ELSEVIER

European Journal of Pharmacology 402 (2000) 181–191

ejp

www.elsevier.nl/locate/ejphar

# Chymase mediates mast cell-induced angiogenesis in hamster sponge granulomas

Michiko Muramatsu [a], Jun Katada [a,b,*], Mari Hattori [b], Izumi Hayashi [a], Masataka Majima [a]

[a] *Department of Pharmacology, Kitasato University School of Medicine, 1-15-1 Kitasato Sagamihara-shi, Kanagawa 228-8555, Japan*
[b] *Life Science Research Center, Advanced Technology Research Laboratories, Nippon Steel Corporation, 3-35-1 Ida Nakahara-ku, Kawasaki 211-0035, Japan*

Received 20 January 2000; received in revised form 26 April 2000; accepted 2 May 2000

**Abstract**

We investigated the contribution of mast cell chymase in mast cell-dependent angiogenesis using the hamster sponge-implant model, where angiogenesis in the granulation tissue surrounding the subcutaneously implanted sponge was evaluated by measuring the hemoglobin content. Daily local injection of compound 48/80 (3–100 μg/site/day), a potent mast cell activator, induced formation of granulomas and angiogenesis in time- and dose-dependent manners. This angiogenic response was inhibited by chymase inhibitors including chymostatin (≥ 1 nmol/site/day), soybean trypsin inhibitor (SBTI; ≥ 1.4 nmol/site/day) and lima bean trypsin inhibitor (LBTI; ≥ 3.3 nmol/site/day), but not by a tryptase inhibitor like leupeptin (≥ 700 nmol/site/day). Although pyrilamine (≥ 2,580 nmol/site/day), a histamine H1 receptor antagonist, and protamine (300 μg/site/day) also inhibited angiogenesis, these effects were much less pronounced than those by chymase inhibitors. Furthermore, antigen-induced angiogenesis in hamsters pre-sensitized with ovalbumin was also inhibited by the chymase inhibitors by 60–70%. Our results suggest that chymase is a major mediator in mast cell-mediated angiogenesis. 

*Keywords:* Chymase; Mast cell; Angiogenesis; Compound 48/80; Granuloma

## 1. Introduction

Angiogenesis is an important physiological phenomenon implicated in integrated biological processes such as wound healing, embryonic development and formation of collateral circulation in ischaemic tissues. It is also involved in the pathogenesis of several diseases, including chronic inflammatory diseases, diabetic retinopathy and tumour growth. Angiogenesis is a multistep event and each step is regulated by different mechanisms. Many mediators and cell types are involved in this complex process, and the progression of angiogenesis may be positively or negatively regulated by numerous factors (Fan and Brem, 1992; Folkman and Shing, 1992).

Mast cells are topographically associated with microvessels (Rakusan et al., 1990; Rhodin and Fujita, 1989) and accumulate in a number of angiogenesis-dependent events such as ovulation, wound healing, synovial proliferation in rheumatoid arthritis and tumour growth (reviewed in Meininger and Zetter, 1992; Norrby and Woolley, 1993). These histological studies implicate mast cells, especially connective tissue type mast cells, in angiogenesis. The role of mast cells in angiogenesis has also been suggested by the results of experiments showing that activation of connective tissue type mast cells in situ by compound 48/80 promoted angiogenesis in several models, such as rat mesenteric microvessels (Norrby et al., 1986) or chick chorioallantoic membrane (Clinton et al., 1988). However, the significance of mast cells in angiogenesis under physiological and/or pathophysiological conditions is still unclear.

Mast cell granules contain numerous substances including histamine, proteoglycans, such as heparin (Metcalfe et al., 1979), and proteases such as tryptase (Schwartz et al., 1981; Hopsu and Glenner, 1963), chymase (Schechter et

* Corresponding author. 2-12-27-107, Naritanishi, Suginami-ku, Tokyo 166-0016, Japan. Tel.: +81-3-3393-2370; fax: +81-3-5347-5176.
*E-mail address:* katada@kt.rim.or.jp (J. Katada).

0014-2999/00/$ - see front matter 
PII: S0014-2999(00)00350-2

al., 1983; Wintroub et al., 1986; Sayama et al., 1987) and carboxypeptidase A (Goldstein et al., 1987). These mediators are released from the granules upon activation of mast cells. Lipid mediators are also produced by activated mast cells (Hogaboam et al., 1992; Lewis et al., 1982; Razin et al., 1982). Although some of these mast cell-derived mediators, such as histamine (Zauberman et al., 1969; Sorbo and Norrby, 1992) and heparin (Jakobsson et al., 1990; Unger et al., 1991; Norrby and Sorbo, 1995), exhibit angiogenic properties both in vitro and in vivo, the precise mechanism and the factors responsible for mast cell-dependent angiogenesis remain to be elucidated.

