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Endostatin Inhibits Adhesion of Endothelial Cells to Collagen I *via* $\alpha_2\beta_1$ Integrin, a Possible Cause of Prevention of Chondrosarcoma Growth[1]

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Endostatin derived from collagen XVIII is a potent endogenous anti-angiogenic factor that induces regression of various tumors of epithelial origin. Endostatin has been shown to inhibit endothelial cell functions, however, its effect remains controversial. We first attempted here to apply the inhibitory effect of recombinant human endostatin on chondrosarcomas, which originate from the mesenchyme, in nude mice. Endostatin induced reduction of chondrosarcoma growth and tumor angiogenesis *in vivo*. However, endostatin showed no effect on the proliferation and migration of chondrosarcoma cells *in vitro*. Next, we investigated the interactions between endostatin and endothelial cells in detail. Endostatin inhibited the migration on and attachment to collagen I but did not affect the proliferation of endothelial cells. Although the migration of endothelial cells was stimulated by angiogenic factors such as basic fibroblast growth factor and vascular endothelial growth factor, endostatin showed similar inhibitory effects on it in the presence and absence of the stimulants. Moreover, the inhibitory effect against endothelial cell attachment to collagen I was attenuated or modulated in the presence of neutralizing antibodies of α_2, $\alpha_5\beta_1$, and $\alpha_V\beta_3$ integrins but not that of α_1 integrin. Our results suggest that endostatin might suppress the $\alpha_2\beta_1$ integrin function of endothelial cells *via* $\alpha_5\beta_1$ or $\alpha_V\beta_3$ integrin. We propose here that endostatin might be effective for anti-angiogenic therapy for human chondrosarcomas through the suppression of $\alpha_2\beta_1$ integrin functions in endothelial cells.

Key words: basic fibroblast growth factor, chondrosarcoma, endostatin, integrin, tumor angiogenesis.

Many endogenous anti-angiogenic molecules such as angiostatin (*1*) and endostatin (*2*) were identified recently. Endostatin, which was first purified from a mouse hemangioendothelioma, is a C-terminal fragment of collagen XVIII (*3*). Similar anti-angiogenic factors also derived from basement membrane collagens include restin (*4*), and the non-collagenous domains of the α2(IV), α3(IV) and α6(IV) chains (*5*). These molecules have also been reported to inhibit tumor growth. O'Reilly *et al.* (*2*) and Boehm *et al.* (*6*) reported that endostatin caused decreases in the volumes of several tumors and induced tumor dormancy. Other investigators showed that endostatin suppressed or delayed the rate of tumor growth by inhibiting the penetration of microvessels (*7–17*). These studies were mainly performed on carcinomas which exhibited high tumor angiogenesis. However, the effects of endostatin on sarcomas have not been fully elucidated yet. Chondrosarcomas derived from avascular cartilaginous tissue also induce tumor angiogenesis (*18*). Actually, Coppola *et al.* have suggested the effectiveness of anti-angiogenic immunotherapy for rat chondrosarcomas (*19*). We therefore examined the anti-angiogenic effect of recombinant human (rh-) endostatin (*9*) on a human chondrosarcoma cell line (OUMS-27) in an *in vivo* tumor model (*20*).

Angiogenesis induced by a tumor, rheumatoid arthritis, or some other disease supports rapid expansion of the vasculature and contributes to the disease progression (*21*). The networks of new blood vessels are regulated by the balance between angiogenic factors and anti-angiogenic molecules (*22*). Basic fibroblast growth factor (bFGF) and vascu-

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Abbreviations bFGF, basic fibroblast growth factor, DMEM, Dulbecco's modified Eagle's medium, ECM, extracellular matrix, FITC, fluorescein isothiocyanate; HUVECs, human umbilical vein endothelial cells, LSGS, low serum growth supplement, M200S, Medium 200S, MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide, PBS, phosphate-buffered saline, rh-, recombinant human, RT-PCR, reverse transcriptase-PCR, VEGF, vascular endothelial growth factor

lar endothelial growth factor (VEGF) are the major cytokines known to strongly induce tumor angiogenesis (*23–25*) Endostatin has been shown to inhibit the blood vessel–forming activities of endothelial cells in the presence of these angiogenic factors However, reports on the relationships between bFGF (or VEGF) and endostatin remain controversial (*8, 9, 13, 26–28*). To examine the previous evidence, we investigated here the inhibitory effects of endostatin in the presence and absence of these angiogenic factors *in vitro*.

Endostatin has been reported to bind $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins (*28*). These integrins mainly interact with Arg-Gly-Asp (RGD)-containing extracellular matrices such as fibronectin (*29*). Rehn *et al.* reported that endostatin showed anti-angiogenic effects by blocking the attachment of endothelial cells to fibronectin *via* $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins (*28*) However, the adhesive activity of endothelial cells as to collagen I, which is ubiquitously expressed as a major component of the extracellular matrix (ECM), in the presence of endostatin is not clear. Collagen I has been reported to be the ligand of many cell surface receptors such as $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins (*30*). Here we investigated the adhesion of human umbilical vein endothelial cells (HUVECs) to collagen I in the presence of rh-endostatin *in vitro* using neutralizing antibodies of integrins.

