—Reviews—

Regulation by Prostaglandin E_2 and Histamine of Angiogenesis in Inflammatory Granulation Tissue

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In an air pouch-type carrageenin-induced inflammation model in rats, the selective cyclooxygenase (COX)-2 inhibitor NS-398 dose dependently inhibited the granulation tissue formation, angiogenesis and the level of vascular endothelial growth factor (VEGF) in the granulation tissue. In culture of the minced granulation tissue, PGE_2 induced VEGF production in a concentration-dependent manner. Histamine also induced VEGF production in the granulation tissue *in vitro*. The H2 receptor antagonist cimetidine, the cAMP antagonist Rp-cAMP and the protein kinase A inhibitor H-89 suppressed the histamine-induced VEGF production in the granulation tissue. However, the H1 receptor antagonist pyrilamine maleate, the H3 receptor antagonist thioperamide, the protein kinase C inhibitors Ro 31–8425 and calphostin C or the tyrosine kinase inhibitor genistein showed no effect. Subcutaneous implantation of a cotton thread in the dorsum of histidine decarboxylase-deficient ($HDC^{-/-}$) mice, but not in mast cell-deficient ($WBB6F_1$-W/W^v) mice, induced less angiogenesis with lower levels of VEGF in the granulation tissue than in their corresponding wild-type ($HDC^{+/+}$ and $WBB6F_1$-$^{+/+}$) mice. In $HDC^{-/-}$ mice, the topical injection of histamine or the H2 receptor agonist dimaprit rescued the defective angiogenesis and granulation tissue formation. In addition, cimetidine but not pyrilamine maleate and thioperamide inhibited the histamine-induced angiogenesis in the granulation tissue in $HDC^{-/-}$ mice. These findings suggest that PGE_2 and histamine augment angiogenesis in the inflammatory granulation tissue by inducing VEGF production, and histamine induces VEGF production possibly through the H2 receptor—cAMP—protein kinase A pathway.

Key words——angiogenesis; cyclooxygenase-2; histamine; granulation tissue; vascular endothelial growth factor

INTRODUCTION

Angiogenesis in a chronic inflammatory state facilitates migration of inflammatory cells to the inflammatory site and supplies nutrients and oxygen to the granulation tissue, thus, the progression of chronic and proliferative inflammation depends on angiogenesis.[1] Therefore, the suppression of angiogenesis is important to suppress chronic inflammatory diseases[2—6] and as well as tumor growth.[7]

Cyclooxygenase (COX) which converts arachidonic acid to prostaglandin (PG) H_2, has two isoforms, COX-1 and COX-2.[8] COX-2 is induced at the inflammatory site in several experimental inflammation models[9—11] and in patients with rheumatoid arthritis.[12] The injection of carrageenin into a subcutaneous air pouch of rats increased the protein levels of COX-2 in the cells lining the inner layer of the pouch and in macrophages infiltrating the pouch fluid.[11] In the recurrence of allergic inflammation model in rats, we reported that COX-2 protein is induced in the granulation tissue by antigen challenge and COX-2-derived PGE_2 participates in the vascular formation and the development of the granulation tissue.[10] It is also reported that E-type PGs, such as PGE_1 and PGE_2, enhance the angiogenesis in rabbit corneas[13] and chorioallantoic membrane of 8-day-old chicken embryos.[14] In addition, COX-2 modulates production of angiogenic factors by colon cancer cells[15] and basic fibroblast growth factor (bFGF)-induced angiogenesis.[16]

Histamine plays a variety of roles as an autacoid which regulates allergic inflammatory reactions,[17,18]

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differentiation of leukocytes precursors[19] and gastric acid secretion,[20] and as a neurotransmitter in the central nervous system.[21] In addition, histamine is produced in rapidly growing tissues, and suggested to promote neoplastic growth and angiogenesis.[17,22—24] Zauberman et al.[25] first reported that histamine is angiogenic in rabbit cornea. However, the roles of PGE_2 and histamine in angiogenesis in chronic inflammation has not been fully clarified. Therefore, in this review, by employing an air pouch-type carrageenin-induced inflammation model in rats, and cotton thread-induced inflammation model in histidine decarboxylase-deficient mice ($HDC^{-/-}$), mast cell-deficient mice ($WBB6F_1$-W/W^V), and their corresponding wild-type mice ($HDC^{+/+}$ and $WBB6F_1$-$^{+/+}$), we describe the regulatory roles of PGE_2 and histamine in angiogenesis in the inflammatory granulation tissue focusing in the production of VEGF.