A major focus of our research is related to the physiological/pathophysiological roles of chymase. Chymase is a serine protease with chymotrypsin-like substrate specificity and is one of the major components of mast cell granules (Lagunoff and Pritzl, 1976). Marked species differences in chymase substrate specificity have been reported. For example, in humans, dogs and hamsters, chymase generates angiotensin II from its inactive precursor angiotensin I, whereas chymase cleaves angiotensin I into inactive fragments in rats, mice or rabbits (Le Trong et al., 1987; Okunishi et al., 1993; Chandrasekharan et al., 1996; Balcells et al., 1997). In this regard, to investigate the physiological and/or pathophysiological roles of mast cell chymase in angiogenesis in humans, hamsters should be used as the experimental animal. Recently, we established a hamster sponge granuloma-angiogenesis model (Muramatsu et al., 2000), which was originally developed in rats by Fan et al. (Andrade et al., 1987; Hu et al., 1996) who measured $^{133}$Xe clearance rate to quantify angiogenesis. In our modified model, the hemoglobin content in the sponge granulomas is used for the quantification of angiogenesis. Using this model, we have demonstrated that chymase acts as a pro-angiogenic factor and that endogenous chymase is involved in basic fibroblast growth factor (FGF)-induced angiogenesis probably via the local generation of angiotensin II (Muramatsu et al., 2000). However, we have not confirmed a source of chymase responsible for angiogenesis under physiological and/or pathophysiological conditions.

The aims of the present study were to elucidate the role of mast cell-derived chymase in angiogenesis using the hamster sponge granuloma model. Our results showed that direct injections of compound 48/80 into sponge implants induced angiogenesis, suggesting that activation of connective tissue type mast cells is sufficient for the induction of angiogenesis in this model. Furthermore, antigen-dependent activation of mast cells also resulted in angiogenesis in sensitized hamsters. These angiogenic responses elicited by activated mast cells were inhibited by treatment with chymase inhibitors including chymostatin and soybean trypsin inhibitor (SBTI). Our results suggest that chymase is one of the factors that mediate mast cell-dependent angiogenesis under certain physiological and/or pathophysiological conditions.

## 2. Materials and methods

### 2.1. Hamster sponge-implant mode

Circular sponge discs, 5-mm thick × 13-mm diameter, weighing 14.2 ± 0.1 mg, were prepared from a polyurethane foam sheet. The discs were soaked in 70% ethanol overnight and then sterilized with an autoclave. Male Syrian hamsters (70–90 g, purchased from Japan SLC) were used in all experiments. The dorsal hair of ether-anaesthetized hamsters was shaved and the skin was sterilized with 70% ethanol. A midline incision was made and sponge discs were implanted in the subcutaneous space. Before suturing, all air present in the sponges and pouches was eliminated. Angiogenesis was induced by daily injection of inducers into the sponges for 7 consecutive days. Test substances, solubilized in sterile phosphate buffered saline (PBS), were directly injected into the sponges 1 h prior to the injection of inducers.

All experimental procedures were approved by the Animal Care Committee of Kitasato University of Medicine and conformed with the international guidelines.

### 2.2. Measurement of hemoglobin content

Measurement of the hemoglobin contents in the sponge implants and surrounding granuloma tissues was performed as described previously (Majima et al., 1997; Muramatsu et al., 2000). Briefly, the sponge implants and surrounding tissue were excised 7 days after subcutaneous implantation and homogenized in distilled water. After centrifugation at 2500 × $g$ for 20 min at 4°C, the supernatant was further centrifugated at 5000 × $g$ for 30 min, and we determined the hemoglobin concentration of the supernatant by using the hemoglobin assay kit (Hemoglobin B test, Wako).

### 2.3. Histology of sponge implants

At the 7th day of the experiment, sponge implants were excised, dissected free of adherent tissue and fixed with 10% formalin. Thin sections (5 μm) were prepared and stained with hematoxylin or toluidine blue, which were then processed for light microscopic examination. For quantitative studies, five randomly selected sections were photographed on 35-mm film using a ×10 objective lens and the number of microvessels was counted. The lumen area of microvessels was also calculated in the same photographs by using NIH image software.

### 2.4. Measurement of chymase activity

To measure chymase activity, each sample was homogenized with a Polytron homogenizer in 10 mM sodium phosphate buffer (pH 7.4) containing 2 M KCl and 0.1% Nonidet P-40. After extraction for 1h with continuous

shaking followed by centrifugation at 20,000 × *g* for 1 h, chymase activity in the supernatant was determined according to the method of Okunishi et al. (1987). Briefly, aliquots of the tissue extract were incubated for 1 h at 37°C with 770 μM of angiotensin I in 48.5 mM borax–borate buffer (pH 8.5), containing 8 mM dipyridyl, 760 μM diisopropyl phosphorofluoridate and 5 mM ethylenediaminetetraacetic acid as inhibitors of angiotensin converting enzyme (ACE) and angiotensinases. Reactions were terminated by addition of 15% cold trichloroacetic acid (which gives 9% of the final concentration), followed by centrifugation at 10,000 × *g* for 5 min. The concentration of His–Leu, an enzymatic cleavage product, in the supernatant was determined from fluorescent intensity as follows. Ten % of *o*-phthaldialdehyde was added to the supernatant in an alkaline condition (pH 12.0) followed by the addition of 6 N HCl (brought to pH 3.0) to stabilize. The fluorescence in the solution was measured at 340 nm of excitation and at 455 nm of emission.

