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Inhibition by acharan sulphate of angiogenesis in experimental inflammation models

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1 The effects of acharan sulphate, a glycosaminoglycan isolated from the giant African snail *Achatina fulica*, on angiogenesis in the granulation tissue were analysed using an air pouch-type carrageenin-induced inflammation model in rats and a cotton thread-induced inflammation model in mice.

2 In the carrageenin-induced inflammation model in rats, intra-pouch injections of acharan sulphate (5 and 50 μg) inhibited the pouch fluid accumulation and the granulation tissue formation as well as the angiogenesis in the granulation tissue at day 6 in a dose-dependent manner.

3 The inhibitory effects of acharan sulphate at 50 μg on the pouch fluid accumulation and the leucocyte infiltration into the pouch fluid was not so effective as that of the cyclo-oxygenase inhibitor indomethacin at 100 μg, but the inhibitory effects of acharan sulphate at 50 μg on the granulation tissue formation and angiogenesis in the granulation tissue were almost the same as those of indomethacin at 100 μg.

4 Acharan sulphate did not affect levels of vascular endothelial growth factor (VEGF) in the granulation tissue and in the pouch fluid at day 6, but indomethacin significantly lowered them.

5 In the cotton thread-induced inflammation model in mice, injections of acharan sulphate (10 μg) at the site of the cotton thread implantation inhibited the granulation tissue formation and angiogenesis as indomethacin (20 μg) did. Acharan sulphate (10 μg) did not affect levels of VEGF in the cotton thread-induced granulation tissue at day 5, but indomethacin (20 μg) significantly lowered them.

6 In culture of human vascular endothelial cells, acharan sulphate at 10 and 100 μg ml^{-1} inhibited VEGF-induced capillary tube formation.

7 These findings suggest that the inhibitory effect of acharan sulphate on angiogenesis in carrageenin- and cotton thread-induced granulation tissues is not due to the inhibition of VEGF protein induction, but is due to the inhibition of VEGF-induced vascular tube formation.

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Abbreviations: bFGF, basic fibroblast growth factor; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor

Introduction

Angiogenesis, the outgrowth of new blood vessels from pre-existing vasculature, is expected to be a promising target for treatment of various diseases including chronic inflammation such as rheumatoid arthritis (Colville-Nash & Scott, 1992), atherosclerosis (Sueishi *et al*., 1997), diabetic retinopathy (Ishibashi *et al*., 1999), psoriasis (Li & Li, 1996) and chronic airway inflammation (Thurston *et al*., 1998), in addition to solid tumour growth (Folkman, 1995). The association of angiogenesis with chronic inflammation promotes the formation of granulation tissue as in pannus formation in rheumatoid arthritis (Colville-Nash & Scott, 1992), and skin in psoriatic diseases (Li & Li, 1996). Angiogenesis in a chronic inflammatory state facilitates migration of inflammatory cells to the inflammatory site and supplies nutrients and oxygen to the granulation tissue (Jackson *et al*., 1997). In fact, the induction and maintenance of these diseases are largely dependent on angiogenesis, and the treatment of these diseases needs the development of new therapeutic agents because of the current lack of satisfactory therapeutic agents.

Glycosaminoglycans are one of the family members of linear anionic polysaccharides that are typically isolated as proteoglycans linked to a protein core. The biological functions of proteoglycans including the regulation of cell growth are expressed by the interaction of the glycosaminoglycan chains in proteoglycans with proteins such as growth factors and their receptors (Hardingham & Fosang, 1992). Acharan sulphate is a glycosaminoglycan which was isolated and purified from the giant African snail *Achatina*

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fulica (Kim *et al*., 1996). It is related to the heparin and heparan sulphate families of glycosaminoglycans, but is distinctly different from all known members of these classes of glycosaminoglycans. Namely, acharan sulphate shows no anti-coagulant activity but inhibits heparin-dependent enhancement of mitogenic activity of basic fibroblast growth factor (bFGF) (Wang *et al*., 1997). Several inhibitors of mitogenic activity of bFGF have been reported including the synthetic polymers, sulphated β-cyclodextrins, sulphated malto-oligosaccharides and suramin (Guimond *et al*., 1993; Venkataraman *et al*., 1996). However, it has been limited to use these inhibitors as bFGF antagonists because they show anti-coagulant activity or toxicity. In cellular level, it is reported that acharan sulphate shows no toxicity at 1 to 5000 ng ml^{-1} in F32 cells (Wang *et al*., 1997).

Vascular endothelial growth factor (VEGF) is a most potent angiogenic factor in physiological and pathological angiogenesis (Breier *et al*., 1992). In a carrageenin-induced air pouch-type inflammation model in rats, we found that indomethacin and cimetidine reduced VEGF production in the granulation tissue, resulting in the suppression of angiogenesis in the granulation tissue (Ghosh *et al*., 2000; 2001). In addition, the angiogenesis in the granulation tissue of a cotton thread-induced inflammation model in mice was highly dependent on VEGF, because the neutralizing antibody of VEGF completely inhibited the angiogenesis (Ghosh *et al*., 2002). Therefore, in this study, by employing the two inflammation models, we analysed the effects of acharan sulphate on VEGF-dependent angiogenesis in the granulation tissue *in vivo* and on VEGF-induced capillary tube formation by human vascular endothelial cells in culture.