MATERIALS AND METHODS

COX-2-Derived PGE_2 Upregulates Angiogenesis in Carrageenin-Induced Granulation Tissue in Rats In an air pouch-type carrageenin-induced inflammation model in rats,[26] we demonstrated that COX-2-derived PGE_2 plays a role in angiogenesis in the developing chronic granulation tissue.[27] From the determination of dye content in the granulation tissue and histological analysis, it was concluded that the selective COX-2 inhibitor NS-398 as well as the COX-1/COX-2 non-specific inhibitor indomethacin inhibit the angiogenesis in the granulation tissue (Fig. 1) and inflammatory responses.[27]

In the granulation tissue in the air pouch-type carrageenin-induced inflammation model in rats, COX-2-derived PGE_2 was suggested to be involved in VEGF production, because NS-398 as well as indomethacin significantly reduced VEGF contents in

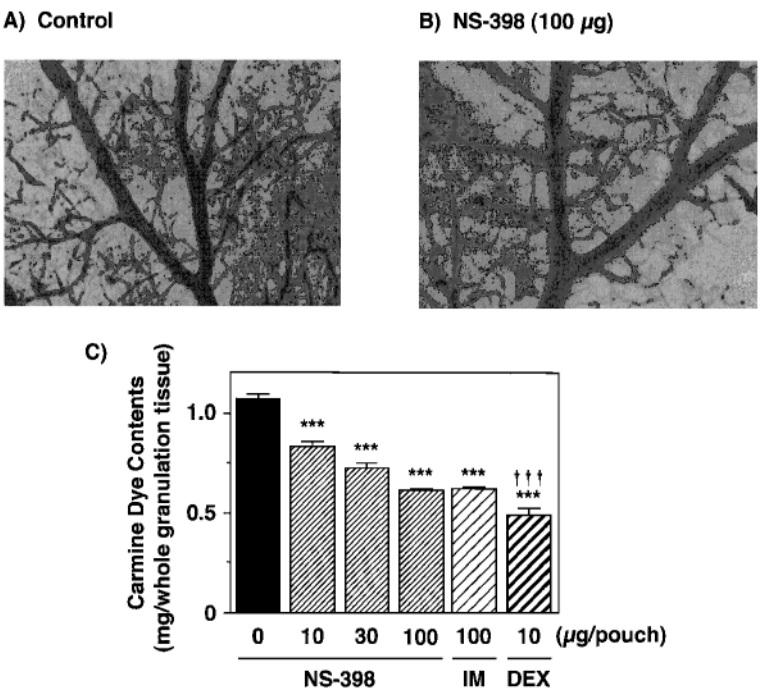

Fig. 1. Effects of NS-398, Indomethacin and Dexamethasone on Angiogenesis in the Granulation Tissue 6 Days after Carrageenin Injection.[27]

Four milliliters of a 2% (w/v) carrageenin solution in saline was injected into the air pouch. NS-398 (10, 30, and 100 μg), indomethacin (IM, 100 μg) or dexamethasone (DEX, 10 μg) dissolved in 500 μl of saline was injected into the pouch 0, 2 and 4 days after carrageenin injection. Six days after carrageenin injection, 3 ml of a 5% (w/v) carmine dye solution in 5% (w/v) gelatin in saline was injected intravenously into each anesthetized rat. (A and B) The granulation tissue was dissected and cleared in cedarwood oil. The angiogenesis in the granulation tissue was observed by a light microscope (40× magnification). (C) The total carmine dye contents in the granulation tissue was determined. Values are the means with S.E.mean shown by vertical bars from nine rats. Statistical significance: $^{***}p<0.001$ versus control and $^{\dagger\dagger\dagger}p<0.001$ versus NS-398 or IM.

