Treatment with the Angiogenesis Inhibitor Endostatin: A Novel Therapy in Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** An endostatin that inhibits angiogenesis dependent tumor growth is being tested as an antitumor agent. The neoangiogenesis condition of cancer is essentially identical to that of rheumatoid arthritis (RA). Thus antiangiogenic treatment has potential for treatment of RA. We investigated the effects of human recombinant endostatin on human RA synovial tissue by use of a novel model of RA, in which human RA tissue is grafted into SCID mice (SCID-HuRAg).

Methods. Ten or 50 mg/kg of human recombinant endostatin was administered by percutaneous direct intrasynovial injection in each of 7 SCID-HuRAg mice. We examined the volume of the grafted tissue mass and the histological changes 7 days after endostatin administration. Six control mice received phosphate buffered saline in the same manner.

Results. The grafted synovial volume of SCID-HuRAg mice was significantly decreased by endostatin administration. The number of inflammatory cells (macrophages and lymphocytes) was also significantly reduced in a dose dependent manner. The number of vessels that were counted by von Willebrand factor VIII and type IV collagen positive cells was decreased, although apoptotic cells were increased in RA synovia.

Conclusion. The results suggest that antiangiogenesis treatment using endostatin represents a potential new therapeutic strategy for RA. (J Rheumatol 2002;29:890–5)

Key Indexing Terms:
ENDOSTATIN RHEUMATOID ARTHRITIS SCID-HuRAg MOUSE
ANGIOGENESIS APOPTOSIS TREATMENT

It is well known that tumors require ongoing angiogenesis to support their growth. Angiogenesis is necessary for a supply of nutrients and oxygen, but also provides a route for metastasis[1]. Therefore, inhibition of angiogenesis by angiostatic factors can be considered for cancer therapy[1,2]. Studies have shown that the angiostatic factor endostatin inhibits endothelial cell proliferation and migration *in vitro* and angiogenesis dependent tumor growth *in vivo*[3,4]. Endostatin, a 20 kDa C-terminal fragment of collagen XVIII, is currently being tested as an antitumor agent. The crystal structure of recombinant human endostatin has been described[5,6]. We recently produced and purified recombinant endostatin from human embryonic kidney cells expressing Epstein-Barr virus nuclear antigen-1 (293-EBNA)[6,7]. This novel endostatin possesses high biological activity and has a possibility for clinical application since it is a human recombinant form[8]. Thus human recombinant endostatin is anticipated to have efficacy as an antitumor agent.

The ability of endostatin to inhibit angiogenesis in tumors suggests that it may be useful for treatment of other pathological neoangiogenic conditions such as rheumatoid arthritis (RA). Exuberant proliferation of new blood vessels is observed in RA synovium and pathological angiogenesis is a crucial aspect of RA[9,10]. Moreover, a variety of angiogenic mediators, including cytokines and growth factors, have been identified in RA joints. The severity of collagen induced arthritis in rats used as an animal model of RA is improved following administration of an angiogenesis inhibitor[11]. Together, these data suggest that antiangiogenic therapy may be effective in treatment of RA. We investigated the effects of human recombinant endostatin on human RA synovial tissue using SCID mice in which human RA tissue has been grafted (SCID-HuRAg)[10,12,13].

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MATERIALS AND METHODS

Preparation of SCID-HuRAg mice and experimental design. The SCID-HuRAg mouse was evaluated as a model for this study. Twenty male SCID mice (CB.17/lcr; Japan Kurea, Tokyo, Japan), 6–7 weeks old, were engrafted subcutaneously with human RA synovial tissue on their backs in accord with our previous studies[10,12,13]. Rheumatoid synovial tissue was obtained from patients with RA at the time of joint surgery. Informed consent was obtained from each patient before surgery. To solve the problem of heterogeneity in individual samples, RA tissue samples for each examination were obtained from the same RA patient. Samples of the same size were implanted into mice. Six weeks after engraftment, mice were divided into 3 groups and treated as follows: Group A (n = 6) was treated with phosphate buffered saline (PBS) alone via percutaneous direct intrasynovial injection; Group B (n = 7) was treated with human endostatin (10 µg/kg) produced as a recombinant protein in 293-EBNA cells[6,7], in PBS; and Group C (n = 7) was treated with 50 µg/kg endostatin in the same manner.