Methods

Carrageenin-induced air pouch-type inflammation in rats

The rats were treated in accordance with procedures approved by the Animal Ethics Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan. Air pouch-type inflammation was induced by carrageenin in male Sprague–Dawley rats, specific pathogen-free and weighing 160–170 g (Charles River Japan, Inc., Kanagawa, Japan) according to the procedure described by Tsurufuji *et al*. (1978). Eight milliliters of air was injected subcutaneously into the back to make an air pouch oval in shape. Twenty-four hours later, 4 ml of a 2% (w v^{-1}) solution of carrageenin (Seakem No. 202, Marine Colloids Inc., Springfield, NJ, U.S.A.) in saline was injected into the air pouch. The carrageenin solution had been sterilized by autoclaving at 121°C for 15 min and supplemented with antibiotics (0.1 mg of penicillin G potassium (Meiji Seika, Tokyo, Japan) and 0.1 mg of dihydrostreptomycin sulphate (Meiji Seika) per ml of the solution) after cooling to 40–45°C.

Cotton thread-induced inflammation in mice

The mice were treated in accordance with procedures approved by the Animal Ethics Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan. Cotton thread (No. 8, Araiwa Co., Sendai, Japan) was washed with ethyl acetate for overnight, dried at room temperature, cut into 1 cm in length (7 mg weight) and sterilized by dry heat at 160°C for 2 h. The cotton thread (1 cm in length) was implanted subcutaneously on the back of male 129 inbred mice (25–28 g) (obtained from Dr H. Ohtsu and Dr T. Watanabe, Graduate School of Medicine, Tohoku University, Sendai, Japan) using a 13 G implant needle (Natsume Co, Ltd, Tokyo, Japan) according to the procedure described by Penn & Ashford (1963) with slight modifications (Ghosh *et al*., 2002).

Drug treatment

Drugs used were acharan sulphate sodium salt isolated and purified from the giant African snail *Achatina fulica* according to the procedure described previously (Kim *et al*., 1996), and indomethacin (Sigma Chemical Co., St Louis, MO, U.S.A.). Acharan sulphate was dissolved in saline, and indomethacin was dissolved in ethanol and diluted with saline. Final concentration of ethanol in these drug solution was adjusted to 0.1% (v v^{-1}). Five-hundred microliters saline containing acharan sulphate (0.5–50 μg) or indomethacin (100 μg) was injected into the pouch of each rat just after carrageenin injection and once a day for following five consecutive days at 24 h interval. In case of mice, 100 μl saline containing acharan sulphate (10 μg) or indomethacin (20 μg) was injected into the site of implantation of a cotton thread in each mouse just after cotton thread implantation and once a day for following four consecutive days at 24 h interval. Control rats and mice received the same amount of saline containing 0.1% (v v^{-1}) ethanol. The doses of acharan sulphate were set according to the preliminary experiment using various doses of acharan sulphate, and the dose of indomethacin was set according to our previous report (Ghosh *et al*., 2000).

Determination of carrageenin-induced pouch fluid accumulation, leucocyte infiltration and granulation tissue formation

Six days after the injection of carrageenin solution, total pouch fluid was collected and its volume measured and the infiltrating leucocytes in the pouch fluid were enumerated using a hemocytometer (Ghosh *et al*., 2000). The entire granulation tissue was dissected and weighed (Ghosh *et al*., 2000).

Determination of angiogenesis in carrageenin-induced granulation tissue

Measurement and visualization of the angiogenesis in the granulation tissue were carried out using carmine dye (Natural Red 4, Sigma Chemical Co.) according to the method described by Kimura *et al*. (1986) and Colville-Nash *et al*. (1995) with slight modifications (Ghosh *et al*., 2000). Six days after injection of the carrageenin solution, 3 ml of a 5% (w v^{-1}) solution of carmine dye in saline containing 5% (w v^{-1}) gelatin (Sankoh Jun-yaku, Tokyo, Japan) at 37°C was injected into the tail vein of each rat. The carcasses were chilled on ice for 3 h, and the entire granulation tissue was dissected and weighed. After being washed with phosphate-buffered saline (PBS, pH 7.4), the granulation tissue was

homogenized in 2 volumes of 0.5 mM sodium hydroxide using a Vir-Tis 45 homogenizer (The Virtis Company, Gardiner, NY, U.S.A.) for 4 min at scale 40 on an ice bed. The tissue homogenate was centrifuged at $10,000 \times g$ and 4°C for 30 min. Five-hundred microliters of the supernatant was diluted 2 fold with 0.5 mM sodium hydroxide and centrifuged again at $14,000 \times g$ and 4°C for 30 min. The dye content in 200 μl of the supernatant was determined spectrophotometrically by measuring absorbance at 490 nm. For the standard curve, known amounts of carmine dye were added to the final supernatant of the granulation tissue homogenates of control rats which had been injected with 3 ml of a 5% (w v^{-1}) gelatin solution in saline without carmine dye, and the absorbance at 490 nm was determined. The amount of carmine dye in the whole granulation tissue was then calculated.