We previously reported that OUMS-27 cells expressed bFGF and VEGF to maintain their proliferative activity (*31*). These findings led us to speculate that anti-angiogenic therapy targeted to bFGF and VEGF may be useful for human chondrosarcomas, and that endostatin can possibily inhibit endothelial cell penetration into the ECM of chondrosarcomas. In the present study, we demonstrated that endostatin inhibits the growth of human chondrosarcomas. Moreover, we suggested that $\alpha_2\beta_1$ integrin on endothelial cells might be regulated through the interactions between endostatin and $\alpha_5\beta_1$ (and/or $\alpha_v\beta_3$) integrin.

MATERIALS AND METHODS

Cells and Cell Culture—The OUMS-27 human chondrosarcoma cell line was maintained and sub-cultured as described previously (*20*). HUVECs were obtained from Kurabo (Osaka), and cultured in Medium 200S (M200S; Cascade Biologics, Portland, OR) containing 2% low serum growth supplement (LSGS; Cascade Biologics) on gelatin-coated dishes (Becton Dickinson, Franklin Lakes, NJ). Cells between passages 2 and 6 were used for experiments and maintained as described in the manufacturer's protocols.

In Vivo Tumor Model—OUMS-27 cells (1×10^7 cells) suspended in 200-μl of Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Rockville, MD) were implanted subcutaneously into the right flanks of 6-week-old male BALB/c nu/nu mice (Clea Japan, Tokyo). The tumor size was determined with calipers and the tumor volume was calculated with a standard formula: width^2 × length × 0.52 (*32*). Three weeks after implantation, the animals were randomly divided into groups, and each group of three mice had tumors of comparable size. The mean tumor volume was 236 mm^3 (range, 205–334 mm^3). Then, treatment was started with recombinant human (rh-) endostatin (each mouse receiving 50 μg/kg/day of rh-endostatin daily by means of subcutaneous injections around the tumor) for a period of 17 days. Control mice received PBS (50 μl) each day. Eighteen days after the first treatment, the animals were sacrificed, and the tumors were removed for histological and immunohistochemical analyses. Rh-endostatin was expressed and purified as described previously (*9*)

Histology, Immunohistochemistry, and Microvessel Counts—Half of a removed tumor xenograft was fixed in a 4% paraformaldehyde-buffered solution, and then embedded in paraffin for safranin O staining and immunohistochemistry for Ki-67 (*33*). The synthesis or depletion of proteoglycans was assessed as the uptake of the safranin O dye (Chroma, Kongen, Germany). Sections were stained in a 0.1% safranin O solution (pH 7.4) for 3 min at room temperature. Proliferative activity was evaluated using mouse anti-human Ki-67 monoclonal antibodies (anti–Ki-67 antibodies) and a HistoMouse-Plus kit (both obtained from Zymed Laboratories, San Francisco, CA) according to the manufacturer's protocol. Tumor sections were pretreated by autoclave heating for antigen retrieval before staining for Ki-67. Phosphate-buffered saline (PBS) without the primary antibodies was used for a negative control. Several fields were assessed randomly, with the exception of necrotic areas, under high-power magnification (×400), and the Ki-67-positive index was determined by scoring the positive cells per total cells in 4 fields, as described (*33*).

The other half of each tumor tissue sample was frozen in liquid nitrogen and stored at –80°C. The synthesis or depletion of collagen I and II were evaluated using mouse anti-human collagen I monoclonal antibodies (I-8H5; Oncogene, San Diego, CA) and mouse anti-human collagen II antibodies (II-4C11; Fuji Chemicals, Toyama) with HistoMouse-Plus kits, respectively. To determine the degree of tumor-induced angiogenesis, 7-μm cryostat sections of tumor xenografts were fixed in acetone for 5 min, air-dried, blocked with 1% BSA for 30 min at room temperature, and then stained with rat anti–mouse CD31 monoclonal antibodies (anti-CD31 antibodies; PharMingen, San Diego, CA) (1:50 dilution in PBS) for 1 hr. PBS without the primary antibodies was used for a negative control. The tissue localization of CD31 was visualized by incubating the sections for 45 min at room temperature with fluorescein-conjugated affinity-purified goat antibodies against rat IgG (ICN Pharmaceuticals, Aurora, Ohio), followed by 5 min incubation in Hoechst 33258 (Wako, Osaka) as a counterstain. Two CD31-immunostained sections were analyzed per tumor. An entire slide was scanned at a magnification of ×100 to determine 4 areas at the periphery of the tumor showing the greatest vascularization A countable microvessel was defined as any stained endothelial cell or cell cluster that was separated from the adjacent microvessels. Neither the presence of vessel lumens nor of red blood cells was necessary to define a microvessel. Branching structures were counted as a single vessel. CD31-immunostained plasma cells were eliminated from the counting. Microvessels in normal subcutaneous tissue adjacent to a tumor were used as an internal control. The final quantification involved the number of microvessels per mm^2 in the 4 fields at a magnification of ×200, as described (*34*).

Assessment of Endogenous Endostatin Expression in OUMS-27 Cells—We performed further analysis to determine whether endostatin was expressed in OUMS-27 cells or not. Total cellular RNAs were extracted from confluent cultured cells and used for RT-PCR, and then Northern blot

analysis was performed as described previously (*31*). A PCR fragment was amplified using the following human endostatin-specific primers: CTGGGAGGCTCTGTTCTCAG and ATCCAGGCGGTGGCTACTTG.

