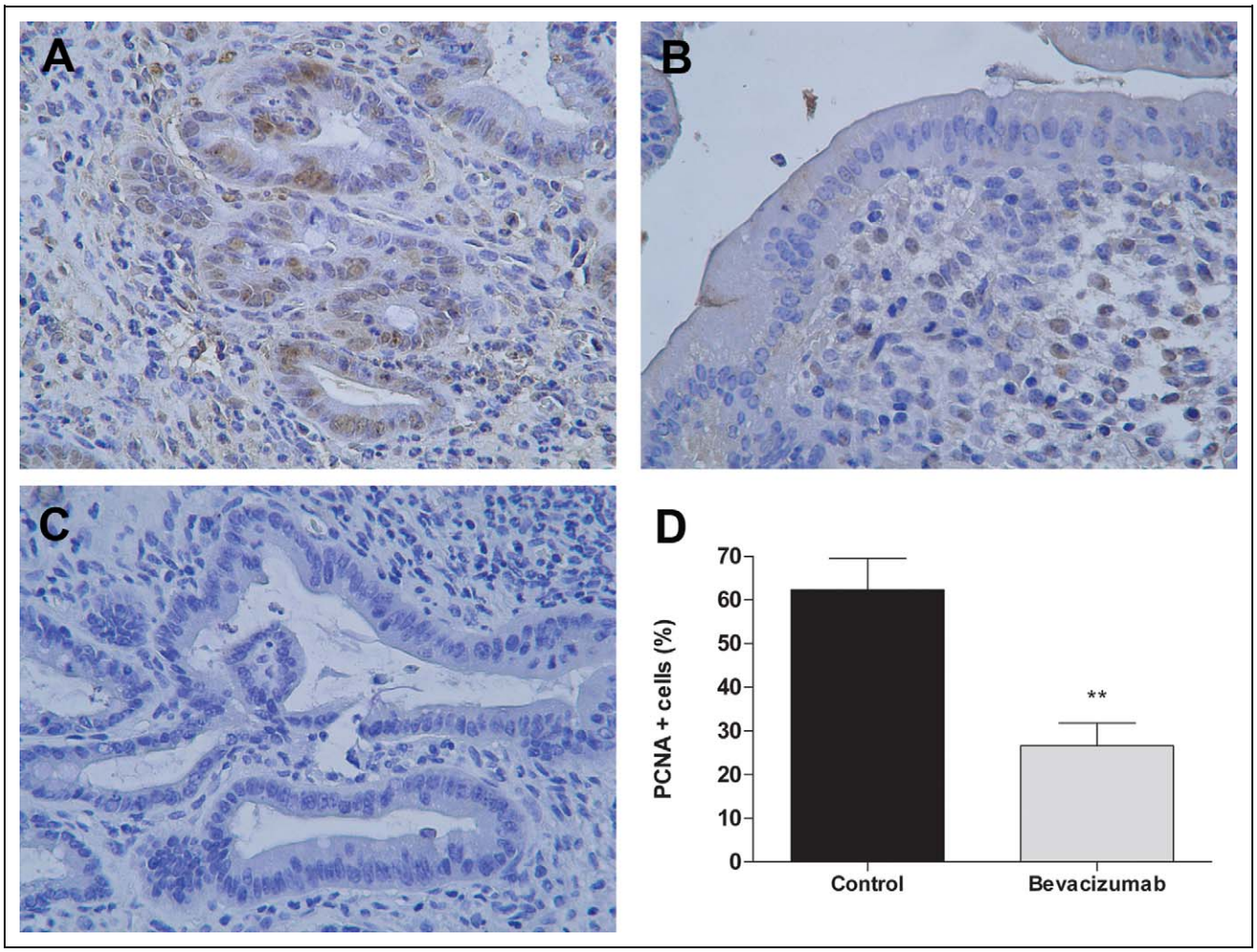

Figure 3. PCNA expression in endometriotic lesions. Mice with surgical induced endometriosis were treated with saline (A, n = 12) or bevacizumab (B, n = 12) starting on post-surgery day 15. After 2 weeks of treatment mice were sacrificed and endometriotic lesions were dissected away, formalin fixed, paraffin-embedded and cut into sections. Cell proliferation was evaluated by immunohistochemistry for PCNA. Control group shows a significantly higher percentage of PCNA positive cells than Bevacizumab. In negative control (C) an immunoglobulin of the same immunoglobulin class and concentration as the primary antibody was used. Cell proliferation was quantified in the epithelial fraction as percentage of PCNA positive cells (D). ** $p < 0.01$ vs. Control. Magnification: 400×.

established. However, size measurement of established lesions in each group revealed that bevacizumab significantly diminished its volume compared with vehicle-treated mice. The lesion size was 25.1 ± 6.2 mm^3 in control group and 10.6 ± 2.3 mm^3 in bevacizumab group ($P < .05$ vs control; Figure 2).