For visualization, the granulation tissues were fixed in 10% (v v^{-1}) formalin in PBS (pH 7.4) for 48 h at 4°C. The samples were dehydrated by continuous immersion in 70% (v v^{-1}) ethanol for 48 h, 90% (v v^{-1}) ethanol for 48 h and pure ethanol for 48 h. After dehydration, the samples were cleared by immersion in cedarwood oil (Sigma Chemical Co.) for 14 days. Retention of carmine dye within the vascular bed was observed with a light microscope ($40 \times$ magnification).

Determination of cotton thread-induced granulation tissue formation and assessment of angiogenesis in the granulation tissue

Mice were sacrificed at specified days after the implantation of cotton thread. The granulation tissue was dissected together with the cotton thread and weighed. Angiogenesis was assessed by taking photographs of vascular network formed around the cotton thread and beneath the skin after dissecting the cotton thread. The granulation tissue with cotton thread was dissected, washed with PBS (pH 7.4), cut into small pieces with scissors, and homogenized in 20 volumes of 0.5 mM sodium hydroxide using a Vir-Tis 45 homogenizer (The Virtis Company) for 4 min at scale 40 on an ice bed. The tissue homogenate was centrifuged at $10,000 \times g$ and 4°C for 30 min. Two-hundred microliters of the supernatant was centrifuged again at $14,000 \times g$ and 4°C for 30 min. The haemoglobin concentration in the supernatant was determined spectrophotometrically by measuring absorbance at 540 nm using a haemoglobin assay kit (Haemoglobin B Test Wako, Wako Pure Chemical Ind., Tokyo, Japan). The content of haemoglobin in the granulation tissue was expressed as μg haemoglobin per mg wet tissue.

Western blot analysis of VEGF levels

Protein levels in the supernatant of the homogenate of the granulation tissue were determined according to the method described by Bradford (1976). Proteins, at 4.6 μg aliquot for the carrageenin-induced granulation tissue and 1.4 μg aliquot for the pouch fluid, and 0.56 μg aliquot for the cotton thread-induced granulation tissue were separated by electrophoresis on a 12% (w v^{-1}) sodium dodecylsulphate-polyacrylamide gel and transferred onto a nitrocellulose membrane (Schleicher and Schuell Inc., Dassel, Germany). The membrane was incubated at 4°C for 12 h with mouse monoclonal anti-VEGF (1 : 200, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), then incubated at 4°C for 3 h with biotinylated anti-mouse IgG (1 : 2000, Vector Laboratories Inc., CA, U.S.A.), and in avidin–biotin–peroxidase complex (Vector Laboratories Inc.) for 30 min at room temperature. The reaction product was visualized by using an ECL kit (ECL System, Amersham, Arlington Heights, IL, U.S.A.).

Determination of capillary tube formation of human vascular endothelial cells in culture

The capillary tube formation was determined by using an angiogenesis kit (Kurabo Co., Osaka, Japan) according to the manufacturer's instruction. The culture medium in each well of a 24-well cluster dish in which human vascular endothelial cells and fibroblasts had been seeded in the optimal condition for the capillary tube formation, was changed with the fresh medium containing human recombinant $VEGF_{165}$ (10 ng ml^{-1}, PeproTech EC Ltd, London, U.K.) and acharan sulphate (0.1–100 μg ml^{-1}) or suramin (5 μM) (Sigma Chemical Co.) at days 1, 4, 7 and 9. At day 11, the capillary tubes formed were detected by immunostaining of CD31. For scoring the capillary tube formation, five pictures at $40 \times$ magnification per each well were taken and the number of points that the capillary tube crossed the grid (11 horizontal lines and 15 vertical lines) was counted. The mean point number from five pictures is expressed as the mean score.

Statistical analysis

The statistical significance of the results was analysed by Dunnett's test for multiple comparisons and Student's *t*-test for unpaired observations.

Results

Effects of acharan sulphate and indomethacin on pouch fluid volume, number of infiltrating leucocytes and granulation tissue weight in carrageenin-induced inflammation in rats

Intra-pouch injections of acharan sulphate (5 and 50 μg) decreased the pouch fluid accumulation (Figure 1A), the leucocyte infiltration into the pouch fluid (Figure 1B), and the granulation tissue formation (Figure 1C) 6 days after carrageenin injection in a dose-dependent manner. The inhibitory effects of acharan sulphate at a dose of 50 μg on the pouch fluid accumulation (Figure 1A), and the leucocyte infiltration into the pouch fluid (Figure 1B) were significantly less than those of the cyclo-oxygenase inhibitor indomethacin at a dose of 100 μg.