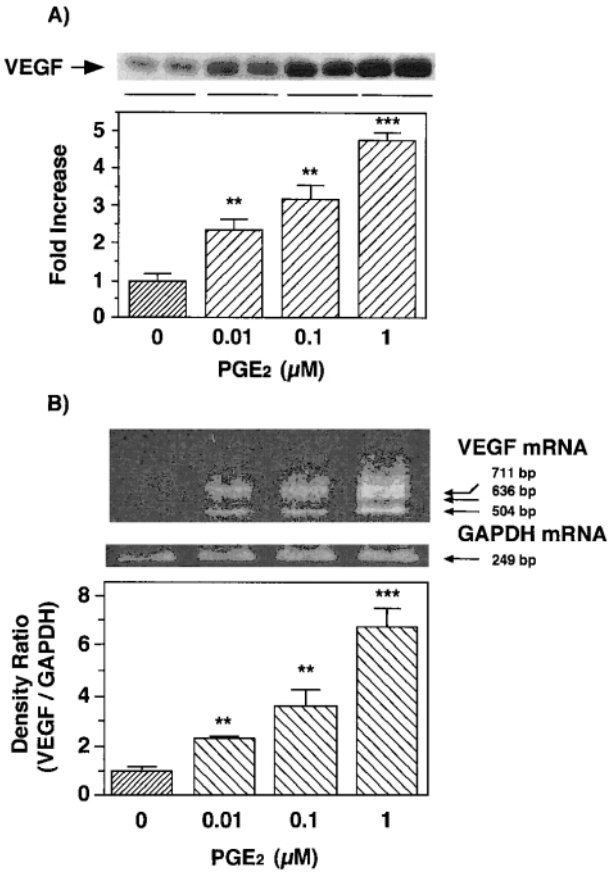

Fig. 2. Effects of Various Concentrations of PGE_2 on VEGF Production in the Minced Granulation Tissue (1 to 2-mm).[27]

The minced granulation tissue (0.4 g) was incubated in 4 ml of EMEM containing 10% (v/v) calf serum at 37℃ for 3 h. After three washes, the tissue was further incubated at 37℃ for 6 h (A) and 1 h (B) in medium containing the indicated concentrations of PGE_2. (A) VEGF protein in the conditioned medium was detected by immunoblotting and analyzed densitometrically. Representative immunoblots from two samples of each group are shown at the top. The mean VEGF protein level in the conditioned medium in the control group is set to 1.0. (B) VEGF mRNA levels in the minced granulation tissue were determined by RT-PCR and analyzed densitometrically. Representative VEGF mRNA bands are shown at the top. The mean density ratio of mRNA for VEGF to that for GAPDH in the control group is set to 1.0. Values are the means from four samples with S.E.mean shown by vertical bars. Statistical significance: $^{**}p<0.01$, $^{***}p<0.001$ versus the corresponding control.

the granulation tissue and in the pouch fluid.[27] To clarify whether PGE_2 is involved in VEGF production, the granulation tissue from the indomethacin-treated rats in which the effect of endogenous PGE_2 on the VEGF production might be minimized, was excised, minced into 1 to 2-mm pieces with a pair of small scissors and incubated in the presence and absence of PGE_2. It was demonstrated that PGE_2 increased both the VEGF mRNA and its protein levels (Fig. 2). These findings suggested that NS-398 reduced angiogenesis by inhibiting COX-2-dependent PGE_2 production resulting in the reduction of VEGF production. NS-398 and indomethacin at 100 μg inhibited PGE_2 production almost completely[27] but VEGF production[27] and angiogenesis were inhibited only about 50% (Fig. 1). Thus, in the carrageenin-induced inflammation model in rats, COX-2-derived PGE_2 partially participates in the angiogenesis in the granulation tissue possibly by stimulating VEGF production.

Regulation by Histamine of VEGF Expression in Carrageenin-Induced Granulation Tissue via H2 Receptors VEGF is a secreted protein[28] with several isoforms translated from alternatively spliced mRNAs.[29] In rats, there are three isoforms of VEGF protein, VEGF188, VEGF164 and VEGF120, of which mRNA are 711, 636 and 504 bp, respectively.[30] In culture of carrageenin-induced granulation tissue, histamine (1 and 10 μM) increased the levels of VEGF protein and three isoforms of VEGF mRNA.[31] In the ovarian epithelial carcinoma tissue in humans, Sowter et al.[32] reported that VEGF164 and VEGF120 play a significant role in the angiogenesis. However, in rats, we could not clarify each role of the three isoforms of VEGF in the angiogenesis in the carrageenin-induced granulation tissue. The VEGF-producing cells in the granulation tissue stimulated by histamine were macrophages, endothelial cells and fibroblasts,[31] as reported in bFGF-induced granulation tissue in rats.[33] In isolated macrophages and fibroblasts in rats, histamine at 1 and 10 μM increased the levels of VEGF mRNA.[31]