Cell Proliferation Assays—To determine the effect of rh-endostatin on tumor growth *in vitro*, we performed proliferation assays with cultured OUMS-27 cells and HUVECs. Cultured OUMS-27 cells were washed with PBS and then dispersed in a 0.1% trypsin, 5 mM EDTA solution. A cell suspension was made with DMEM (containing fetal bovine serum, FBS; Sigma), plated at 1×10^4 cells/well onto 96-well tissue culture plates (Coster, Cambridge, MA), and then incubated (37°C, 5% CO_2) for 24 h. After the cells had been washed with PBS, the medium was replaced with serum-free M200S containing 0.1% BSA. Cultured HUVECs were washed with PBS and then dispersed in a 0.25% trypsin, 1 mM EDTA solution. A cell suspension was made in M200S (LSGS+), plated at 1×10^4 cells/well onto 96-well plates, and then incubated (37°C, 5% CO_2) for 24 h. After the cultures had been washed with PBS, the medium was replaced with LSGS-free M200S containing 0.1% BSA. Medium containing rh-bFGF (10 ng/ml; Pepro Tech, London, UK) or rh-$VEGF_{165}$ (rh-VEGF, 5 ng/ml, R&D Systems, Minneapolis, MN) was also used. Rh-endostatin was added to this fresh medium at several concentrations (0, 1, 10, and 100 ng/ml). In some cases, neutralizing antibodies (rabbit anti-human bFGF polyclonal antibodies, anti-bFGF antibodies; Pepro Tech; or goat anti-human VEGF polyclonal antibodies, anti-VEGF antibodies; R&D Systems) were added to the medium to a final concentration of 10 μg/ml, and the cells were incubated for 1 h at room temperature before the proliferation assays. The plates were incubated (37°C, 5% CO_2) for 24 h for proliferation and then the cells were incubated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; Chemicon, Temecula, CA] according to the manufacturer's protocol. Each of the test samples was added to 4 wells ($n = 4$). The optical density was measured with a Model 550 microplate reader (Bio-Rad) at a wavelength of 595 nm. The data were evaluated as relative cell proliferation, which is the percentage compared with the mean value for the medium containing only BSA. The assays were performed in quadruplicate and similar results were obtained.

Cell Migration Assays—Cell migration assays were performed in a modified Boyden chamber (48-well chemotaxis chamber AP48; Neuro Probe, Gaithersburg, MD) Polycarbonate membranes (8-μm pore, 25 × 80 mm size, polyvinylpyrrolidine-free, Neuro Probe) were coated with 100 μg/ml of rat tail collagen I in 0.2 N acetic acid (Collaborative Biomedical Products, Bedford, MA) for 2 days and then air-dried. Each membrane was placed over the bottom chamber filled with M200S containing 0.1% BSA ($n = 4$). Rh-bFGF (10 ng/ml), rh-$VEGF_{165}$ (5 ng/ml), or neutralizing antibodies were added to the medium (see above). Cells were suspended in M200S with the indicated concentrations of rh-endostatin for 30 min and 1×10^4 cells in 50 μl were added to the upper chamber of each well. The chamber was incubated for 6 h at 37°C with 5% CO_2 to allow cells to migrate through the collagen-coated polycarbonate membrane. Non-migrating cells on the upper surface of the membrane were removed with a wiper tool (Neuro Probe), and then the membrane was stained with Diff-Quik (VWR Scientific Products, Bridgeport, NJ). The total number of cells with nuclei that migrated per well was determined as described (*9*). The data were evaluated as relative cell migration, which is the percentage compared with the mean cell number for the medium containing only BSA. The assays were performed in quadruplicate.

Cell Attachment Assays—The attachment assays were performed in 96-well plates coated with 10 μg/ml of rat tail collagen I. The plates were coated with collagen I overnight at 4°C, blocked with 0.1% BSA in PBS for 30 min at room temperature, and then washed twice with PBS. Cells were suspended in LSGS-free M200S containing 0.1% BSA. Neutralizing antibodies against human α_1 (FB12), α_2 (P1E6), α_5 (NKI-SAM-1), $\alpha_5\beta1$ (JBS5), and $\alpha_V\beta_3$ (LM609) integrins (all obtained from Chemicon, Temecula, CA) were reacted with the cell suspension at 1:100 dilution for 15 min at room temperature. Non-functional blocking antibodies of β_1 integrin (P4G11, Chemicon) were used as a control IgG Incubation with the indicated concentrations of rh-endostatin was performed at 37°C for 30 min before seeding. Rh-bFGF (10 ng/ml) or rh-$VEGF_{165}$ (5 ng/ml) was added after 30 min preincubation at 37°C, and the cells (20,000) were plated into wells (4 wells/sample) and then allowed to become attached at 37°C for 30 min. The adherent cells were fixed with a 4% paraformaldehyde-buffered solution and then stained for 5 min with 1% toluidine blue. Stained cells were eluted with 1% sodium dodecyl sulfate and the absorbance was measured at 595 nm. The data were evaluated as relative cell attachment, which is the percentage compared with the mean value for the medium containing only BSA. The assays were performed in quadruplicate.

Statistical Analyses—Data were expressed as means ± SD. Differences among groups were compared by means of the Mann-Whitney *U* test Statistical significance was established at $p < 0.05$.