Effects of acharan sulphate and indomethacin on angiogenesis in granulation tissue in carrageenin-induced inflammation in rats

Acharan sulphate decreased the carmine dye content in the whole granulation tissue in a dose-dependent manner (Figure 2). The inhibition by acharan sulphate (50 μg) was almost the same as that by indomethacin (100 μg) (Figure 2).

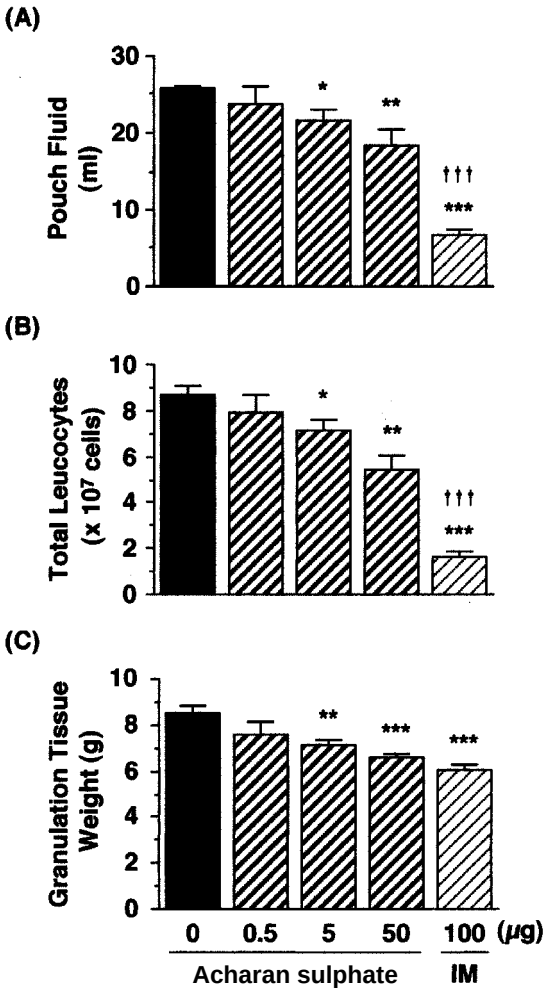

Figure 1 Effects of acharan sulphate and indomethacin on pouch fluid volume, number of infiltrating leucocytes, and granulation tissue weight 6 days after carrageenin injection. Four milliliters of a 2% (w v^{-1}) carrageenin solution in saline was injected into the air pouch. Acharan sulphate (0.5, 5, and 50 μg) or indomethacin (IM, 100 μg) dissolved in 500 μl of saline was injected into the pouch of each rat just after carrageenin injection and once a day for following five consecutive days. Pouch fluid volume (A), total number of leucocytes in the pouch fluid (B), and granulation tissue weight (C) were determined 6 days after injection of the carrageenin solution. Values are the means from six rats with s.e.mean shown by vertical bars. Statistical significance: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ *versus* the corresponding control (Dunnett's test), and $\dagger\dagger\dagger P<0.001$ *versus* acharan sulphate (50 μg) (Student's *t*-test).

Vascular network formation as examined by a light microscope was also suppressed by acharan sulphate (50 μg), and the effect of acharan sulphate at a dose of 50 μg was almost the same as that of indomethacin at a dose of 100 μg (Figure 3).

Effects of acharan sulphate and indomethacin on VEGF levels in the granulation tissue and the pouch fluid in carrageenin-induced inflammation in rats

Treatment with acharan sulphate (0.5–50 μg) did not lower the VEGF levels in the granulation tissue (Figure 4A) and in the pouch fluid (Figure 4B) at day 6, but indomethacin (100 μg) significantly lowered the levels both in the granulation tissue (Figure 4B) and in the pouch fluid (Figure 4B).

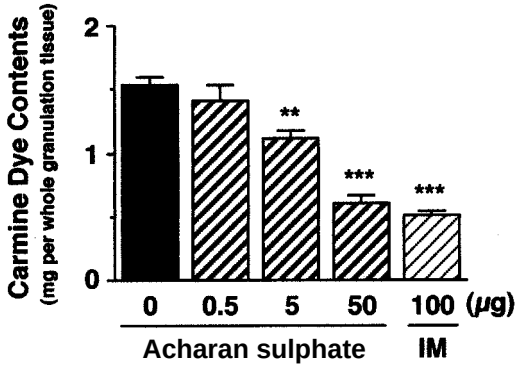

Figure 2 Effects of acharan sulphate and indomethacin on angiogenesis in the granulation tissue 6 days after carrageenin injection. Four milliliters of a 2% (w v^{-1}) carrageenin solution in saline was injected into the air pouch. Acharan sulphate (0.5, 5 and 50 μg) or indomethacin (IM, 100 μg) dissolved in 500 μl of saline was injected into the pouch of each rat just after carrageenin injection and once a day for following five consecutive days. Six days after injection of the carrageenin solution, 3 ml of prewarmed saline at 37°C containing 5% (w v^{-1}) carmine dye and 5% (w v^{-1}) gelatin was injected intravenously into each anaesthetized rat. The granulation tissue was dissected, and total carmine dye content was determined as described in Methods. Values are the means from six rats with s.e.mean shown by vertical bars. Statistical significance: $^{**}P<0.01$ and $^{***}P<0.001$ *versus* control (Dunnett's test).