In the carrageenin-induced granulation tissue, histamine induced expression of VEGF protein through the H2 receptor, because the H2 receptor antagonist cimetidine (Fig. 3B) but not the H1 receptor antagonist pyrilamine or the H3 receptor antagonist thioperamide, inhibited the histamine-induced VEGF production.[31] The possibility that histamine induces the production of VEGF by increasing PGE_2 production by the minced granulation tissue (1 to 2-mm) was excluded because indomethacin at 1 μM, high enough to inhibit PGE_2 production by macrophages

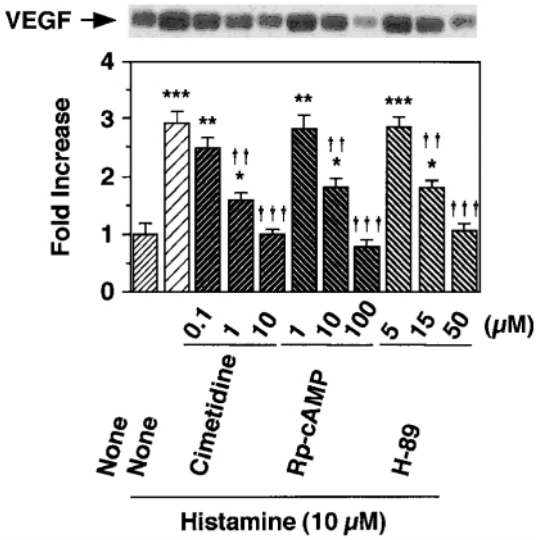

Fig. 3. Effects of Various Concentrations of Cimetidine, RpcAMP and H-89 on Histamine-Induced VEGF Production[32]

The minced granulation tissue (1 to 2-mm, 0.4 g) was incubated in 4 ml of EMEM containing 10% (v/v) calf serum at 37℃ for 3 h. After three washes, the tissue was further incubated at 37℃ for 6 h in medium containing histamine (10 μM) in the presence of cimetidine (0.1—10 μM), Rp-cAMP (1—100 μM) or H-89 (5—50 μM). VEGF protein in the conditioned medium was detected by immunoblotting and analyzed densitometrically. Representative immunoblots are shown at the top. The mean VEGF protein level in the conditioned medium in the control group is set to 1.0. Values are the means from four samples with S.E.mean shown by vertical bars. Statistical significance: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ versus control and $\dagger\dagger p<0.01$, $\dagger\dagger\dagger p<0.001$ versus histamine (10 μM) alone.

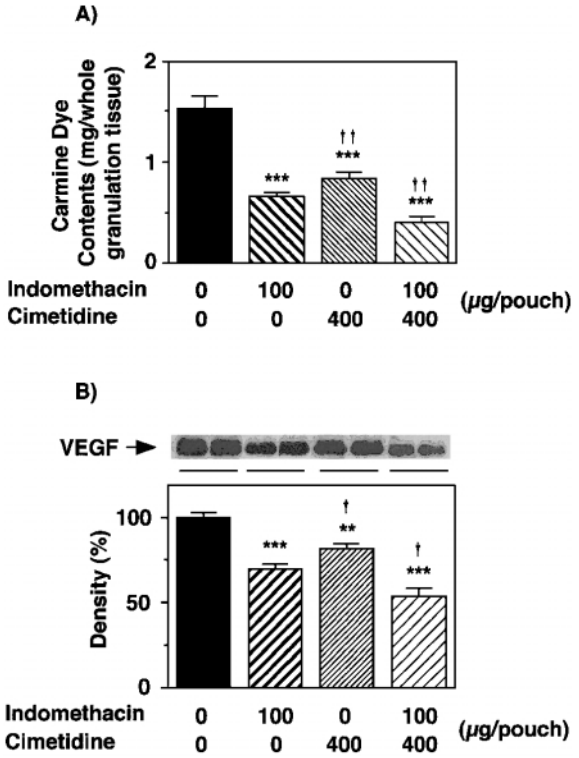

Fig. 4. Effects of Cimetidine and Indomethacin on Angiogenesis and VEGF Protein Levels in the Granulation Tissue 6 Days after Carrageenin Injection[32]