### 2.5. Enzymatic activities

The in vitro effects of inhibitors of chymase activity were examined using hamster chymase purified from the cheek pouches according to the method described previously (Muramatsu et al., 2000) and the chromogenic substrate, *N*-suc-Ala-Ala-Pro-Phe-pNA (1.5 mM). Tryptase was also purified from hamster cheek pouches as described previously (Schwartz, 1994). Tryptase activity was measured by adding 2 mM *N*-*p*-tosyl-Gly-Pro-Lys-pNA as the substrate. The enzymes and substrates used for the in vitro enzyme assay of other serine proteases were as follows: *N*-succinyl-Ala-Ala-Pro-Phe-pNA (3 mM) for human leukocyte cathepsin G (2.5 U/ml); *N*-succinyl-Ala-Ala-Ala-pNA (1 mM) for porcine pancreatic elastase (0.8 μg/ml); and Bz-L-Arg-pNA (1 mM) for porcine pancreatic trypsin (1 U/ml).

### 2.6. Sensitization and antigen challenge

Male Syrian hamsters, weighing 70–90 g, were sensitized by intraperitoneal injection of ovalbumin (20 μg/hamster) absorbed with aluminium hydroxide (10 μg ovalbumin/mg) on days 0, 7 and 14. Seven to ten days after the last injection, sensitization in each hamster was confirmed by challenging with aerosolized ovalbumin. Briefly, hamsters anaesthetized with pentobarbital sodium (25 mg/kg i.p.) were intubated using polyethylene tubing (PE 240). The inspiratory and expiratory tubes of a rodent ventilator (model 360; Harvard Apparatus, South Natick, MA) were attached to the tracheal cannula through a Y connector. Animals were challenged with aerosolized ovalbumin (6% solution in PBS, pH 7.4) through the inspiratory tube, and changes in pulmonary resistance were monitored by using a bronchospasm transducer (Ugo Basile, Comerio, Italy). Only hamsters that showed apparent responsiveness to ovalbumin within 5 min were used for the experiments. Non-sensitized control hamsters were treated with aluminium hydroxide alone.

The angiogenic effects of ovalbumin on sensitized animals were examined by direct injection of ovalbumin into the implanted sponges. Sterile ovalbumin solution (100 μg/ml in PBS) was injected into the sponge (100 μl/site) once a day in sensitized and non-sensitized hamsters (non-sensitized control). In the vehicle control group, PBS alone was injected in sensitized animals. The sponges were excised with the surrounding granuloma tissues on day 2, 4, 7 or 11, and hemoglobin contents were determined.

### 2.7. Agents

Compound 48/80, chymostatin, pyrilamine, protamine, ovalbumin (grade VI), lima bean trypsin inhibitor (LBTI) and angiotensin I were purchased from Sigma (St. Louis, MO). Leupeptin, diisopropyl phosphorofluoridate, aluminium hydroxide, SBTI and Hemoglobin B test WAKO® were purchased from Wako (Osaka, Japan). Dipyridyl was purchased from Kanto Chemicals (Tokyo, Japan). Enzyme substrates, *N*-succinyl-Ala-Ala-Pro-Phe-pNA, *N*-succinyl-Ala-Ala-Ala-pNA and *p*-tosyl-Gly-Pro-Lys-pNA were obtained from Sigma, and Bz-L-Arg-pNA was from Peptide Institute (Osaka, Japan). Cathepsin G (Elastin Products), elastase (Type III; Sigma) and porcine pancreatic trypsin (Wako) were also used. *N*-(*o*-fluoro-benzyloxycarbonyl)-L-phenylalanine chloromethyl ketone (CI-21) was a synthetic chloromethylketone derivative developed as a specific human chymase inhibitor described by Hayashi et al. (manuscript submitted). This compound was a kind gift from Dr. Yoshio Hayashi (Kyoto Pharmaceutical University).

### 2.8. Statistical analysis

All data were expressed as mean ± S.E.M. Differences between groups were examined for statistical significance using the Kruskal–Wallis test followed by the Scheffé's *F*-test. A $p$ value less than 0.05 denoted the presence of a statistically significant difference.

## 3. Results

### 3.1. Compound 48/80-induced angiogenesis

In this hamster sponge-implant model, spontaneous angiogenesis (PBS-injected controls in Fig. 1A) occurred after day 7, as evident by a gradual increase in hemoglobin contents. Daily injection of compound 48/80 significantly induced angiogenesis in the sponge implants. As shown in Fig. 1A, the hemoglobin contents in compound 48/80