After 7 days, mice were euthanized with methoxyflurane, and the grafted synovial tissues were removed for histological analysis. Synovial volume was measured with a dial-caliper, volumes were determined using the formula width^2 × length × 0.52, in accord with our report[5].

Histological analysis. To detect microvessels, synovial tissues were embedded in paraffin, sectioned (2 µm), and were immunostained with anti-human CD34 Mab (Nichirei Co., Tokyo, Japan), anti-human type IV collagen Mab (Dako, Hamburg, Germany), and anti-human von Willebrand factor VIII (vWF VIII) Mab (Dako) according to the avidin-biotin-peroxidase complex method, using the Vectastain ABC kit (Vector, Burlingame, CA, USA) as described[12,13]. Positive staining of the microvessels was confirmed when both an endothelial cell nucleus and lumen were seen at high power (×400). The vessel density was determined by counting the number of capillary blood vessels per high power field in sections.

To examine immunocytes in the synovial tissue, hematoxylin and eosin staining and immunohistochemical staining were performed as described[13]. The sections were stained with the following anti-human Mab: HLA-DR (LN-3; Nichirei Co.), CD4, leukocyte common antigen (Nichirei Co.), T cell (UCHL-1; Dako), B cell (L26; Dako), and macrophage (CD68; Dako). In addition, to identify the mouse tissue, samples were stained with the following anti-mouse Mab: mouse MHC class II (Southern Biotechnology Associates, Birmingham, AL, USA) and mouse blood vessels (CD34; PharMingen, San Diego, CA, USA). The number of positive staining cells per 300 cells was counted in each tissue sample. Apoptotic cells in the synovial tissue were detected utilizing the Apop Tag *in situ* apoptosis detection kit (Intergen Co., Gaithersburg, MD, USA) following the manufacturer's protocol. This technique is based on the enzymatic addition of digoxigenin-nucleotide to the nicked DNA by terminal deoxynucleotidyl transferase (TUNEL staining). The sections were also stained with anti-Fas (Medical & Biological Laboratories Co., Nagoya, Japan) or anti-Fas ligand (FasL) Mab (PharMingen) as described[10,11,14]. Apoptotic cells were counted under light microscopy at 400-fold magnification.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). For each variable, the numbers of positive cells in each tissue were compared between groups, using the Mann-Whitney U test. P values < 0.05 were considered significant.

RESULTS

Human blood vessels in grafted tissue. To investigate whether the human cells were preserved in the implanted tissues, we analyzed the grafted tissues for human and mouse cell markers using species-specific HLA-DR Mab and MHC class II Mab. Both human and mouse-specific cells were observed in implanted tissues 7 weeks post-transplantation (Figure 1A). These results were in agreement with our previous reports[12,13]. When we analyzed whether the human vasculature was preserved in the implanted tissues following transplantation, using blood vessel-specific Mab, both human and mouse blood vessels were observed in the grafted tissues. In some instances human blood vessels were intermingled with mouse blood vessels (Figure 1B). Our results showing the grafted human vessels directly connected to the murine vessels, were almost the same as the previous report. It has also been reported that human vessels in implanted tissue connect directly to mouse vessels and were functional for human vessels[15].

Reduction of rheumatoid synovial tissue volume after endostatin. Animal weights were monitored as a health indicator throughout this experiment. All the animals remained healthy and were available for analysis. The grafted synovial tissue volume of SCID-HuRAg mice was potently reduced by endostatin. As a result of endostatin treatment, the grafted synovial volume was significantly decreased at a dose of 10 µg/kg compared to control mice treated with PBS alone (Figures 2 and 3). At a dose of 50 µg/kg, further regression of synovial volume was observed (50 µg/kg group: 77.8% reduction compared to controls; 10 µg/kg group: 45.6% reduction compared to controls). Therefore, increasing doses of endostatin were associated with improved efficacy.