Four milliliters of a 2% (w/v) carrageenin solution in saline was injected into the air pouch. Five-hundred microliters saline containing 400 μg cimetidine, 100 μg indomethacin or both was injected into the pouch every day after the injection of carrageenin. (A) Six days after injection of the carrageenin solution, 3 ml of prewarmed saline at 37℃ containing 5% (w/v) carmine dye and 5% (w/v) gelatin was injected intravenously into each anesthetized rat. The granulation tissue was dissected, homogenized, centrifuged and total carmine dye content was determined. (B) VEGF protein levels in the supernatant fraction of the homogenate of the granulation tissue was immunoblotted and analyzed densitometrically. The immunoblots of VEGF proteins in the granulation tissue two rats in each group are shown on the top. Values are the means with S.E.mean shown by vertical bars from five to six rats. The mean density in the control group is set to 100% (B). Statistical significance: $^{**}p<0.01$, $^{***}p<0.001$ versus control and $\dagger p<0.05$, $\dagger\dagger p<0.01$ versus indomethacin (100 μg).

almost completely,[34] did not inhibit the histamine-induced VEGF production.[31] In addition, although histamine induces PGE_2 production in various cells, the action is not mediated by H2 receptors.[35] Therefore, it is likely that histamine directly induces VEGF production via H2 receptors. In general, it is accepted that the stimulation of H2 receptor increases cAMP levels.[36] We found that the induction of VEGF protein by histamine in the granulation tissue is cAMP-mediated, because the histamine-induced VEGF production was markedly inhibited by the cAMP antagonist Rp-cAMP and the PKA inhibitor H-89 (Fig. 3). In contrast, PKC and the protein tyrosine kinase do not play critical roles in the VEGF induction by histamine because the PKC inhibitors Ro 31-8425 (10 μM) and calphostin C (10 μM), and the protein tyrosine kinase inhibitor genistein (30 μM) did not inhibit the histamine-induced VEGF production.[31] These findings suggest that the histamine-induced production of VEGF in the granulation tissue is dominantly mediated by the cAMP—PKA pathway. This hypothesis is supported by the observation that histamine modulates the expression of *c-fos* through the production of cAMP via H2 receptors in a human promonocytic cell line U937[36] and *c-fos* participates in the induction of VEGF.[37] PGE_2 also induces VEGF production in a human monocytic cell line and lung tissues through the cAMP—PKA pathway.[38] Therefore, histamine and PGE_2 might activate a common signal pathway for VEGF production.

We also indicated that endogenous histamine plays

a significant role in the angiogenesis in the carrageenin-induced granulation tissue in rats. The intrapouch injection of indomethacin and cimetidine resulted in the decrease in the granulation tissue weight,[31] the angiogenesis in the granulation tissue (Fig. 4A), and VEGF protein levels both in the granulation tissue (Fig. 4B) and in the pouch fluid.[31] Consistent with our previous findings that treatment with cimetidine, 6 and 12 h after the injection of carrageenin solution, increased the number of infiltrating neutrophils in the pouch fluid at 24 h,[39] the intrapouch injection of cimetidine once a day for 6 consecutive days also increased the number of infiltrating neutrophils at day 6.[31] Although cimetidine increased neutrophil infiltration, it decreased infiltration of macrophages and lymphocytes, pouch fluid accumulation and angiogenesis.[31] Since indomethacin inhibits neutrophil infiltration and vascular permeability increase at the acute phase of this model,[40] the inhibition of angiogenesis by indomethacin might be caused both by inhibition of VEGF production and by inhibition of acute inflammatory responses. Cimetidine also inhibited pouch fluid accumulation, infiltration of macrophages and granulation tissue formation.[31] Therefore, the possibility remains that the inhibition of angiogenesis by cimetidine was also caused both by inhibition of histamine-induced VEGF production and by inhibition of the infiltration of macrophages in which VEGF mRNA levels were increased by histamine.[31] Because cimetidine and indomethacin showed additive inhibitory effects on VEGF production and angiogenesis,[31] it is suggested that histamine in addition to PGE_2 participates in VEGF production and angiogenesis in the granulation tissue in carrageenin-induced inflammation in rats.