Effect of Bevacizumab on Cell Proliferation

After treatment with bevacizumab, mice were sacrificed and endometriotic lesions were dissected away, formalin fixed, paraffin-embedded, and cut into sections. Cell proliferation was evaluated by immunohistochemistry for PCNA (Figure 3A-C). Treatment with bevacizumab caused a decrease in epithelial cell proliferation compared to control group. The percentage of PCNA-positive cells was 62.3% ± 7.2% in the control group and it was decreased to 26.6% ± 5.2% after treatment with bevacizumab ($P < .01$ vs control; Figure 3D).

Effect of Bevacizumab on Apoptosis

Complementary to the observed results for cell proliferation, treatment with bevacizumab increased the apoptotic index in epithelial cells of endometriotic-like lesions (Figure 4A-C), as assessed by TUNEL technique. The percentage of apoptotic cells was 2.2% ± 0.8% in the control group and 12.1% ± 1.4% in the lesions from mice treated with bevacizumab ($P < .001$ vs control; Figure 4D).

Effect of Bevacizumab on Vascular Density

To determine vascular density, blood vessels in endometriotic lesions were immunostained with CD34 antibody, and the

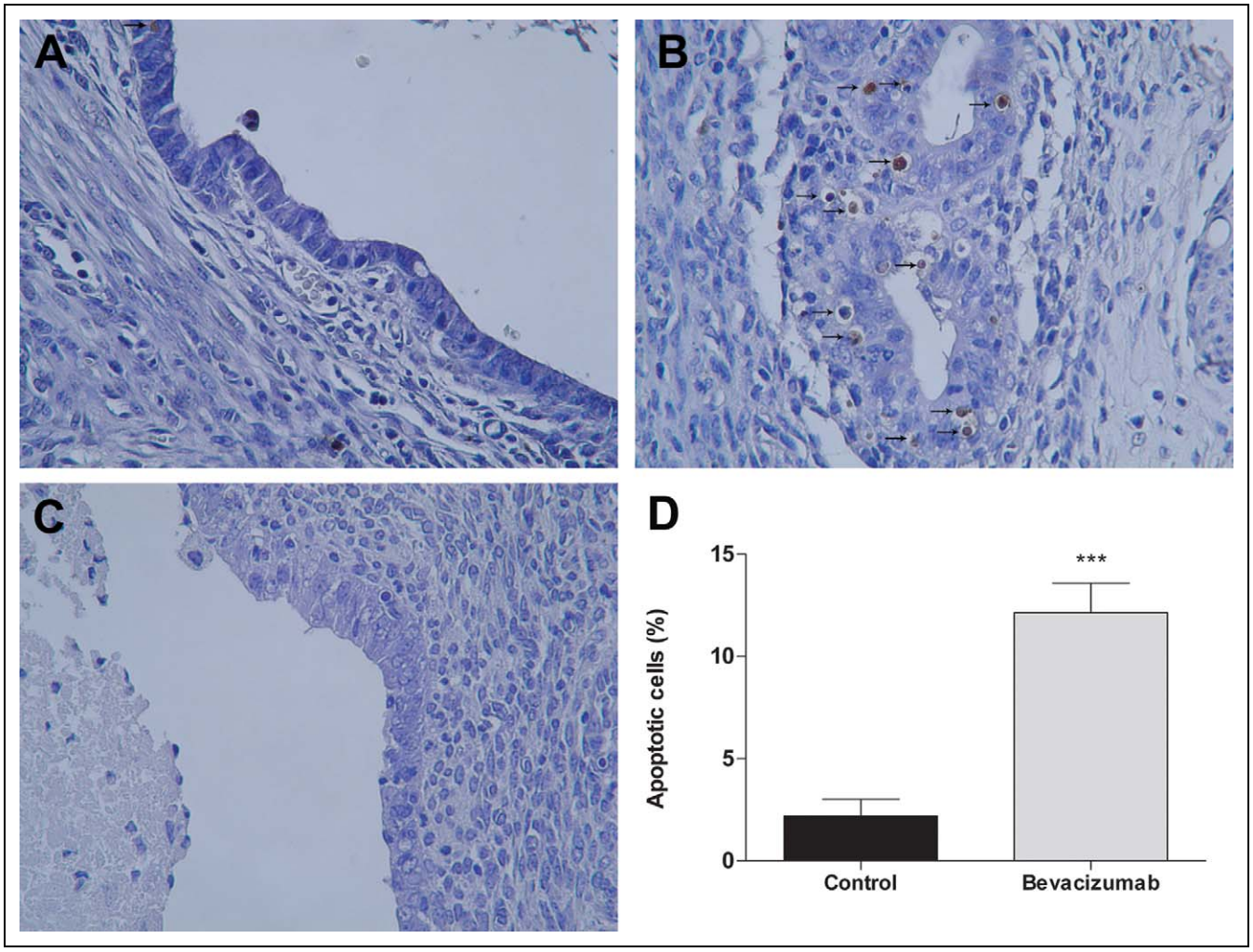

Figure 4. Apoptosis in endometriotic lesions. Mice with surgical induced endometriosis were treated with saline (A, n = 12) or bevacizumab (B, n = 12) starting on post-surgery day 15. After 2 weeks of treatment mice were sacrificed and endometriotic lesions were dissected away, formalin fixed, paraffin-embedded and cut into sections. Apoptosis was assessed by TUNEL technique. Arrow heads indicate apoptotic cells in the epithelial fraction. Control group presents a significantly lower percentage of apoptotic cells than Bevacizumab. In negative control (C) sections were incubated in absence of TdT. Apoptosis was quantified in the epithelial fraction as percentage of apoptotic cells (D). *** $p < 0.001$ vs. Control. Magnification: 400×.