Inhibition of angiogenesis in synovial tissue. Histological analysis of the synovial tissue treated with endostatin indicated a potent inhibition of angiogenesis as well as reduction in cell infiltration (Figure 4). The density of microvessel stained with anti-type IV collagen was markedly reduced even at a dose of 10 µg/kg endostatin. Further inhibition of angiogenesis was evident at 50 µg/kg endostatin. Similarly, microvessel density determined by anti-vWF VIII staining was significantly reduced by the endostatin treatment (Table 1).

Endostatin reduced inflammatory cell infiltration in the synovial tissue. A large number of inflammatory cells including CD4+ T cells were observed in the rheumatoid synovial tissue of SCID-HuRAg mice. B cells located mainly in the lymphoid follicle, and macrophages infiltrated widely into the synovial tissue were also observed (Figure 5A). These histological features of implanted tissue in the SCID-HuRAg mouse appeared to be very similar to the features of synovial tissue in donor RA. Endostatin treatment significantly reduced the number of infiltrating leukocytes, as shown in Figures 4B and 4C. This reduction was seen not only in CD4+ T cells but also in B cells and macrophages (Figure 5 and Table 1).

TUNEL positive cells and Fas/FasL expression. TUNEL staining of the synovial tissue of the mice indicated the presence of apoptotic cells. Endostatin treated synovial tissue seemed to increase the number of TUNEL positive

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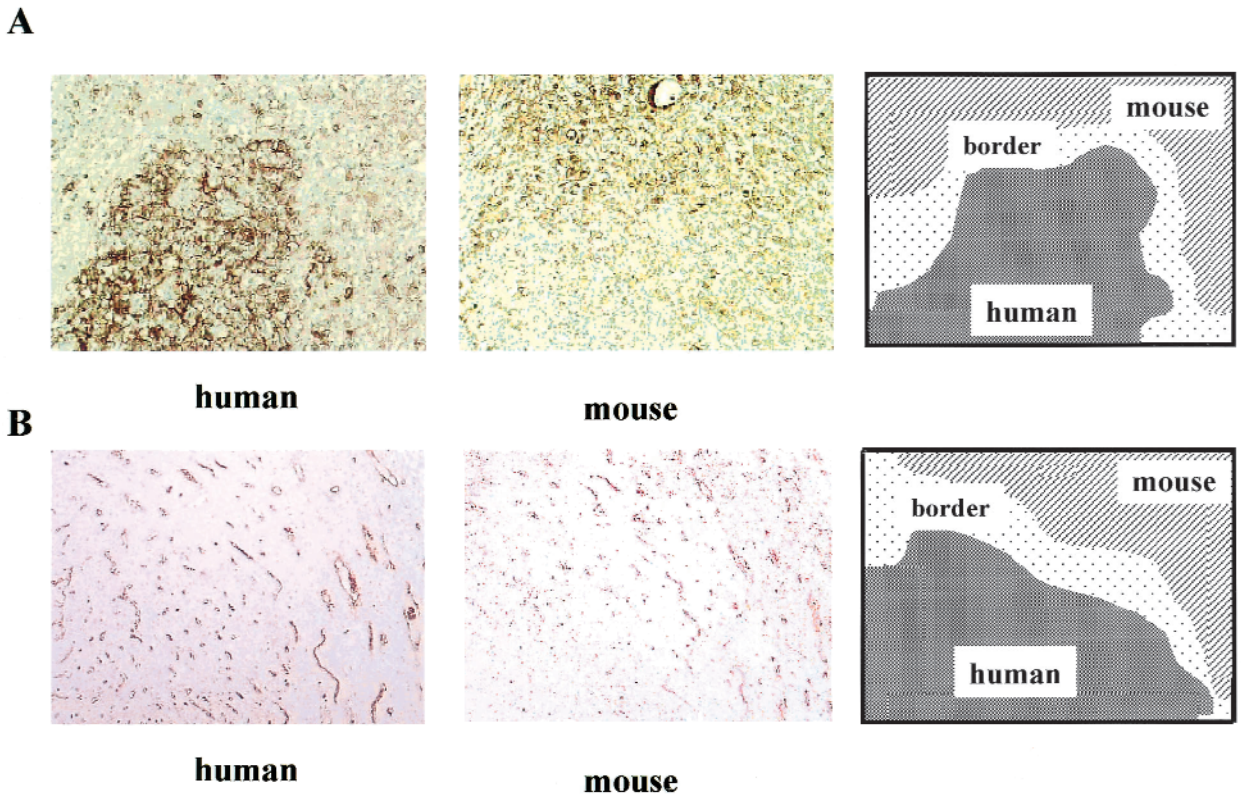