Effects of acharan sulphate and indomethacin on the formation of granulation tissue, VEGF levels, and angiogenesis in cotton thread-induced inflammation in mice

Local injections of acharan sulphate (10 μg) inhibited the formation of granulation tissue (Figure 5A) and angiogenesis in the granulation tissue (Figure 5B,D) at day 5. The inhibitory effects of acharan sulphate (10 μg) on the formation of granulation tissue and angiogenesis in the granulation tissue were almost same as those of indomethacin (20 μg) (Figure 5A,B,D). Treatment with acharan sulphate (10 μg) did not lower the VEGF levels at day 5 in the granulation tissue (Figure 5C), but indomethacin (20 μg) significantly lowered the levels (Figure 5C).

Effects of acharan sulphate and suramin on the VEGF-induced capillary tube formation of human vascular endothelial cells in culture

The capillary tube formation at day 11 was significantly increased by VEGF at 10 ng ml^{-1} (Figure 6). Acharan sulphate decreased the VEGF-induced capillary tube formation in a concentration-dependent manner at 10 to 100 μg ml^{-1} (Figure 6). The inhibitory effects of acharan sulphate at 100 μg ml^{-1} was almost the same with that of suramin at 5 μM (Figure 6). The effects of acharan sulphate at 10–100 μg ml^{-1} and suramin at 5 μM on the viability of the human dermal microvascular endothelial cells (TaKaRa Biomedicals, Tokyo, Japan) were examined by the ability of mitochondrial succinate dehydrogenase to cleave 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

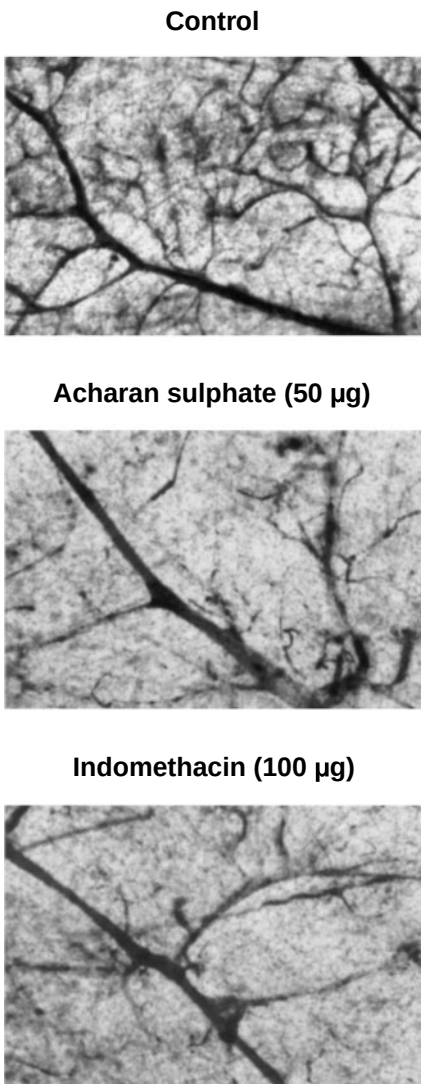

Figure 3 Effects of acharan sulphate and indomethacin on angiogenesis in the granulation tissue 6 days after carrageenin injection. Four milliliters of a 2% (w v^{-1}) carrageenin solution in saline was injected into the air pouch. Acharan sulphate (50 μg) or indomethacin (100 μg) dissolved in 500 μl of saline was injected into the pouch of each rat just after carrageenin injection and once a day for following five consecutive days. Six days after injection of the carrageenin solution, 3 ml of prewarmed saline at 37°C containing 5% (w v^{-1}) carmine dye and 5% (w v^{-1}) gelatin was injected intravenously into each anaesthetized rat. The granulation tissue was dissected and cleared in cedarwood oil as described in Methods. The angiogenesis in the granulation tissue was observed by a light microscope (40 × magnification). Representative micrographs are shown from four rats.