RESULTS

Endostatin Suppresses OUMS-27 Growth and Neovascularization In Vivo—As endostatin has been used as an inhibitory reagent for epithelial-derived cancers (*2, 6–17*), we were prompted to determine if the growth of chondrosarcomas of mesenchymal origin could be inhibited by injecting rh-endostatin in an *in vivo* tumor model. Figure 1 shows the inhibitory effect of rh-endostatin on chondrosarcoma growth *in vivo*. After 18 days, the tumor volumes in PBS-injected mice reached 216% (range, 164–243%) of the size on the first PBS-injection. However, the tumor volumes in endostatin-injected mice did not change (mean, 109%; range, 100–115%). Moreover, we observed that the number of tumor lobules in endostatin-injected mice was lower than that in PBS-injected mice (Fig. 2, A and B). No significant differences in the stainability with the safranin O dye, which reflect the amount of proteoglycan, were found and the ECM organization of the chondrosarcomas was not affected by endostatin treatment (Fig. 2, C and D). To assess the viabilities of OUMS-27 cells *in vivo*, we stained tumor sections with anti–Ki-67 antibodies (Fig. 2, E and F). Although rh-endostatin induced tumor dormancy in OUMS-27 cells-injected mice, no significant differences in the rates of Ki-67 positive cells between the PBS- and endostatin-injected groups were found (Table I). We observed that the ECM produced by OUMS-27 cells *in vivo*

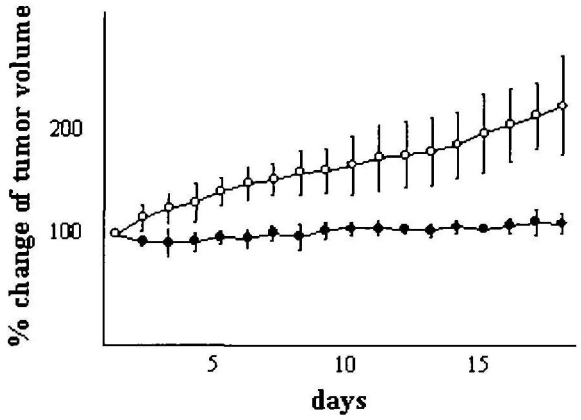

Fig. 1. **Rh-endostatin inhibited the growth of OUMS-27 cells in an *in vivo* tumor model.** Three weeks after tumor inoculation, endostatin treatment (50 μg/kg/day) was started. The effect of endostatin was evaluated in terms of the % change in tumor volume. Each xenograft at the initiation of treatment was used as a 100% control. Significant differences ($p < 0.05$) were assessed by means of the Mann-Whitney U test ($n = 3$) for days 2–18. Open and filled circles indicate the mean % changes in PBS-injected and endostatin-injected mice, respectively. Error bars, SD.

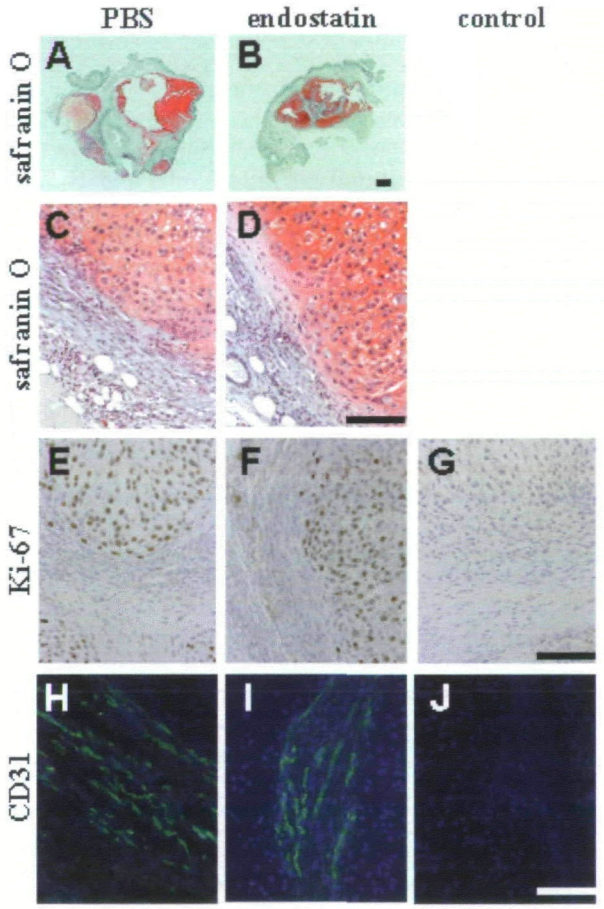

Fig. 2. **Histological and immunohistochemical analyses of tumor specimens.** Paraffin-embedded tumor sections were stained with safranin O (A–D). PBS-injected tumor tissue expanded in the subcutaneous space and formed several lobules (A). The red areas show the proteoglycans produced by OUMS-27 cells. Note that rh-endostatin suppressed tumor growth (B). The structure of the ECM synthesized by tumor cells was not affected by endostatin injections (C and D). Serial sections stained with anti-Ki-67 antibodies are shown (E–G). The brown spots in panels E and F indicate Ki-67–positive cells. Cryostat sections stained with anti-CD31 antibodies were analyzed by fluorescence microscopy (H–J). Serial sections from the PBS-injected group were stained without primary antibodies as negative controls (G and J). CD31-positive endothelial cells are shown in panels H and I (green). The nuclei stained with Hoechst 33258 are shown in panels H-J (blue). Scale bars, 1 mm (B), 100 μm (D, G, and J).

contained collagen I and collagen II, but no significant differences in their stainabilities between the two groups were found (data not shown). In contrast, the number of CD31-positive cells decreased in endostatin-injected mice (Fig. 2, H and I). Table I demonstrates the significant differences in the microvessel counts ($p < 0.05$). These findings indicate that the proliferative activities of OUMS-27 cells are not directly affected by endostatin treatment.