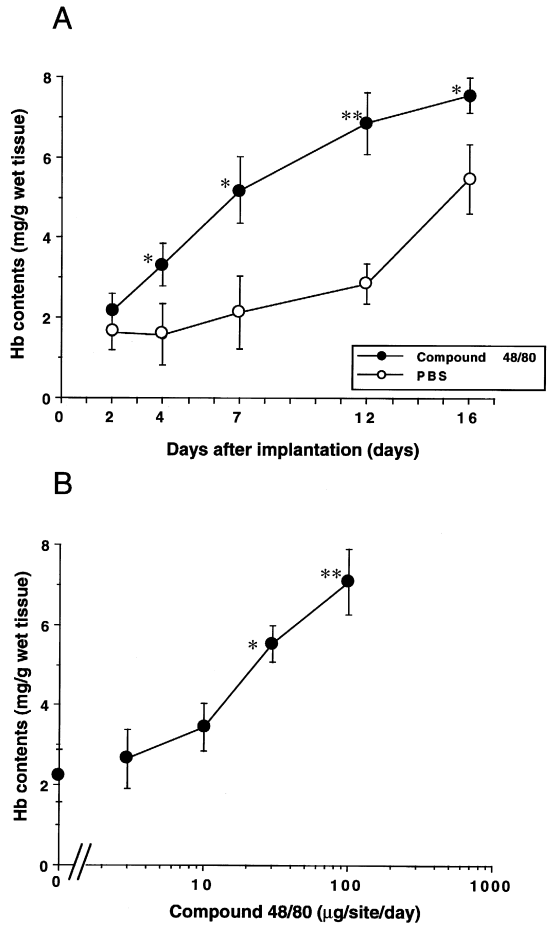


Fig. 1. Compound 48/80-induced angiogenesis in hamster sponge granulomas. Compound 48/80 was injected daily into the sponge implanted in the subcutaneous pouch. After a predetermined period, sponges were excised together with the surrounding granulomas, and the hemoglobin content in each sponge granuloma was determined as an index of angiogenesis. (A) Time-course of compound 48/80 (100 μg/site/day)-induced angiogenesis. Closed circles: compound 48/80-injected group; open circles: PBS-injected control. Each symbol represents the mean ± S.E.M. of six samples. $^{*}P < 0.05$, $^{**}P < 0.01$ versus corresponding PBS-injected control. (B) Dose-dependent curve of compound 48/80-induced angiogenic response. Compound 48/80 at various concentrations was injected daily into the sponge, and the hemoglobin content was determined at day 7. Each symbol represents the mean ± S.E.M. of 10 samples. $^{*}P < 0.05$, $^{**}P < 0.01$ versus PBS-injected control.

(100 μg/site/day)-injected sponge granulomas increased progressively from day 2 (2.2 ± 0.17 mg/g of wet tissue) to day 16 (7.5 ± 0.42 mg/g of wet tissue). The hemoglobin contents were significantly higher in compound 48/80-treated group compared to PBS-injected control rats from day 2.

Furthermore, the effect of compound 48/80 on angiogenesis was dose-dependent (Fig. 1B). At a higher dose (1000 μg/site/day), compound 48/80 caused bleeding around the sponges and damage of the back skin. Since local injection of compound 48/80 at a dose of 100 μg/site/day for 7 days induced significant and reproducible angiogenesis in our hamster model, this dosage was used in the following experiments.

### 3.2. Histology of compound 48/80-treated sponges

The formation of new microvessels in sponge granulomas was confirmed by microscopic examination of paraffin sections prepared from sponges treated with compound 48/80 for 7 days. As shown in Fig. 2, the sponge was surrounded by a well-developed granuloma tissue characterized by the presence of fibroblasts at a high density, together with matrix formation. The space between the sponge matrix was infiltrated with fibrous stroma, in which numerous leukocytes including macrophages, neutrophils or lymphocytes also existed. Most microvessels (arrows) were localized within the granulomas encapsulating the sponges, and several microvessels were observed in the stroma. These microvessels were not uniformly distributed in the granulomas and a cluster of vessels was often observed. As we previously showed in basic FGF-treated sponges (Muramatsu et al., 2000), the hemoglobin content correlated significantly with the number of microvessels in each sponge granuloma treated with compound 48/80 or vehicle solution (Fig. 3A, $r = 0.778$, $P < 0.001$). As shown in Fig. 3B, there was also a linear relationship between lumen area of the microvessels and hemoglobin contents ($r = 0.837$, $P < 0.001$).

Toluidine blue stained sections, which were prepared from sponge granulomas 24 hr after the final injection of compound 48/80, showed the presence of a relatively large number of mast cells. In most mast cells, the cytoplasm was weakly stained, suggesting that degranulation had occurred (data not shown).

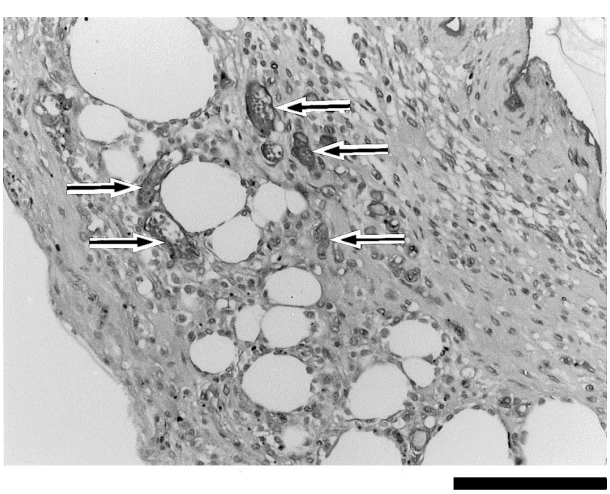

Fig. 2. Histological sections of 7-day old sponge granulomas treated with compound 48/80. Sections of the sponge and surrounding granulomas stained with hematoxylin. Microvessels (arrows), which appear as tubular structures often filled with erythrocytes, are localized in a cluster within the granuloma encapsulating the sponge. Bar scale: 100 μm.