Figure 1. Human and mouse blood vessels in the implanted tissue after transplantation. A. Both human HLA-DR positive cells and mouse MHC class II positive cells were observed in the implanted tissues 7 weeks post-transplantation. B. Human and mouse blood vessels (CD34+ cells) are also observed in the implanted tissue. In some instances the human blood vessels were intermingled with mouse blood vessels (original magnification ×50).

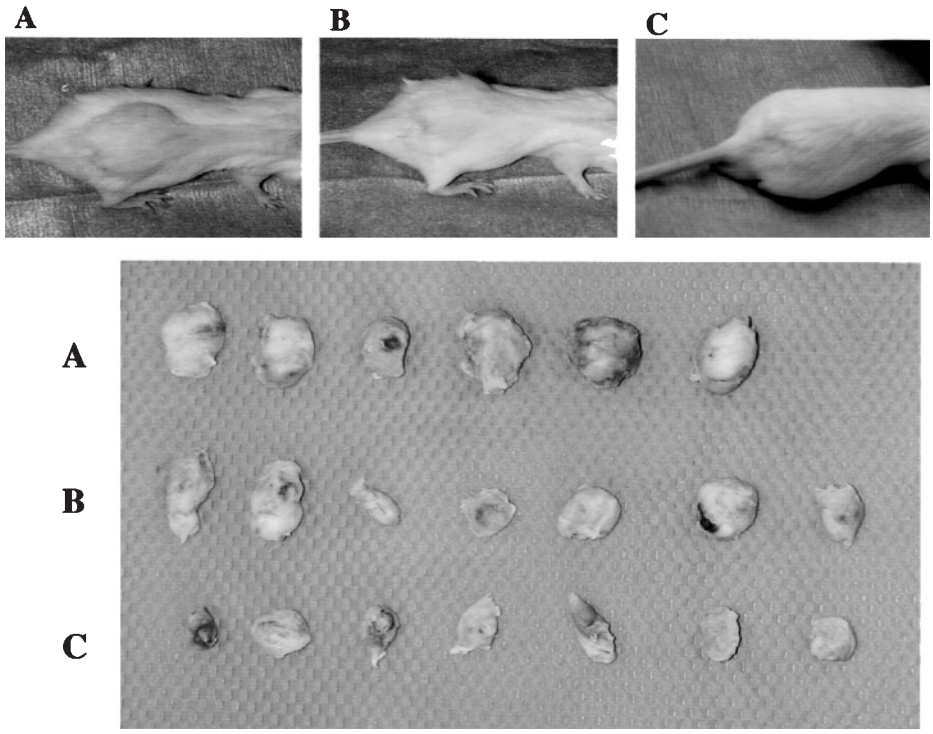

Figure 2. Human recombinant endostatin treated SCID-HuRAg mice. Upper photographs show representative control and treated mice after 7 days of injection therapy. Lower panel shows surgically removed specimens from each treated mouse. A. Grafted RA synovial tissue was observed on the back of SCID-HuRAg control mouse. Graft volume was decreased by endostatin treatment in a dose dependent manner: (B) 10 µg/kg endostatin treatment; (C) 50 µg/kg endostatin treatment.