Endostatin Shows No Effect on OUMS-27 Cells In Vitro—To assess the endogenous production of endostatin, we first performed RT-PCR and Northern blot analysis using cultured OUMS-27 cells. Neither endogenous endostatin nor collagen XVIII was detected in OUMS-27 cells (data not shown). Next we examined the effects of rh-endostatin on the activities of OUMS-27 cells *in vitro*. The proliferation and migration of tumor cells were not affected by rh-endostatin at several concentrations (1, 10, and 100 ng/ml) (Fig. 3, A and B). We previously reported that rh-bFGF (10 ng/ml) stimulates the proliferation and migration of OUMS-27 cells up to 1.4- and 3.1-fold the control levels, respectively, but rh-VEGF (5 ng/ml) has no effect (*33*). In this study, we further analyzed the effect of endostatin in the presence and absence of bFGF and VEGF on OUMS-27 cells. The augmentation of the proliferative and migratory activities of OUMS-27 cells was abolished by anti-bFGF antibodies and not by anti-VEGF antibodies (data not shown). However, rh-endostatin showed no inhibition in these cases (Fig. 3, A and B). These results showed that endostatin played no role in the proliferative and migratory activities of OUMS-27 cells in either the presence or absence of growth factors.

Endostatin Inhibits the Migration but Not the Proliferation of HUVECs—To explain the inhibition of OUMS-27 growth by endostatin *in vivo*, we performed *in vitro* analyses using HUVECs as representative endothelial cells. We previously reported and confirmed that endostatin inhibited the endothelial cell migration stimulated by VEGF (*9* and Fig. 3D). Under the same experimental conditions, the migration of HUVECs was inhibited by rh-endostatin without VEGF stimulation (Fig. 3D). Endostatin had similar

TABLE I **The rates of Ki-67 positive cells and numbers of CD31-positive cells in tumor sections.**

	PBS injection	Endostatin injection	Internal control
Ki-67 positive (%)	58.6 ± 9.5	55.1 ± 8.5	
CD31-positive (cells/mm²)	169.7 ± 8.0*†	116.7 ± 9.3*	99.3 ± 2.5

Values are means ± SD for 3 tumor samples. The Ki-67 positive rate was determined as the percentage of stained cells in 4 fields per sample. CD31-positive cells were counted in 4 fields/sample at a magnification of ×200. * and † indicate $p < 0.05$ compared with an internal control and the endostatin-injected group, respectively, using the Mann-Whitney U test.

inhibitory effects on migration on the addition of bFGF (Fig. 3D). However, the viability of HUVECs was not affected by the indicated concentrations of rh-endostatin and we did not find that endostatin inhibited bFGF- or VEGF-induced endothelial cell proliferation (Fig. 3C). These results mostly agree with previous observations on the endothelial cell-specific functions of endostatin.

Endostatin Firstly Inhibits the Attachment of HUVECs to Collagen I—Cell migration is a process of coordinated actions such as polarization, membrane extension, cell-substratum attachment, contractile force and release of attachments (*35*) In the migration assays described above, cell attachment to collagen I is firstly required for cellular motility. To clarify the inhibition of migration caused by endostatin in the absence of growth factors or chemoattractants, we further analyzed the interactions between endostatin and HUVECs in terms of cell attachment. Rh-Endostatin showed an inhibitory effect on HUVECs attachment to collagen I without a stimulant (Fig. 4A). Approximately 30 and 40% inhibition was induced by endostatin treatment with concentrations of 1 and 10 (100) ng/ml, respectively. The attachment was not affected by rh-bFGF or rh-VEGF within 30 min but rh-endostatin prevented it to a similar degree (Fig. 4A). Endostatin thus exerted a partial effect on the attachment phase *in vitro*, which might be the reason for the finding that endostatin inhibited the migration of HUVECs in the absence of any angiogenic factors. Endostatin has been reported to suppress endothelial cell attachment to fibronectin *via* $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins (*28*). In our case, Fig. 4A demonstrates that endostatin inhibits HUVEC attachment to collagen I in a dose-dependent manner. However, collagen I has been reported to bind $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins but not to $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins (*30*). We therefore examined whether these results were the result of interactions between endostatin and $\alpha_1\beta_1$ (or $\alpha_2\beta_1$) integrin. The attachment of HUVECs and the effects of rh-endostatin were not affected by the neutralizing antibodies of α_1 integrin (Fig. 4B). However, anti–α_2 integrin antibodies inhibited HUVECs attachment on collagen I–coated wells to 50% of the control level. These data showed that endostatin inhibits endothelial cell attachment to collagen I, and that this inhibitory effect is attenuated or modulated in the presence of neutralizing antibodies of α_2, $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins but not that of α_1 integrin (Fig. 4, B and C). These findings suggest that endostatin might have an inhibitory effect on $\alpha_2\beta_1$ integrin function in HUVEC attachment to collagen I but not on $\alpha_1\beta_1$ integrin.