percentage of vascular area was quantified (Figure 5A-C). Treatment effect was clearly associated with an important decrease in vascular density. The percentage of vascular area was 12.42% ± 1.02% in the control group and 2.06% ± 0.44% in mice treated with bevacizumab ($P < .001$ vs control; Figure 5D).

Effect of Bevacizumab on VEGF Levels

After treatment with bevacizumab, mice were sacrificed, peritoneal fluid was collected, and levels of VEGF were assessed by ELISA.

Treatment with bevacizumab decreased VEGF levels in the peritoneal fluid (Figure 6). The concentration of VEGF was 13.1 ± 3.8 pg/mL in control group and 2.6 ± 0.5 pg/mL in mice treated with bevacizumab ($P < .05$ vs control).

Discussion

Current medical therapies for endometriosis are aimed at surgical removal of endometriotic lesions and at suppression of ovarian steroid production, but the outcome is still highly unsatisfactory because of the high recurrence rate of symptoms and the adverse effect profiles of the different treatments.[4,21]

Angiogenesis is essential for the pathogenesis of many diseases such as cancer, atherosclerosis, chronic inflammation, and endometriosis.[22] It has been shown that endometriotic lesions are highly vascularized and it is widely accepted that angiogenesis is critical for their establishment and growth in ectopic sites.[23,24]

Extensive studies performed over the last decade have established the critical role of VEGF in the regulation of physiological and pathological angiogenesis.[25] Various methods of inhibiting vascularization in the metastatic cascade of cancer