Although there are several reports describing that cimetidine delays wound healing,[41,42] the mechanism of action has not been clarified. However, it is possible that cimetidine inhibits the histamine-mediated upregulation of angiogenesis, thus delays wound healing.

Non-Mast Cell-Derived Histamine Responsible for Angiogenesis in Cotton Thread-Induced Granulation Tissue in Mice Subcutaneous implantation of a cotton thread in the dorsum of mouse induced the rapid formation of granulation tissue with an apparent angiogenesis.[43] The angiogenesis was highly dependent on VEGF production, as it was strongly inhibited by goat anti-VEGF IgG.[43]

Using $HDC^{-/-}$ mice, we found that the development of angiogenesis especially in the early phase (3 to 5 days after cotton thread implantation) depends on endogenous histamine.[43] The possibility that the functioning of hemangioblasts and endothelial cells is defective due to the destruction of the HDC gene was ruled out because the injection of histamine or dimaprit rescued the defective ganulation tissue formation (Fig. 5B) and angiogenesis in $HDC^{-/-}$ mice (Figs. 5A and C). The production of VEGF in $HDC^{-/-}$ mice was significantly less than that in $HDC^{+/+}$ mice,[43] and the injection of dimaprit or histamine increased the VEGF levels in the granulation tissue (Fig. 5D). In addition, topical injection of cimetidine inhibited the histamine-induced angiogenesis in $HDC^{-/-}$ mice (Fig. 5E). Therefore, we concluded that endogenous histamine enhances VEGF production via H2 receptors.

Although it is known that histamine production is increased in rapidly growing tissues,[39,44] the histamine-producing cells have not yet been identified. It has been reported that the number of mast cells in the rapidly growing tissues increases[45] and mast cell-derived histamine is angiogenic.[46] However, we found that HDC activity in the tissue surrounding the implanted cotton thread including the skin, cutaneous muscle layer, subcutaneous tissues and the granulation tissue increased even in mast cell-deficient ($WBB6F_1$-W/W^V) mice (Fig. 6A and B). In addition, histochemical analysis of the granulation tissue dissected 5 days after the cotton thread implantation indicated the absence of mast cells in the granulation tissue.[43] In contrast, HDC-producing cells in the granulation tissue were identified as infiltrating macrophages (Fig. 6C). Histamine production by non-mast cells was observed in mouse skin