We hypothesized that endostatin might inhibit endothelial cell attachment to collagen I *via* $\alpha_2\beta_1$ integrin through the binding to $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins. To clarify this hy-

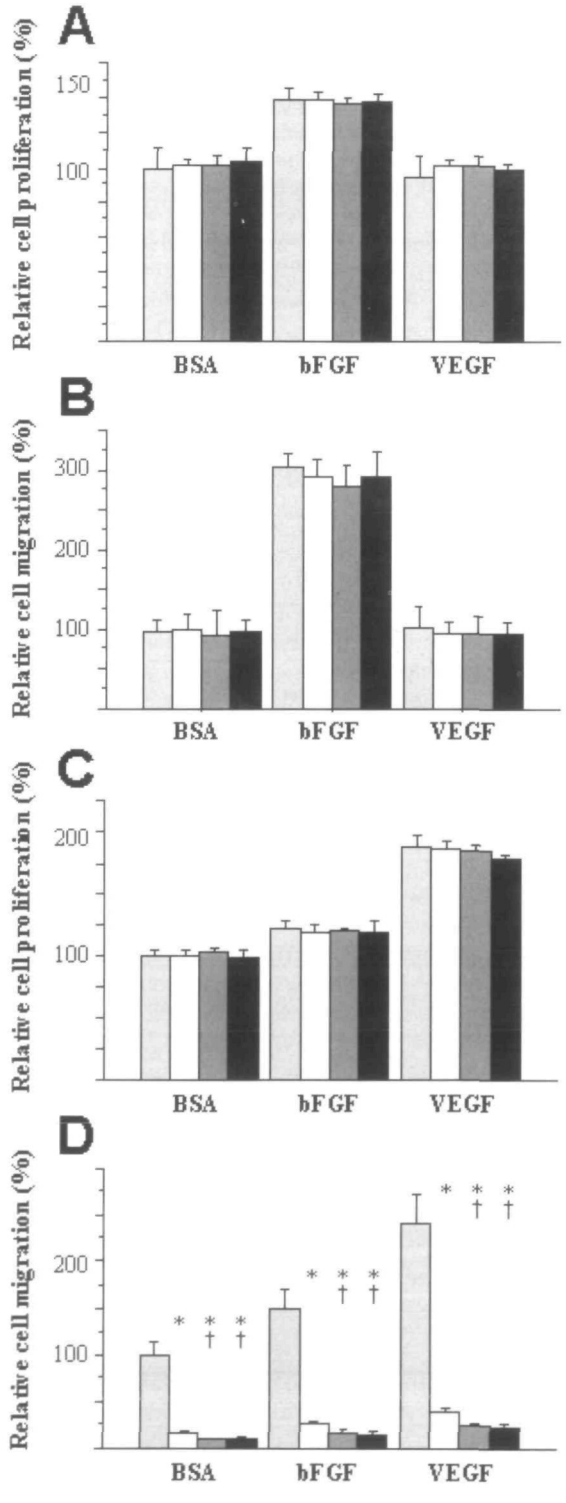

Fig 3 **Effects of endostatin on the proliferation and migration of OUMS-27 cells and HUVECs *in vitro*.** (A) The proliferation of OUMS-27 cells increased up to approximately 140% of the control level with only 0 1% BSA on rh-bFGF (5 ng/ml) treatment. Rh-VEGF (5 ng/ml) had no effect on their proliferation Significant differences ($p < 0\ 05$), compared with BSA controls, were observed for the bFGF-added group Rh-endostatin had no effect at any concentration (0, 1, 10, and 100 ng/ml) on the proliferation of OUMS-27 cells (B) Rh-bFGF stimulated the migration of OUMS-27 cells up to approximately 300% of the control levels ($p < 0\ 05$) Rh-VEGF and rh-endostatin had no effect on the migration of OUMS-27 cells (C) The proliferation of HUVECs was augmented up to approximately 120 and 190% of the BSA control levels by rh-bFGF and rh-VEGF, respectively ($p < 0\ 05$) Their proliferation was not affected by rh-endostatin (D) Rh-bFGF and rh-VEGF stimulated the migration of HUVECs up to approximately 150 and 240% of the control levels, respectively ($p < 0\ 05$) Rh-endostatin inhibited their migration in a dose-dependent manner in all groups. Light-gray, open, dark-gray and filled bars indicate the percentages of relative cell proliferation or migration with rh-endostatin concentrations of 0, 1, 10, and 100 ng/ml, respectively Error bars, SD $*p < 0\ 05$ *vs.* rh-endostatin at 0 ng/ml, $^{\dagger}p < 0\ 05$ *vs.* rh-endostatin at 1 ng/ml, with the Mann-Whitney *U* test