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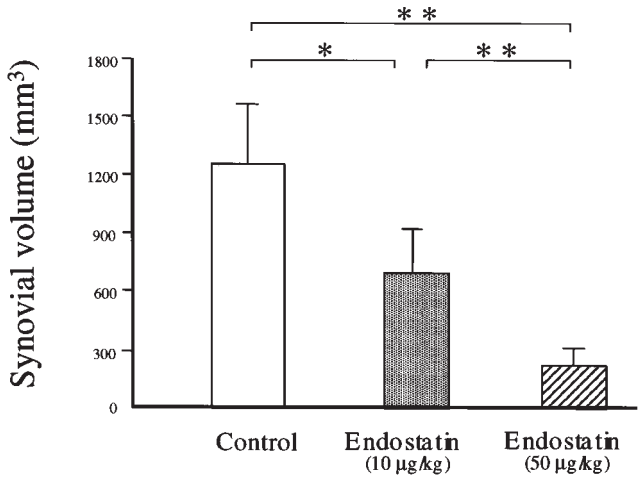

Figure 3. Reduction in grafted synovial volume in SCID-HuRAg mice. The grafted synovial tissue was reduced in size in endostatin treated mice (10 µg/kg) compared to controls (mean volumes 725.9 mm^3 and 1254.4 mm^3, respectively). In group treated with 50 µg/kg endostatin, marked regression of the synovial volume was observed (mean volume 278.5 mm^3). Statistical significance was also observed between 10 µg/kg and 50 µg/kg endostatin treated groups. Values are means ± SD. *$p < 0.05$, **$p < 0.01$.

synovial fibroblasts compared to the control mice. Further, the numbers of Fas and FasL positive synovial fibroblasts in the endostatin treated mice were also increased compared to controls (Figure 5 and Table 1).

DISCUSSION

It has been well established that angiogenesis, or new blood vessel growth, has an important role at sites of fibrovascular disorder, such as wound repair, tumor growth, and RA. Accordingly, there has been much interest in the development of antiangiogenic drugs for cancer treatment[1]. Recently, we and others have isolated human recombinant endostatin, produced as recombinant protein in human 293-EBNA cells, and examined the effect of it *in vitro* and *in vivo*[5-8]. As the results indicate, this novel endostatin has a high biological activity against the migration of human umbilical vein endothelial cells (HUVEC) in response to vascular endothelial growth factor (VEGF), and also inhibits tumor growth[5,8]. The other report focuses attention on the apoptosis induction effect of endostatin[16]. These findings indicate that endostatin is a powerful physiological antiangiogenic agent. The exuberant proliferation of new blood vessels is crucial to the development of RA synovium, and VEGF, which induces angiogenesis via protein kinase C, is an important mediator of angiogenesis in RA[9]. In addition, apoptosis plays an important role in synovial proliferation in RA, since RA synovitis can be treated by exogenous apoptosis induction[10,12,16]. If human recombinant endostatin can inhibit pathologic angiogenesis and induce apoptosis on RA synovia, this treatment strategy should be a viable approach for RA therapy.