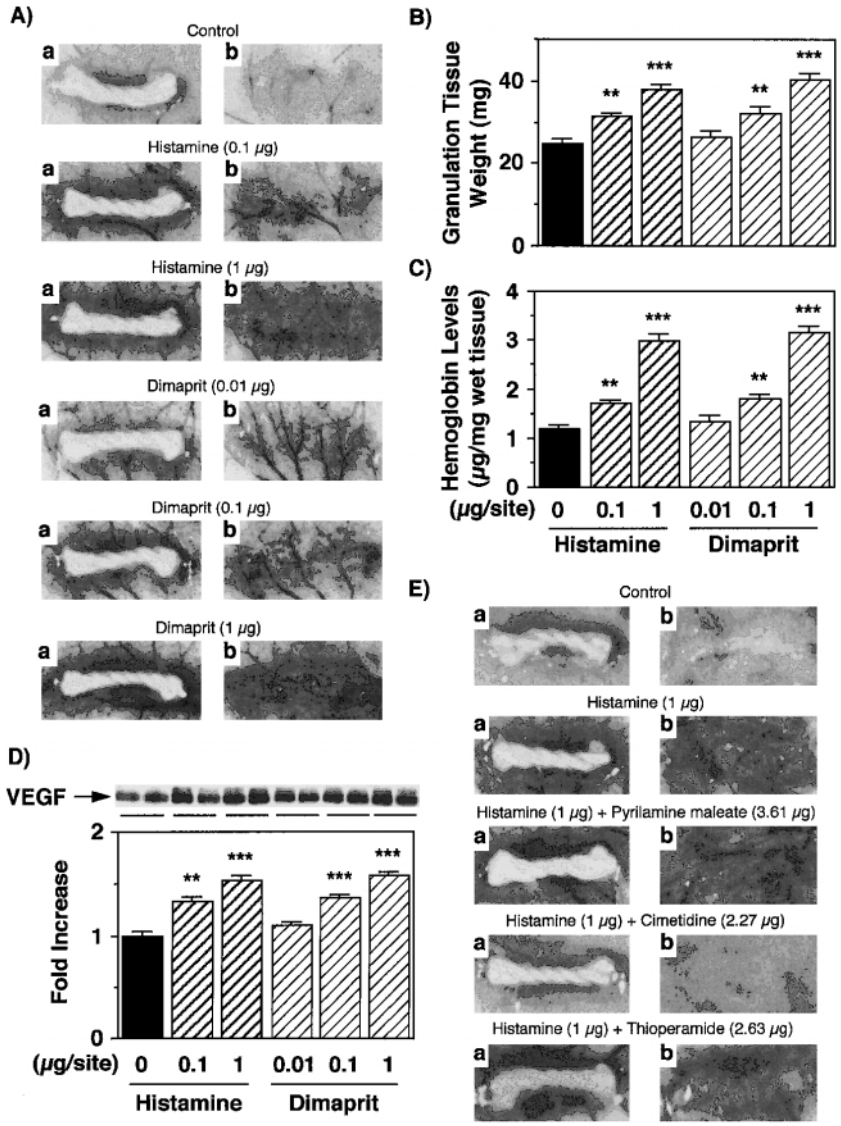

Fig. 5. Effects of Histamine and Dimaprit on Angiogenesis in HDC$^{-/-}$ Mice[43]

A cotton thread (1 cm, 7 mg) was implanted subcutaneously in the dorsum of each mouse. Histamine (0, 0.1 and 1 μg), pyrilamine maleate (3.61 μg), cimetidine (2.27 μg), thioperamide (2.63 μg) or dimaprit (0.01, 0.1 and 1 μg) dissolved in 100 μl sterile saline were injected subcutaneously at the site of cotton thread implantation just after the implantation and then once a day on consecutive days. The mice were sacrificed 5 days after cotton thread implantation. (A and E) The vascular network formation around the cotton thread (a) and the subcutaneous tissue beneath the cotton thread (b). (B) The granulation tissue weight. (C) Hemoglobin levels in the granulation tissue. (D) VEGF protein levels in the granulation tissue. VEGF protein levels in the granulation tissue were determined by immunoblotting and analyzed densitometrically. Representative immunoblots from two mice in each group are shown at the top of (D). The mean VEGF protein level in the granulation tissue in control mice is set to 1.0. Values are the means from five mice with S.E.mean shown by vertical bars. Statistical significance: $^{**}p<0.01$, $^{***}p<0.001$ compared with values in control group.

treated with PMA,[47] in various tissues such as liver and lung in IL-1-treated mice,[48] and in the infiltrating leukocytes in allergic inflammation in rats.[49] Our findings did not exclude the possibility that mast cells in the granulation tissue and the surrounding tissues release histamine. Because the cotton thread implantation induced an apparent angiogenesis in WBB6F$_1$-W/W^V mice as well as in WBB6F$_1$-$^{+/+}$ mice,[43] we concluded that histamine from infiltrating macrophages play a significant role in angiogenesis of the granulation tissue.

It is reported that cimetidine, an H2 antagonist, delays wound healing especially in peptic ulcer,[42] but its mechanism of action has not been clarified. We found that cimetidine reduces VEGF production in the carrageenin-induced inflammation model in