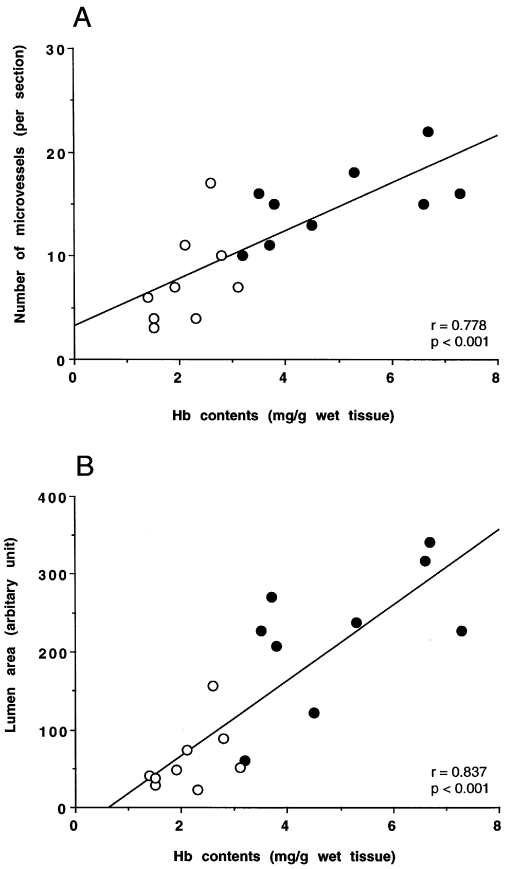


Fig. 3. Hemoglobin contents in sponge granulomas as a parameter for angiogenesis. PBS or compound 48/80 (100 μg/site/day) was injected once a day for 7 days. The sponge granulomas were excised at day 7 and divided into two halves, one for measuring the haemoglobin contents, while the other was used for histological studies. (A) Relationship between hemoglobin contents and number of microvessels. Open circles: PBS-injected sponges; closed circles: compound 48/80-injected sponges. The regression line was drawn by the least-square method. (B) Relationship between the hemoglobin contents and lumen area of microvessels. Open circles: PBS-injected sponges; closed circles: compound 48/80-injected sponges. The regression line was drawn by the least-square method.

### 3.3. Effects of serine protease inhibitors

Several serine proteases, such as tryptase, chymase, cathepsin G and carboxypeptidase A, are stored in the secretary granules of mast cells. We had previously confirmed that mast cells in the hamster skin and sponge granulomas treated with basic FGF or angiotensin II are connective tissue type (Muramatsu et al., 2000). Because tryptase and chymase are major proteases in the connective tissue mast cells, we investigated the roles of these proteases in compound 48/80-induced angiogenesis. As shown in Table 1, leupeptin is an inhibitor of hamster tryptase with an $IC_{50}$ value of 8.3 μM. It did not inhibit hamster chymase even at 1 mM. Local injection of leupeptin, combined with compound 48/80 (100 μg/site/day), did not affect compound 48/80-induced angiogenesis, even at a dose of 700 nmol/site/day, which was the highest dose examined in this series of experiments (Fig. 4A). Leupeptin itself did not have any angiogenic effects at 700 nmol/site/day and it also had no effects on hemoglobin contents in the PBS-treated controls at day 7 (data not shown).

Chymostatin is an inhibitor of chymotrypsin-like serine proteases and inhibits hamster chymase ($IC_{50} = 0.24$ μM), but not hamster tryptase ($IC_{50} > 100$ μM) (Table 1). Daily injections of chymostatin 1 h prior to the injection of compound 48/80 dose-dependently inhibited angiogenesis (Fig. 4B). Significant inhibition was observed at doses higher than 1 nmol/site/day, and 65% inhibition was obtained at a dose of 10 nmol/site/day, which was the highest dose examined because of insolubility of this compound. Chymostatin treatment did not cause bleeding or tissue damage. Treatment with chymostatin alone (10 nmol/site/day) in PBS-treated controls for 7 days did not produce any effects (data not shown).

SBTI is a potent inhibitor of trypsin ($IC_{50} = 0.14$ μM) although its inhibitory action is less potent on chymotrypsin ($IC_{50} = 22$ μM) and elastase ($IC_{50} = 340$ μM). SBTI inhibited hamster chymase and tryptase at submicromolar concentrations (Table 1). Local injection of SBTI into the sponges also inhibited compound 48/80-induced angiogenesis in a dose-dependent manner. Such inhibition was significant at 0.14–14 nmol/site/day, and maximum inhibition was noted at 4.7 nmol/site/day, where 75% inhibition was observed (Fig. 4C).

Table 1
Inhibitory activities of serine protease inhibitors

| Inhibitors | $IC_{50}$ values | | | | | |
|---|---|---|---|---|---|---|
| | Chymase (μM) | Tryptase (μM) | Chymotrypsin (μM) | Cathepsin G (μM) | Elastase (μM) | Trypsin (μM) |
| Leupeptin | > 1000 | 8.3 ± 0.74 | > 1000 | > 1000 | > 1000 | 0.33 ± 0.028 |
| SBTI | 0.023 ± 0.002 | 0.021 ± 0.002 | 1.0 ± 0.13 | 1.3 ± 0.11 | 16 ± 1.8 | 0.007 ± 0.0005 |
| LBTI | 0.019 ± 0.002 | 0.080 ± 0.009 | 0.46 ± 0.057 | 1.4 ± 0.11 | > 110 | 0.021 ± 0.002 |
| Chymostatin | 0.24 ± 0.018 | > 100 | 0.018 ± 0.0015 | 0.022 ± 0.0019 | 0.21 ± 0.017 | > 100 |
| CI-21 | 0.31 ± 0.033 | > 100 | 34 ± 1.6 | > 100 | 310 ± 27 | > 100 |

Concentrations inhibiting 50% of the activities of the corresponding enzymes were listed. SBTI: soybean trypsin inhibitor; LBTI: lima bean trypsin inhibitor. The origins of the enzymes and the enzyme assay conditions are described in detail in Section 2. The values are means ± S.E.M. from three independent experiments. Molecular weights of SBTI and LBTI used here were 21,500 and 9000, respectively.