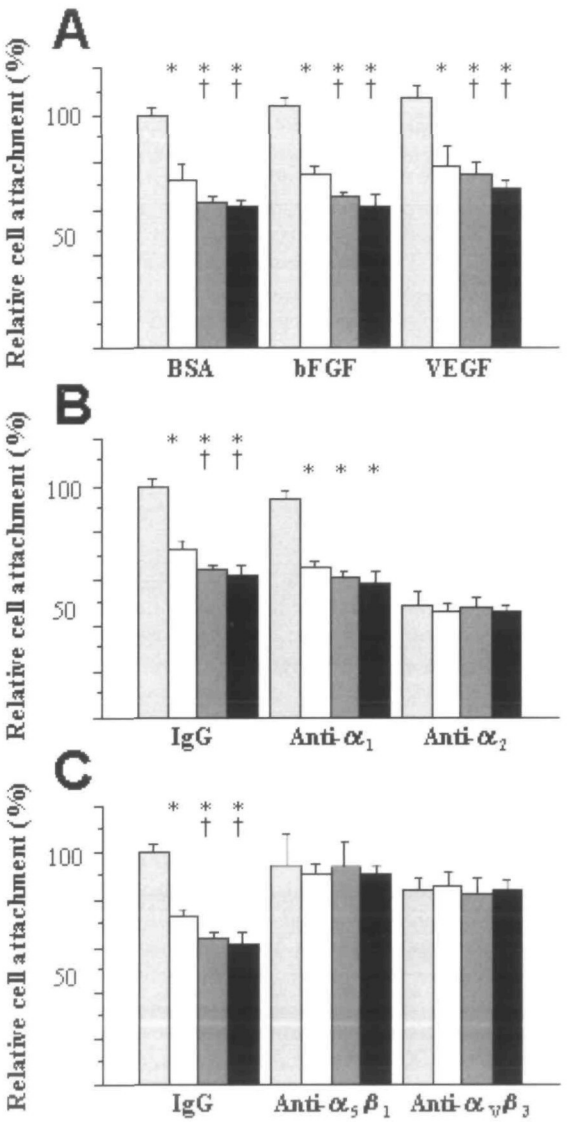

Fig 4 **Role of endostatin in the attachment of HUVECs to collagen I.** Cell attachment assays on collagen I–coated plates were performed as described under "MATERIALS AND METHODS." (A) The attachment of HUVECs was prevented by rh-endostatin (0, 1, 10, and 100 ng/ml) in a dose-dependent manner without any angiogenic factors. Similar results were obtained under rh-bFGF (10 ng/ml)– and rh-VEGF (5 ng/ml)–stimulated conditions. No significant differences were found among the groups. (B) Control IgG (P4G11) and anti-α_1 integrin antibodies (FB12) did not prevent HUVECs adhesion to collagen I The attachment was inhibited by anti-α_2 integrin antibodies (P1E6) ($p < 0.05$; compared with each control IgG column) Note the decrease in the rh-endostatin effects with anti-α_2 integrin antibodies. (C) The attachment of HUVECs was slightly affected by anti-$\alpha_v\beta_3$ integrin antibodies (LM609) but not by anti-$\alpha_5\beta_1$ integrin antibodies (JBS5) ($p < 0.05$) These antibodies blocked the inhibitory effects of rh-endostatin on HUVEC attachment Light-gray, open, dark-gray, and filled bars indicate the percentages of relative cell attachment with rh-endostatin concentrations of 0, 1, 10, and 100 ng/ml, respectively Error bars, SD. *$p < 0.05$ *vs.* rh-endostatin at 0 ng/ml, †$p < 0.05$ *vs.* rh-endostatin at 1 ng/ml, with the Mann-Whitney *U* test.

pothesis, we assessed HUVEC attachment using the neutralizing antibodies of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins. Cell attachment to collagen I was not dramatically prevented by these antibodies. However, the inhibitory effects of rh-endostatin were blocked by both antibodies (Fig 4C) We propose here that endostatin might prevent the function of $\alpha_2\beta_1$ integrin by modulating $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins on endothelial cells.

DISCUSSION

In this study, we demonstrated that rh-endostatin inhibits the growth of human chondrosarcomas (OUMS-27) *in vivo*. Several recombinant proteins of endostatin have been reported to induce similar inhibition of tumor growth (*7–17*). However, their origins were quite different, such as yeast and mammalian cells. The single dose of rh-endostatin used in this experiment was lower than that in other studies. Our recombinant protein was purified from human embryonic kidney cells (*9*), so its folding system seems to be more physiological than in the case of the others (*7, 8, 15*). We previously reported that OUMS-27 cells induced high tumor angiogenesis in spite of their avascular origin (*31*). Hayami *et al.* reported that marked reduction of an anti-angiogenic molecule (chondromodulin-I) in OUMS-27 cells was involved in the loss of the anti-angiogenic property of cartilage (*32*). Our rh-endostatin showed no direct inhibition of the viability of OUMS-27 cells *in vivo* (Fig. 2, E and F, and Table I) or *in vitro* (Fig. 3A). The amount of proteglycan (Fig. 2, C and D), collagen I and collagen II (data not shown) in tumor sections, and the migration of OUMS-27 cells (Fig. 3B) were also not affected by rh-endostatin treatment. However, the growth of tumors was prevented by a low dose injection of rh-endostatin (Fig. 1) We observed a reduction of microvessel counts (Fig. 2, H and I, and Table I) and the inhibition of HUVEC migration (Fig. 3D) on rh-endostatin treatment. These findings suggest the indirect effect of endostatin on tumors *via* endothelial cells and are consistent with previous reports (*7–17*). We propose here that additional treatment with rh-endostatin might be useful for tumor dormant therapy after wide margin tumor resection of human chondrosarcomas.