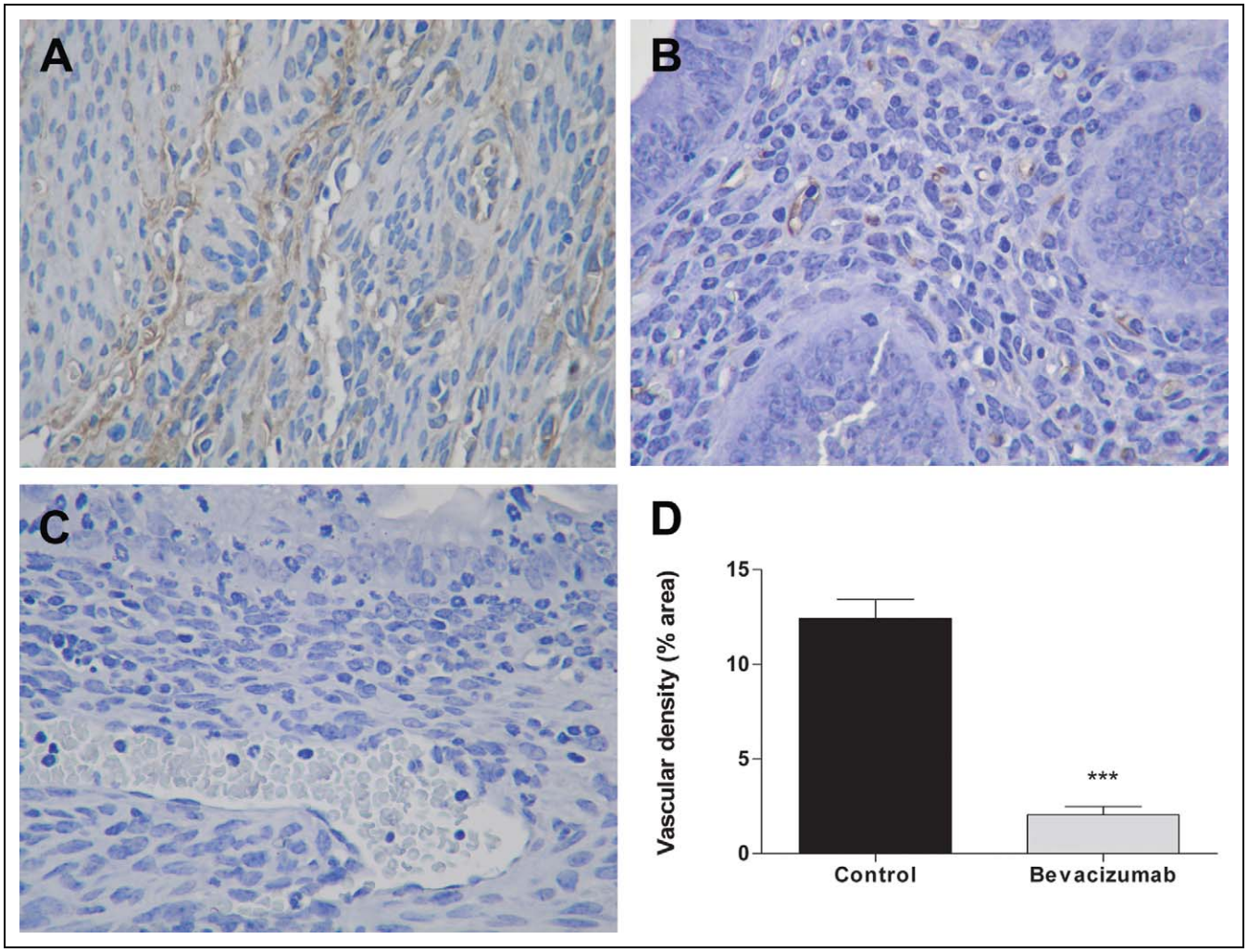

Figure 5. Vascular density in endometriotic lesions. Mice with surgical induced endometriosis were treated with saline (A, n = 12) or bevacizumab (B, n = 12) starting on post-surgery day 15. After 2 weeks of treatment mice were sacrificed and endometriotic lesions were dissected away, formalin fixed, paraffin-embedded and cut into sections. Control group shows a significantly higher vascular density than Bevacizumab. In negative control (C) an immunoglobulin of the same immunoglobulin class and concentration as the primary antibody was used. Vessels were identified using CD34 staining of vascular endothelium and quantified as percentage area (D). *** $p < 0.001$ vs. Control. Magnification: 400×.

have been examined with encouraging results.[26-29] Vascular endothelial growth factor is probably the most studied angiogenic factor and it has been demonstrated that it is involved in the pathophysiology of endometriosis. Hence, the suppression of blood vessel development by inhibition of VEGF may be a novel therapeutic approach for the treatment of endometriosis.[11,12,30,31]

Several studies have assessed the effect of numerous inhibitory agents, such as SU5416, SU6668, TNP-470, endostatin, anginex, and cabergoline,[12,30,32,33] with different results. As mentioned earlier, most of these studies have been developed in nude mice in which human endometrium were implanted. In the model used in the present study, one uterus horn is autotransplanted in the peritoneal cavity. As we know, the limitations of murine models are the obvious lack of menstruation and subsequent spontaneous endometriosis. Additionally, the use of entire uterus including myometrium in this autologue suture model does not exactly mimic the situation of human endometriotic lesions. Despite these limitations, considering that immune system is of pivotal importance in the pathogenesis of endometriosis,[34-36] we decided to study the antiangiogenic therapy in a widely accepted experimental endometriosis model using BALB/c mice.[16]

The aim of the present study was to confirm whether the maintenance and growth of endometriotic lesions depend on angiogenesis and whether angiogenesis inhibition could be a therapeutic approach for endometriosis treatment. For this purpose, we evaluated the effect of an antiangiogenic therapy with bevacizumab during the development of established endometriotic lesions. We found that the treatment with bevacizumab inhibited endometriotic lesion development by diminishing cell proliferation and increasing apoptotic levels. In addition, we demonstrated that this treatment decreased VEGF levels in peritoneal fluid and consequently decreased vascular density in the lesions.