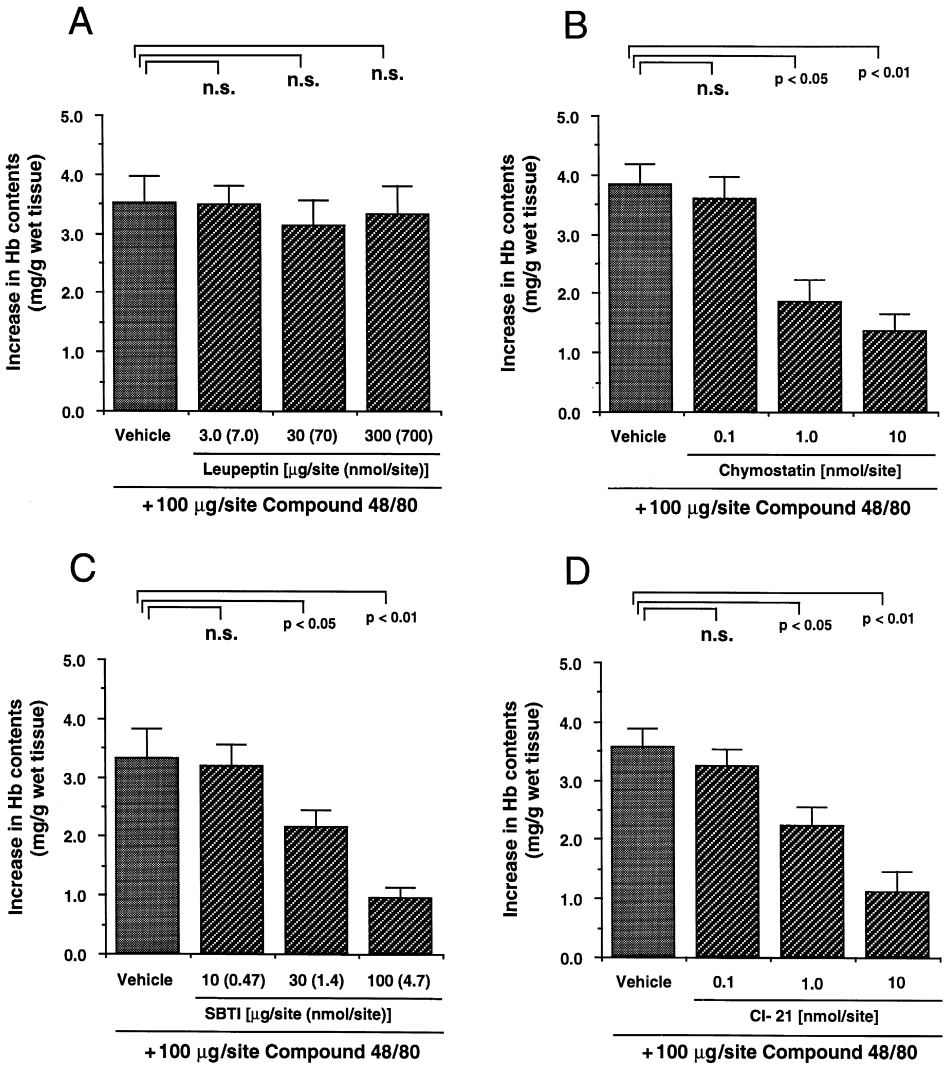


Fig. 4. Inhibition of compound 48/80-induced angiogenesis by serine protease inhibitors. Compound 48/80 (100 μg/site/day) was injected into the sponges 1 h after injection of leupeptin (A), chymostatin (B) or SBTI (C) at various concentrations for 7 days. PBS was used as vehicle to solubilize leupeptin and SBTI, and PBS containing 0.1% DMSO was used for chymostatin. The mean hemoglobin content at day 7 of each experimental group was compared with the vehicle-treated control. Each column represents the mean ± S.E.M. of eight samples.

We also examined the effects of LBTI, another potent inhibitor of chymase but a less potent inhibitor of hamster tryptase. LBTI also inhibited compound 48/80-induced angiogenesis and the degree of the inhibition was almost similar to that of SBTI (about 70% inhibition at 11.1 nmol/site/day, data not shown).

CI-21 was a synthetic chloromethylketone derivative that was a relatively specific inhibitor for chymase (Table 1). In in vitro enzyme assays, its inhibitory activity on elastase, cathepsin G, trypsin or tryptase was at least 1000 times less potent as compared with the activity on hamster chymase. As shown in Fig. 4, the administration of this compound directly into the implanted sponges resulted in a dose-dependent inhibition of compound 48/80-induced angiogenesis.

These results suggest that chymase derived from activated mast cells may be implicated in compound 48/80-induced angiogenesis.