For the aforementioned reasons, we examined the effect

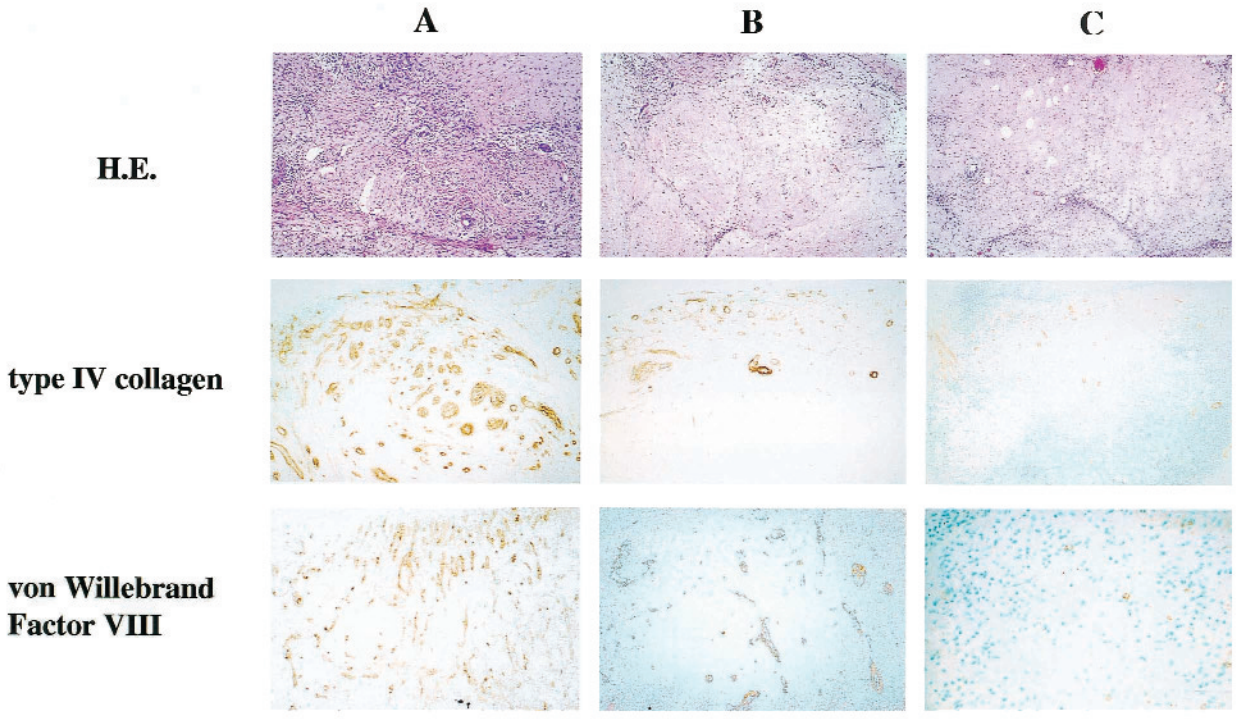

Figure 4. Microvessels in synovial tissue after endostatin treatment. Histological sections were analyzed by standard H&E staining and immunohistochemistry with anti-type IV collagen and anti-vWF VIII. Striking reduction in the number of microvessels in synovial tissue was observed after endostatin treatment. A. Control. B. 10 µg/kg endostatin group. C. 50 µg/kg endostatin group. Original magnification ×50.

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Table 1. Immunological findings in RA synovium after endostatin treatment. Values are mean ± standard deviation (SD).

	Control	Endostatin 10µg/kg	Endostatin 50 µg/kg
Microvessel staining			
Type IV collagen positive vessels/field	40.0 ± 13.5	18.3 ± 7.1**	10.8 ± 4.4**
vWF VIII positive vessels/field	18.9 ± 5.9	9.2 ± 2.4**	6.1 ± 2.4**
Immunocyte staining			
Number of positive cells per 300 cells			
LCA	165.9 ± 52.1	47.4 ± 21.3**	9.9 ± 7.5**
T cell	46.6 ± 23.1	27.9 ± 21.4**	17.0 ± 3.9**
B cell	57.5 ± 22.5	18.9 ± 14.3**	17.3 ± 12.7**
Macrophage	103.9 ± 27.5	72.9 ± 22.9**	57.8 ± 20.8**
Apoptotic cell staining			
Positive cells/field			
TUNEL	11.1 ± 4.9	16.8 ± 9.9 **	15.9 ± 7.4**
Fas	80.7 ± 19.8	104.1 ± 33.3 **	141.4 ± 45.2 **
FasL	84.3 ± 22.7	98.9 ± 41.4 *	127.8 ± 43.7 **

*p < 0.05 versus control mice;** p < 0.01 versus control mice.
LCA: leukocyte common antigen. vWF VIII: von Willebrand factor VIII.