In this work, we failed to observe a significant effect of bevacizumab on the number of endometriotic lesions developed

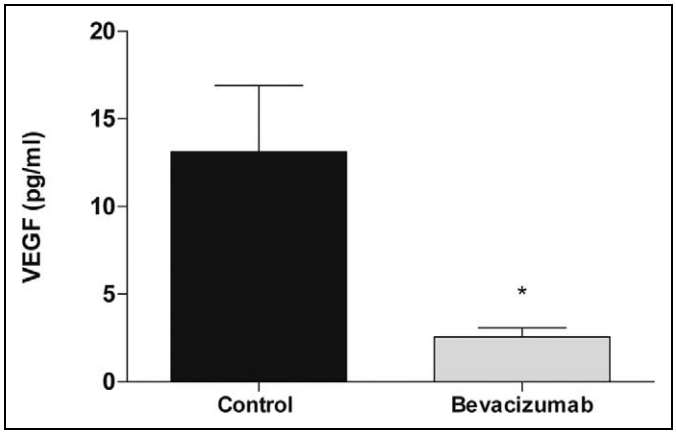

Figure 6. VEGF peritoneal fluid levels. Mice with surgical induced endometriosis were treated with saline (control, n = 12) or bevacizumab (n = 12) starting on post-surgery day 15. After 2 weeks of treatment mice were sacrificed and the peritoneal fluid was collected. Levels of VEGF were assessed by ELISA. * $p < 0.05$ vs. Control.

per mouse. Nevertheless, in a nude mouse model Nap et al found that some angiostatic agents significantly reduced the number of endometriotic lesions,[12] even though they initiated treatment 3 weeks after surgery. However, the size of the endometriotic lesions or the mechanisms by which the number of lesions was reduced were not assessed.

On the other hand, Laschke et al demonstrated that vascularization of endometriotic lesions in hamsters was significantly downregulated by combined inhibition of VEGF, fibroblast growth factor, and platelet-derived growth factor, but not by antagonizing VEGF alone.[30] Moreover, the authors did not observe a significant reduction of the graft size with either treatment. However, in our model, we found that the number of blood vessels and the size of endometriotic lesions in bevacizumab group were significantly reduced compared to the control group; this suggests that antiangiogenic and antiproliferative effects may result from blocking VEGF.

It has been recently shown that VEGF protects human endothelial and epithelial endometrial cells from apoptosis.[37,38] In addition, inhibition of VEGF expression induces apoptosis and reduces cell proliferation in several models, both in vitro and in vivo.[39,40] In agreement with these observations, in our work, we found that the antiangiogenic treatment increases apoptotic levels and reduces cell proliferation in the epithelial fraction of the endometriotic lesion. This could be suggesting a possible mechanism for the reduction of the endometriotic lesion size observed.

Recently, the inhibitory and neutralizing properties of bevacizumab against VEGF was successfully demonstrated in a variety of in vitro models.[41] Although Ferrara et al reported that bevacizumab binds to and neutralizes all human VEGF-A isoforms, but not murine or rat VEGF,[15] other groups have demonstrated that bevacizumab binds to murine VEGF, albeit with low affinity.[17] This topic is controversial and many studies have been developed to clarify the antibody affinity.[17,42] In the present study, we were able to support Bock et al[17] by finding that bevacizumab binds to murine VEGF, since we found a significant effect of bevacizumab on the inhibition of endometriotic lesion growth in the BALB/c endometriosis model. Moreover, Nakamura et al exposed that human VEGF-A shows a high degree of homology to mouse VEGF-A.[43]

The ability of bevacizumab to inhibit angiogenesis has been assessed in oncology patients with success.[15,44] In this work, we could demonstrate its effectiveness in endometriosis and it leads to the idea of a new potential treatment. However, it is important to clarify potential adverse events that can occasionally occur, such as proteinuria, hypertension, or hemorrhages.[45]

In summary, our work confirms that angiogenesis is a prerequisite for the maintenance and growth of endometriosis and that antiangiogenic therapy effectively inhibits the development of endometriotic lesions in mice.

In spite of recent advances in this field, there is still limited knowledge about the mechanisms regulating the complex dynamic process of blood vessel development in endometriotic lesions.[46] Further investigation is necessary to evaluate whether bevacizumab could be a useful drug and to find the accurate antiangiogenic compound to treat women who suffer from endometriosis.

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Declaration of Conflicting Interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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