### *3.4. Roles of histamine and heparin*

It has been demonstrated that histamine and heparin are angiogenic in several angiogenesis models (Zauberman et al., 1969; Jakobsson et al., 1990; Unger et al., 1991; Sorbo and Norrby, 1992; Norrby and Sorbo, 1995). Because connective tissue mast cells contain relatively large amounts of histamine and heparin in their granules, we also examined their roles in compound 48/80-induced angiogenesis in hamsters. We examined the effects of pyrilamine, a histamine H1 receptor antagonist on angiogenesis, and found that only a weak inhibition was noted

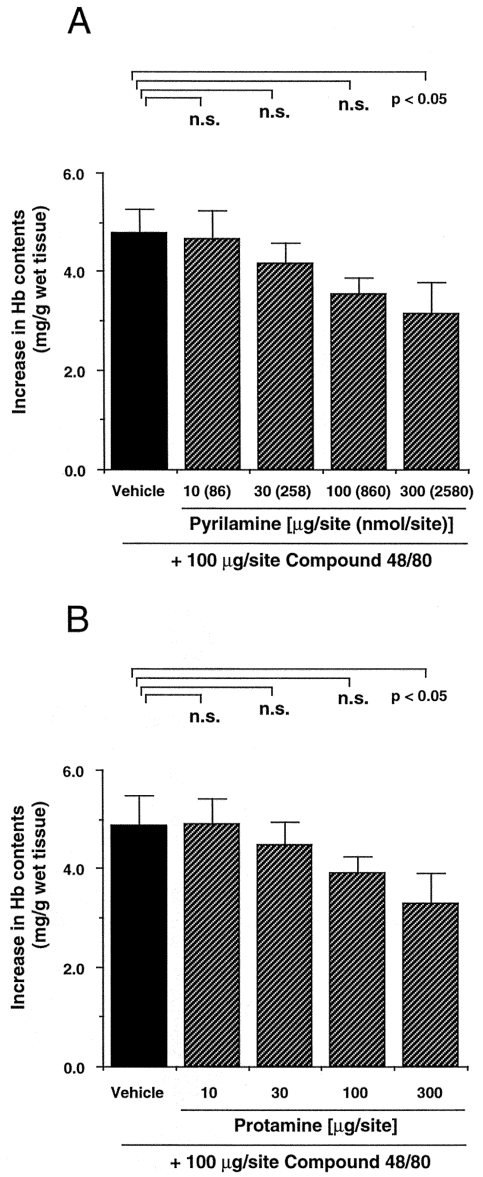


Fig. 5. Inhibition of compound 48/80-induced angiogenesis by pyrilamine or protamine. Compound 48/80 (100 μg/site/day) was injected into the sponges 1 h after injection of pyrilamine (A) or protamine (B) at various concentrations for 7 days. PBS was used as vehicle to solubilize these inhibitors. The hemoglobin content of each experimental group at day 7 was compared with the vehicle-treated control. Each column represents the mean ± S.E.M. of eight samples.

even at a dose more than 860 nmol/site/day. Administration of pyrilamine alone in the absence of compound 48/80 did not have any effect even at the highest dose (2580 nmol/site/day), compared with the control group (data not shown). These results suggest that histamine is only partly involved in compound 48/80-stimulated angiogenesis via histamine H1 receptor. We also investigated the effects of protamine, an inhibitor of heparin, on compound 48/80-induced angiogenesis, as shown in Fig. 5B. Protamine combined with compound 48/80 inhibited compound 48/80-induced angiogenesis, resulting in 35% reduction in hemoglobin contents at 100 μg/site/day. Protamine had no effects when administered alone even at the highest dose (data not shown).

### 3.5. Chymase in sponge granulomas

Chymase activity, which was determined from His–Leu release from angiotensin I, in the sponge granulomas was detected from day 2 and gradually increased to 0.58 ± 0.15 μg of His–Leu/min/g wet tissue at day 7. The time course of this increment was almost parallel to that of hemoglobin contents (data not shown). As shown in Fig. 6, His–Leu release correlated with the hemoglobin contents in the sponge granulomas. These results suggest that chymase activity in the sponge granulomas may play a significant role in the progression of angiogenesis. Immunohistochemical studies using an anti-hamster chymase antibody were also carried out. In frozen sections prepared from sponge granulomas at day 7 (24 h after the final injection of compound 48/80), almost all mast cells in the granulomas were positively stained with the antibody (data not shown). In several preparations, the fibrous matrix in close proximity to mast cells was only slightly stained.

### 3.6. Antigen-induced angiogenesis in sensitized hamsters

To examine whether activation of mast cells through antigen- and IgE-dependent pathways can also evoke the angiogenic response, we determined the effects of local injection of ovalbumin into the sponges implanted in hamsters that had been sensitized with ovalbumin. As shown in Fig. 7A, daily injection of ovalbumin (10 μg/site) significantly increased hemoglobin contents in the sponge granulomas from day 2 to day 11 in sensitized hamsters. Morphologically, developed granulomas encapsulating the

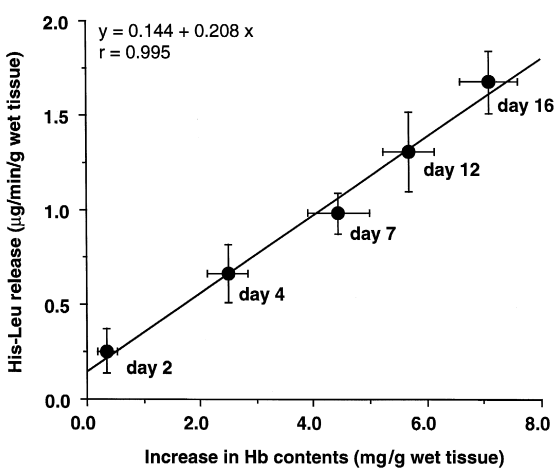


Fig. 6. Relationship between hemoglobin contents and chymase activity in each sponge sample. PBS or compound 48/80 (100 μg/site/day) was injected each day and the sponge granulomas were excised at predetermined experimental days (at days 2, 4, 7, 12 or 16) to measure the hemoglobin contents and His–Leu releasing activity from angiotensin I as described in Section 2. Each symbol represents the mean ± S.E.M. of the given experimental day ($n = 6$). The regression line was drawn by the least-square method.