of human recombinant endostatin on patients with RA, using a SCID-HuRAg mouse model that closely resembles human RA. Due to its substantial similarity, the SCID-HuRAg mouse has been considered a model for screening antirheumatic drugs[10,12,13]. As a result of endostatin treatment, grafted synovial volume in SCID-HuRAg mice was reduced in a dose dependent manner. The numbers of inflammatory cells, such as macrophages, CD4 positive T cells, and B cells, were significantly decreased by the endostatin treated RA synovial tissues, and this effect was also dose dependent. The numbers of vessels that were counted upon vWF VIII and type IV collagen staining were also significantly decreased. It has been described that endostatin induced vascular endothelial cell apoptosis via intracellular protease caspase-3 activation and inhibition of the expression of BcL-2 and BcL-x antiapoptotic protein[14]. Therefore, it is likely that the decreased angiogenesis we observed in synovial tissue was caused, at least in part, by vascular endothelial cell apoptosis. It is noteworthy, however, that the TUNEL staining and Fas/FasL staining was also present and increased in the synovial fibroblasts after endostatin treatment. The endostatin induced endothelial cell apoptosis was described *in vitro*[14]; however, we could not confirm this in our study. Probably, the endothelial cell apoptosis appeared soon after endostatin administration, since the number of vessels was already decreased at our observation time. The ischemic changes of synovial tissue were induced by the vessel apoptosis, consequently; the synovial fibroblasts might similarly undergo apoptosis. As well, the activated Fas/FasL system may contribute to reduction of the synovial volume. Indeed, as we reported, Fas/FasL activation by agonistic anti-Fas Mab or FasL gene transfer induced apoptosis in the

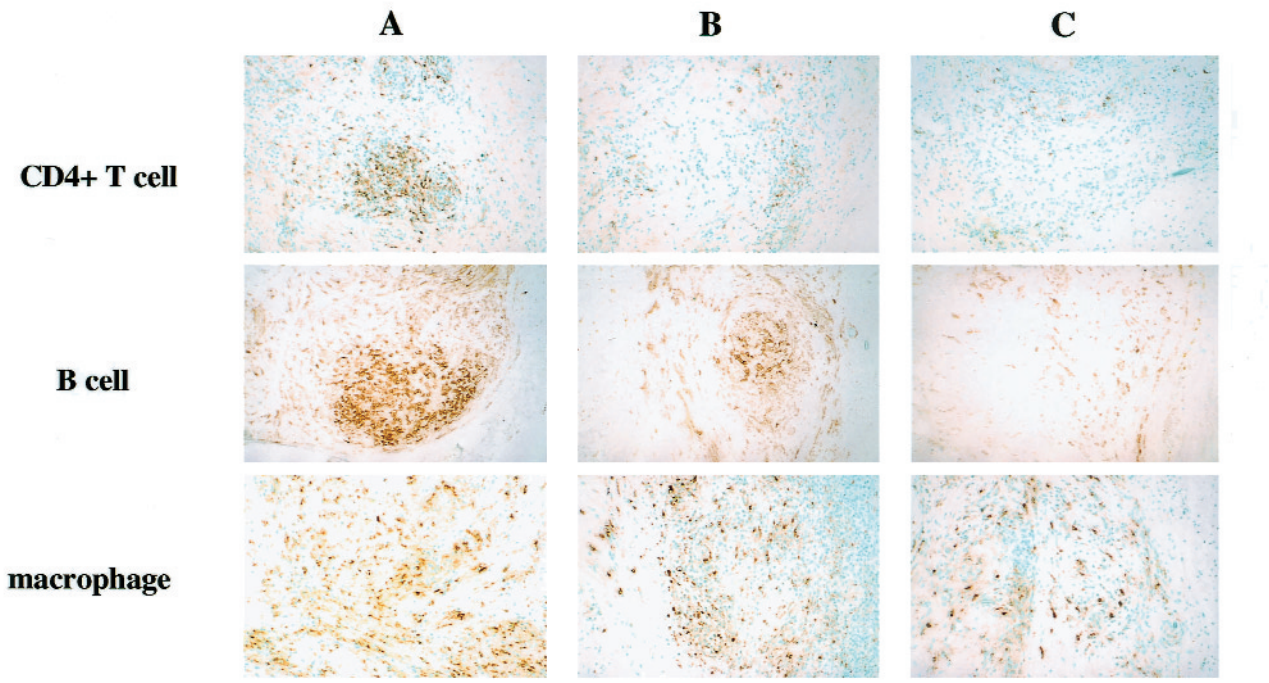

Figure 5. Inflammatory cells and endostatin treatment. A large number of inflammatory cells (CD4+ T cells, B cells, and macrophages) were observed in synovial tissue in the control mice. In contrast, the number of cells was significantly decreased in the endostatin treated mice. A. Control. B. 10 µg/kg endostatin group. C. 50 µg/kg endostatin group. Original magnification ×50.