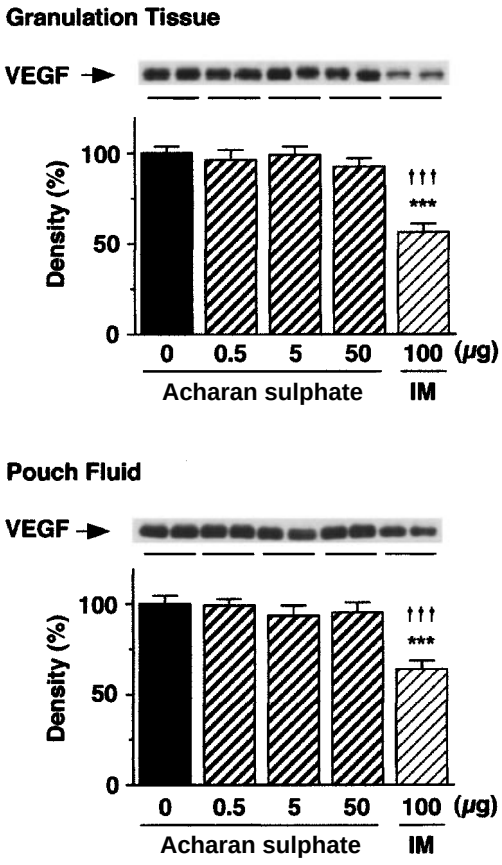

Figure 4 Effects of acharan sulphate and indomethacin on VEGF levels in the granulation tissue and in the pouch fluid 6 days after carrageenin injection. Four milliliters of a 2% (w v^{-1}) carrageenin solution in saline was injected into the air pouch. Acharan sulphate (0.5, 5, and 50 μg) or indomethacin (IM, 100 μg) dissolved in 500 μl of saline was injected into the pouch of each rat just after carrageenin injection and once a day for following five consecutive days. The granulation tissue 6 days after injection of the carrageenin solution was dissected, homogenized and centrifuged as described in Methods. VEGF levels in the supernatant of the homogenate of the granulation tissue (A) and the pouch fluid (B) were immunoblotted and analysed densitometrically. The immunoblots of VEGF in the granulation tissue and the pouch fluid from two rats in each group are shown at the top, respectively. Values are the means from six rats with s.e.mean shown by vertical bars. The mean density in the control group is set to 100%. Statistical significance: ***$P<0.001$ *versus* the corresponding control (Dunnett's test), and †††$P<0.001$ versus acharan sulphate (50 μg) (Student's *t*-test).

(MTT, Sigma Chemical Co.) to the blue compound formazan (Mosmann, 1983), and no significant changes in the viability of the cells was observed (data not shown).

Discussion

In this study, using an air pouch-type carrageenin-induced inflammation model in rats and a cotton thread-induced inflammation model in mice, we demonstrated that the new glycosaminoglycan, acharan sulphate from *Achatina fulica*, inhibits angiogenesis in the proliferative inflammatory granulation tissue. Injection of the carrageenin solution into an air pouch induced gradual increases in the pouch fluid volume and the granulation tissue weight as well as the angiogenesis in the granulation tissue (Ghosh *et al*., 2000). The increase in the dye content in the granulation tissue correlates with the increase in the capillary density (Ghosh *et al*., 2000).

The intra-pouch injections of acharan sulphate or indomethacin resulted in the decrease in the pouch fluid accumulation, the leucocyte infiltration into the pouch fluid, the granulation tissue weight, and the angiogenesis in the granulation tissue in rats (Figures 1, 2 and 3). The anti-inflammatory effects of acharan sulphate at doses examined (5 and 50 μg) were less than those of indomethacin at