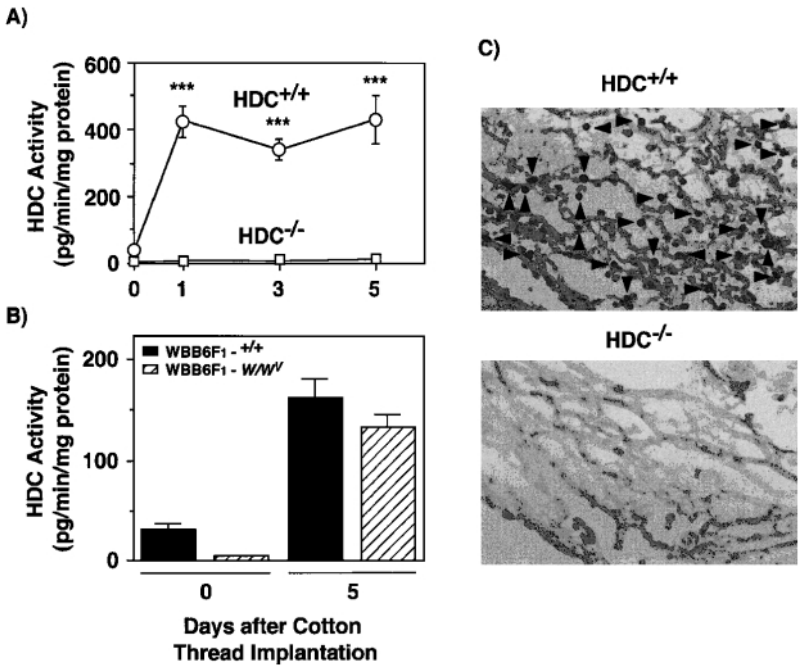

Fig. 6. HDC Activity in the Tissue Surrounding the Implanted Cotton Thread[43]

A cotton thread (1 cm, 7 mg) was implanted subcutaneously in the dorsum of each mice. (A) $HDC^{+/+}$ and $HDC^{-/-}$ mice were sacrificed and a piece of the tissue, 2 cm in diameter surrounding the implanted cotton thread including the skin, cutaneous muscle layer, subcutaneous tissues, the granulation tissue together with the cotton thread, was dissected 0, 1, 3 and 5 days after cotton thread implantation. The dissected tissue and cotton thread were homogenized and the HDC activity in the supernatant was determined. Values are the means from five to six mice with S.E.mean shown by vertical bars. Statistical significance: $***p<0.001$ compared with the HDC activity in the tissue dissected just after cotton thread implantation in $HDC^{+/+}$ mice. (B) $WBB6F_1$-$^{+/+}$ and $WBB6F_1$-W/W^V mice were sacrificed and a piece of the tissue, 2 cm in diameter surrounding the implanted cotton thread including the skin, cutaneous muscle layer, subcutaneous tissues, the granulation tissue together with the cotton thread, was dissected 0 and 5 days after cotton thread implantation. The dissected tissue and cotton thread were homogenized and the HDC activity in the supernatant was determined. (C) HDC-expressing cells in the granulation tissue dissected 5 days after cotton thread implantation in $HDC^{+/+}$ and $HDC^{-/-}$ mice were detected immunohistochemically using rabbit polyclonal anti-HDC, and observed with a light microscope (magnification: ×400). HDC-producing cells are mainly macrophages and are indicated by closed arrowheads. Representative micrographs are shown from four samples.

rats.[31] In the cotton thread-induced inflammation model in mice,[43] treatment with cimetidine showed partial but significant inhibition of angiogenesis and VEGF production. Therefore, we hypothesized that cimetidine delays wound healing by inhibiting histamine-mediated VEGF production and angiogenesis. In addition, because cimetidine inhibits tumor growth *in vivo*,[41] there is a possibility that the growth of some tumors may also be regulated by histamine-mediated angiogenesis.

Our findings that the defective angiogenesis in the inflammatory granulation tissue in histidine decarboxylase-deficient mice but not in mast cell-deficient mice, suggest that histamine derived from non-mast cells plays a significant role in the angiogenesis in the inflammatory granulation tissue.

Regulation of angiogenesis is a new approach to treat chronic inflammation as well as solid tumor growth because anti-angiogenic therapy is demonstrated as much safer and better tolerated compared to conventional chemotherapy.

Therefore, the approach to investigate the roles of chemical mediators of inflammation in regulation of angiogenesis in inflammatory granulation tissue might be hold a great promise to treat angiogenesis-dependent inflammatory diseases.

CONCLUSION

The chemical mediators of inflammation, PGE_2 and histamine regulate angiogenesis in inflammatory granulation tissue by directly inducing VEGF production. In addition, it was suggested that H2 receptor antagonists as well as COX-2 inhibitors, are useful for the suppression of angiogenesis-dependent formation of inflammatory granulation tissue, and conversely, H2 receptor agonists enhance angiogenesis in wound healing and several ischemic diseases.

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