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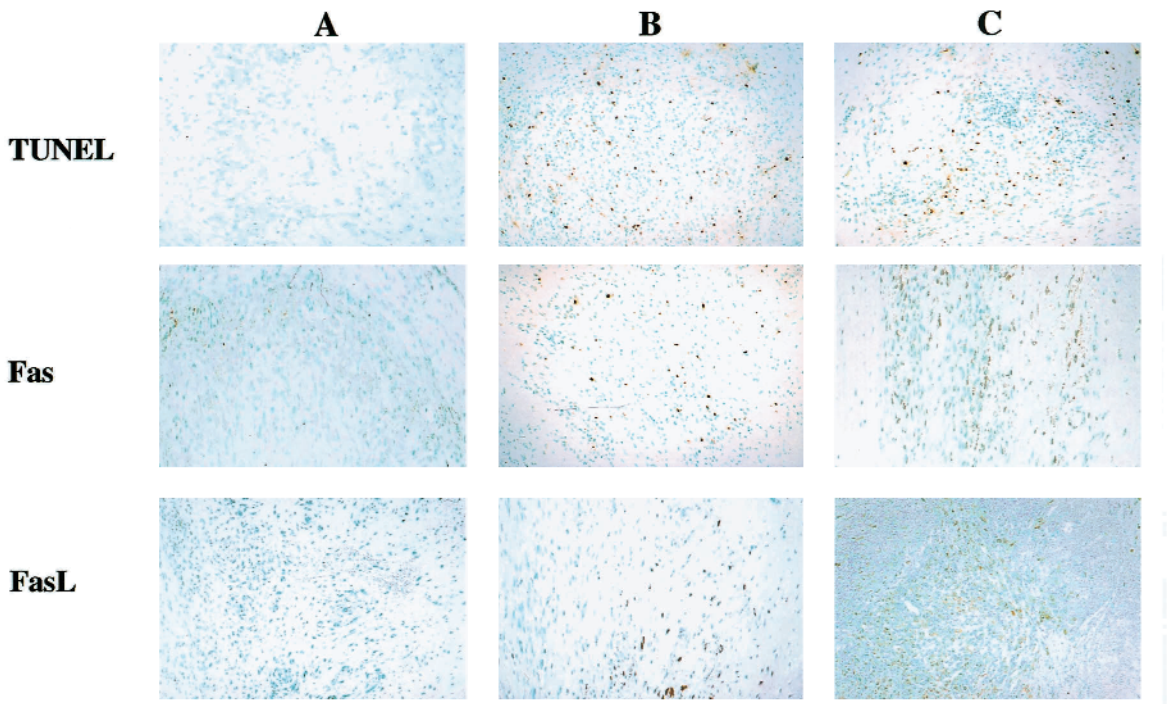

Figure 6. TUNEL, Fas, and FasL staining in the synovial tissue. Treatment of the SCID-HuRAg mice with endostatin caused a dose dependent increase in TUNEL, Fas, and FasL positive synovial fibroblasts in the synovial tissue. A. Control. B. 10 µg/kg endostatin group. C. 50 µg/kg endostatin group. Original magnification ×50.

reduction of synovial volume[10,12,17]. However, the significance of the Fas/FasL system after endostatin treatment should be clarified by further experiments.

The other endostatin mechanism is an inhibitory effect against immunocytes in RA synovitis. This decrease of infiltrating immunocytes would be an indirect effect of endostatin through inhibition of neovascularization. This apparent indirect effect of endostatin would suppress RA inflammation, since the pathogenesis of RA is mediated by immunocytes. Further, it is possible that our recombinant human endostatin is well suited to clinical application of antiangiogenesis treatment for RA because it is produced and purified from human cells as recombinant protein and its biological activity is significant.

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