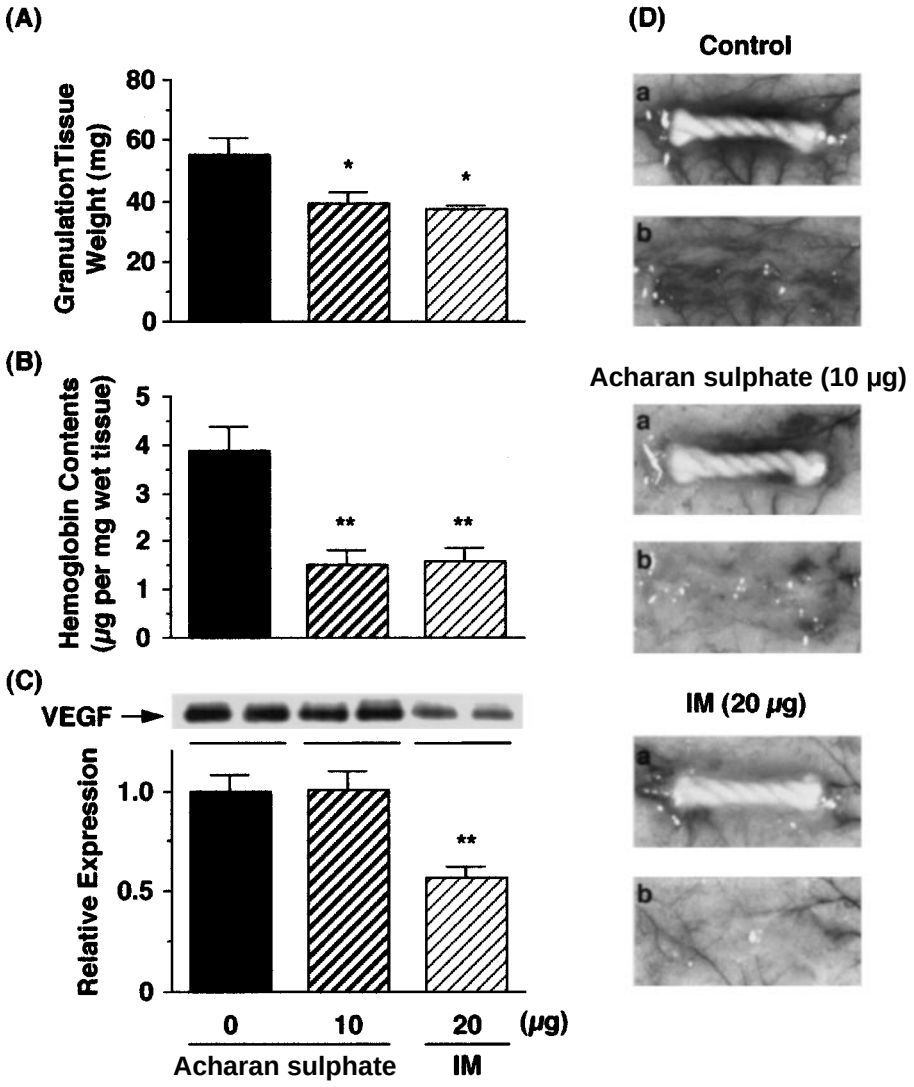

Figure 5 Effects of acharan sulphate and indomethacin on granulation tissue formation, angiogenesis and VEGF levels in the granulation tissue 5 days after the cotton thread implantation. A cotton thread (1 cm, 7 mg) was implanted subcutaneously in the dorsum of mice. Acharan sulphate (10 μg) or indomethacin (IM, 20 μg) dissolved in 100 μl of saline was injected into the site of the cotton thread implantation just after the implantation and once a day for following four consecutive days. The mice were sacrificed 5 days after cotton thread implantation. The granulation tissue weight (A), haemoglobin content in the granulation tissue (B), VEGF levels in the granulation tissue (C), and the vascular network formation around the cotton thread (D,a) and beneath the skin after cotton thread dissection (D,b), were determined. VEGF levels in the granulation tissue were determined by immunoblotting and analysed densitometrically. The values are expressed as relative expression to the control. Representative immunoblots from two mice in each group are shown at the top of (C). The mean VEGF levels in the granulation tissue in control mice is set to 1.0. Values are the means from four mice with s.e.mean shown by vertical bars. Statistical significance: $*P<0.05$ and $**P<0.01$ *versus* control (Dunnett's test).

100 μg, but the inhibitory effect of acharan sulphate at 50 μg on angiogenesis was as effective as that of indomethacin at 100 μg (Figures 1, 2 and 3). These findings indicate that the inhibitory effect of acharan sulphate on angiogenesis is more prominent than that of its anti-inflammatory effect. In addition, acharan sulphate (10 μg) showed similar inhibitory effects on granulation tissue formation and VEGF-dependent angiogenesis in the cotton thread-induced inflammation in mice as indomethacin (20 μg) did (Figure 5). These findings indicate that acharan sulphate has an anti-angiogenic activity both in carrageenin-induced granulation tissue in rats and in cotton thread-induced granulation tissue in mice.

Indomethacin significantly reduced the level of VEGF in the granulation tissue and in the pouch fluid at day 6 of carrageenin-induced inflammation in rats (Figure 4) and in the cotton thread-induced granulation tissue in mice at day 5 (Figure 5C), but acharan sulphate did not (Figures 4 and 5C). We reported that indomethacin inhibits the angiogenesis in the carrageenin-induced granulation tissue in rats by inhibiting cyclo-oxygenase-2-dependent production of prostaglandin E_2 which mediates VEGF production (Ghosh *et al*., 2000). These findings suggest that acharan sulphate inhibits the activity of VEGF without affecting its production. It is reported that the dimer of bFGF, a mitogenically active form, expresses the binding surface for glycosaminoglycans