The inhibitory effects of endostatin have been reported to be endothelial cell–specific (*36*). However, the interactions between endostatin and the endothelial cell function remain controversial. Dhanabal *et al.* reported that recombinant endostatin produced in yeast inhibits the proliferation and migration of endothelial cells in response to stimulation by bFGF (*8*), and indicated that endostatin treatment of bovine pulmonary artery endothelial cells causes apoptosis through marked reductions of the Bcl-2 and Bcl-X_L anti-apoptotic proteins (*37*). However, we could not obtain any such data suggesting that endostatin inhibits cell proliferation (Fig. 3C), or induces apoptotic changes in HUVECs (data not shown). Shichiri and Hirata reported that endostatin did not induce apoptosis of endothelial cells and suggested that endostatin appears to trigger gene suppression signals specifically in growing endothelial cells, resulting in a potent anti-migratory effect (*38*). We consider the difference between our results and previous ones to be due to the following reasons. (i) It appears that the use of different expression systems or species of endostatin results in different outcomes. (ii) The concentrations of rh-endostatin could be low to prevent HUVEC proliferation in this *in vitro* model. (iii) Although 30 min preincubation of HUVECs with rh-endostatin is known to be important (*9*), we could not use this preincubation time in this cell prolifera-

tion assay model. (iv) The cell proliferation assays were performed under serum-supplemented conditions for 48 h The different effects of endostatin in various endothelial cells have been previously reported (*38, 39*). Further studies to investigate the effect of endostatin on endothelial cell proliferation are required.

Endostatin has been reported to be an inhibitor of endothelial cell migration stimulated by bFGF (*8, 13, 26–28*) or VEGF (*9, 13*). One paper by Rehn *et al* (*28*) described that the migration of endothelial cells is prevented by endostatin only with bFGF-, *i.e.* not VEGF-, stimulation, and another by Sasaki *et al.* (*27*) that the anti-angiogenic effect of endostatin on VEGF-induced angiogenesis is little. Kim *et al.* suggested that endostatin inhibits the invasion of endothelial cells in an agonist-independent manner (*13*). The present study indicated that rh-endostatin suppressed the endothelial cell migration not only in the presence of bFGF and VEGF, but also in the case of no growth factor (Fig. 3B). Moreover, we detected a large increase in relative cell migration with no rh-endostatin in the presence of such angiogenic factors. Senger *et al.* reported that VEGF promoted cell spreading on collagen I through the increase in $\alpha_1\beta1$ and $\alpha_2\beta_1$ integrins (*40*). These findings suggest that the significant difference on cell migration assaying with no stimulator might have been hard to detect in previous studies.

The interaction between cells and substrates is recognized to be a key role for many cellular functions (*35, 41*). Our results demonstrated that HUVEC migration on collagen I is prevented to 20% of the control level by rh-endostatin (Fig. 3D), and that endostatin treatment firstly affects HUVEC adhesion to collagen I. In fact, the relative attachment of HUVECs to collagen I was suppressed to 60% of the control level by rh-endostatin (Fig. 4A). We obtained similar results to those shown in Fig. 4A in cell attachment assays involving a modified Boyden chamber (data not shown). Although endostatin has been reported to have no effect on cell migration on collagen I (*28*), we previously demonstrated the inhibition of HUVEC migration on collagen I by rh-endostatin (*9*) and confirmed the results in this study (Fig. 3D). Moreover, we first propose here that endostatin inhibits the HUVEC attachment to collagen I. These results might reflect the prevention of OUMS-27 cell growth in the *in vivo* tumor model We previously detected the expression of the α1(I) chain in OUMS-27 cells (*20*), and observed it in tumor sections by using an anti-collagen I antibody (data not shown). Therefore, the penetration of microvessels into the ECM of chondrosarcomas might be dependent on the endothelial cell attachment and/or migration on collagen I as a scaffold. Our results suggest that endostatin might regulate endothelial cell functions on collagen I through two phase effects: (i) a short time effect on attachment and (ii) a middle time effect on migration

Rehn *et al.* have reported that soluble endostatin inhibited endothelial cell attachment to fibronectin through interactions with $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins (*28*). Although fibronectin and collagens contain the RGD motif (*29, 42*), native collagen I has been reported to be recognized by conformation-dependent (RGD-independent) integrins such as $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (*43*). This recognition site is the GFOGER (O = hydroxyproline) sequence corresponding to residues 502–507 of the α1(I) chain (*44*). In our study, endostatin inhibited the attachment of HUVEC to collagen I and the suppression was mainly dependent on $\alpha_2\beta_1$ integrin function (Fig. 4B). The present study indicated that the inhibitory effects of endostatin on cellular attachment were attenuated by the neutralizing antibodies of $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_V\beta_3$ integrins (Fig. 4, B and C). Figure 4C suggests that the integrin-antibody complex on $\alpha_5\beta_1$ or $\alpha_V\beta_3$ integrin probably prevents the binding between endostatin and these integrins. We considered that endostatin might negatively regulate the $\alpha_2\beta_1$ integrin function by binding with $\alpha_5\beta_1/\alpha_V\beta_3$ integrins, and that the binding between $\alpha_5\beta_1/\alpha_V\beta_3$ integrins and endostatin might be quite different from the association between $\alpha_5\beta_1/\alpha_V\beta_3$ integrins and their antibodies. However, we could observe neither the co-localization of rh-endostatin with $\alpha_2\beta_1$ integrin nor that with $\alpha_5\beta_1$ (or $\alpha_V\beta_3$) integrin on immunohistochemical analyses using their antibodies (data not shown). In addition, several cases of outside-in signaling derived from $\alpha_5\beta_1$ or $\alpha_V\beta_3$ integrin have been reported (*45*), but the inside-out signaling to $\alpha_2\beta_1$ integrin expressed on the cell surface is not clear. Further study of these signals may shed more light on the anti-angiogenic mechanism of endostatin.

In conclusion, we suggest that endostatin inhibits endothelial cell attachment to collagen I *via* $\alpha_2\beta_1$ integrin to suppress tumor angiogenesis induced by chondrosarcomas.

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