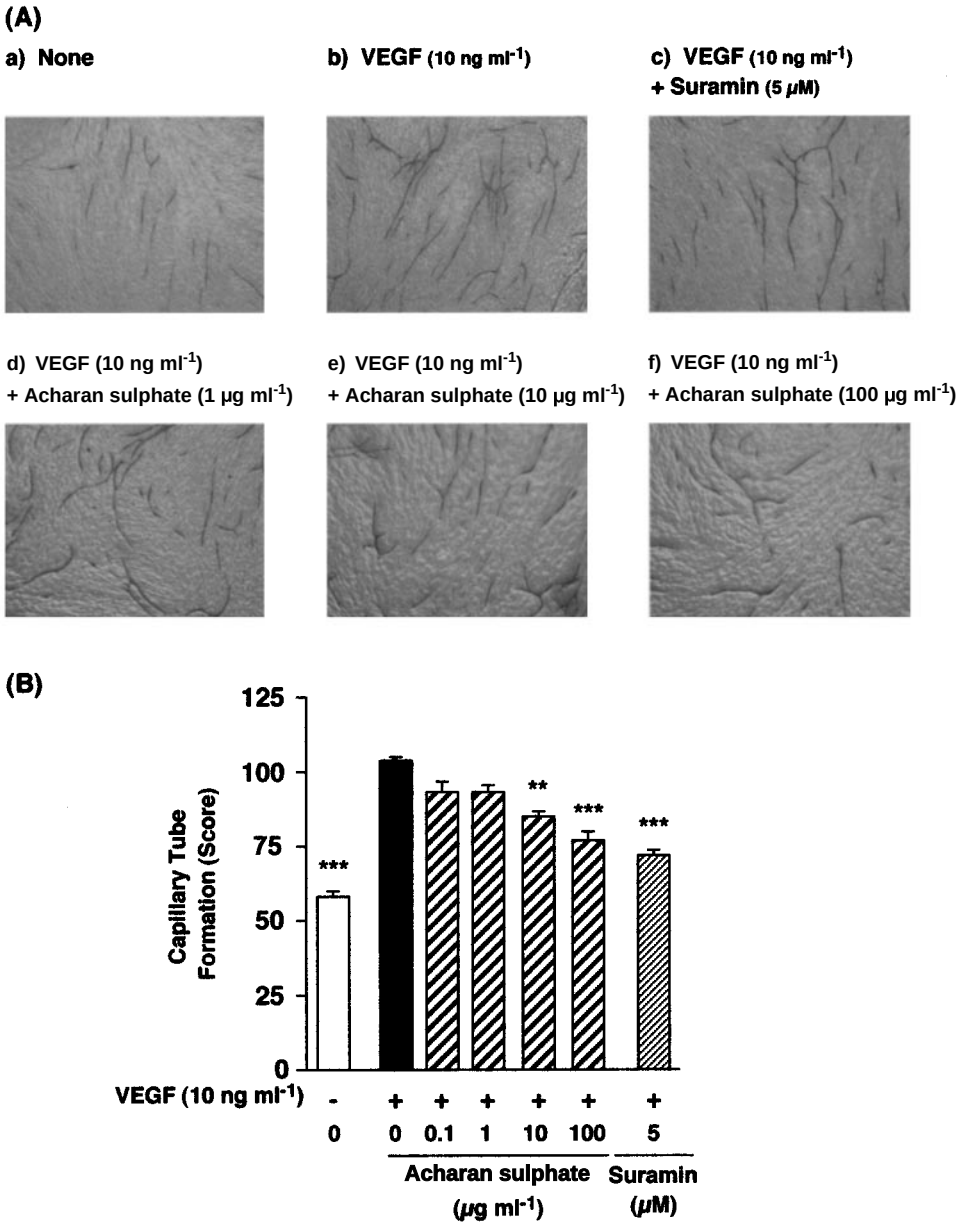

Figure 6 Effects of acharan sulphate and suramin on the VEGF-induced capillary tube formation of human vascular endothelial cells. The coculture of human vascular endothelial cells and fibroblasts was stimulated by human recombinant $VEGF_{165}$ (10 ng ml^{-1}). The medium was changed with fresh medium containing $VEGF_{165}$ (10 ng ml^{-1}) and the indicated concentration of acharan sulphate or suramin (5 μM) at days 1, 4, 7 and 9. At day 11, the capillary tube formation was detected by immunostaining of CD31 (A). Representative micrographs are shown from five samples. The capillary tube formation was scored as described in Methods (B). Values are the means from five samples with s.e.mean shown by vertical bars. Statistical significance: $^{**}P<0.01$ and $^{***}P<0.001$ *versus* VEGF (10 ng ml^{-1}) (Dunnett's test).

when stabilized with heparin (Faham *et al*., 1996), and the interaction of acharan sulphate with the binding surface inhibits the mitogenic activity of bFGF (Wang *et al*., 1997). Because the dimer of VEGF was also stabilized by heparin (Fairbrother *et al*., 1998), it is possible that acharan sulphate binds the dimer of VEGF and inhibits the mitogenic activity. The finding that acharan sulphate inhibited the VEGF-induced capillary tube formation in culture (Figure 6) might suggest that acharan sulphate inhibits the mitogenic activity of VEGF on vascular endothelial cells. As we have not examined the effect of indomethacin on the VEGF-induced capillary tube formation, the role of arachidonate metabolites in the VEGF-induced capillary tube formation remains to be elucidated.

In conclusion, acharan sulphate inhibits angiogenesis in the inflammatory granulation tissues. It is not due to the inhibition of VEGF production, but possibly due to the inhibition of capillary tube formation of vascular endothelial cells.

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