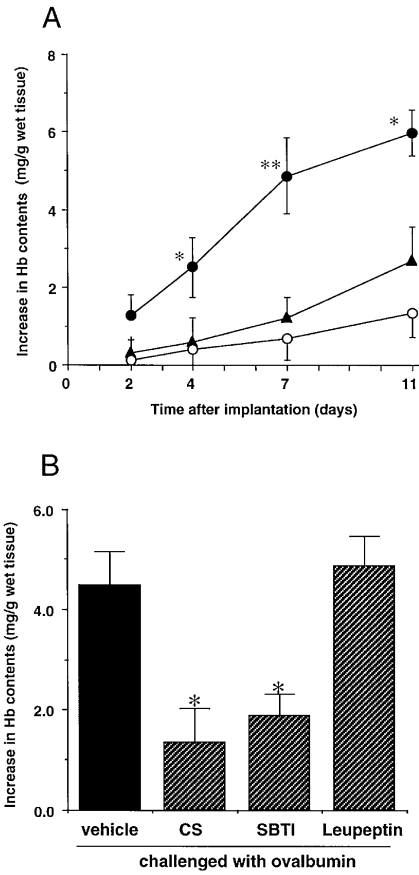


Fig. 7. Antigen-induced angiogenesis in hamsters pre-sensitized with ovalbumin. (A) Time course of increment of haemoglobin contents in sponge granulomas. Ovalbumin (10 μg/site/day) or PBS was injected into the sponges and the hemoglobin contents were determined at days 2, 4, 7 and 11. Closed circles: ovalbumin-injected sponges in the pre-sensitized hamsters; open circles: PBS-injected sponges in the pre-sensitized hamsters; closed triangles: ovalbumin-injected sponges in non-sensitized hamsters. Each symbol represents the mean ± S.E.M. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus PBS-injected control. (B) Effects of chymase inhibitors on angiogenesis induced by ovalbumin. Ovalbumin (10 μg/site/day) was injected for 7 days and sponge granulomas were excised at day 7 to determine the hemoglobin contents. Each inhibitor was injected into the sponge 1 h prior to the injection of ovalbumin. CS: 10 nmol/site/day chymostatin; SBTI: 100 μg/site/day SBTI; Leupeptin: 100 μg/site/day. Data are the mean ± S.E.M. of four samples in each group. $^{*}P < 0.05$ versus vehicle control.

implanted sponge were observed from day 4 and microvessels were seen both in the granulomas and stroma infiltrating into the sponge matrix (data not shown). In control hamsters injected with the vehicle only, no increment in hemoglobin contents was observed throughout the experimental period. Injection of ovalbumin in non-sensitized hamsters induced a slight increase in hemoglobin contents only at day 11. Finally, we examined the involvement of mast cell chymase in ovalbumin-induced angiogenesis at day 7 after sponge implantation by using inhibitors of chymase (Fig. 7B). Direct injection of chymostatin (10 nmol/site/day) or SBTI (100 μg (4.7 nmol)/site/day) significantly inhibited the increment of hemoglobin contents elicited by ovalbumin injections by 71% and 59%, respectively. Leupeptin, a tryptase inhibitor but not chymase inhibitor, did not show any inhibitory effects at 100 μg/site/day. A slight, but significant inhibition was observed by treatment with pyrilamine (860 nmol/site/day; data not shown).

## 4. Discussion

In the present study, we showed that chymase, derived from activated mast cells, was involved in mast cell-mediated angiogenesis. This finding complements our recent study (Muramatsu et al., 2000), in which we confirmed that hamster chymase is angiogenic when injected directly into the subcutaneously implanted sponges. While our previous study suggested that basic FGF-induced angiogenesis might be partly mediated through the endogenous chymase, the exact origin of chymase in sponge granulomas was not elucidated.

The significant role of mast cell chymase in angiogenesis was demonstrated in the present study where several kinds of chymase inhibitors including chymostatin, SBTI and LBTI inhibited angiogenesis induced by compound 48/80. As shown in Table 1 and also in many previous studies, chymostatin is a potent inhibitor of serine proteases with chymotrypsin-like substrate selectivity such as chymase, cathepsin G or chymotrypsin or elastase. SBTI and LBTI are also potent inhibitors of hamster chymase at submicromolar concentrations. It is likely that cathepsin G, which are contained in neutrophils or mast cells (Schechter et al., 1990) or leukocyte-derived elastase may be implicated in new blood vessel formation because mast cells, neutrophils and macrophages are often observed in the sponge granulomas or stroma. However, not only chymostatin, but also SBTI and LBTI, much weaker inhibitors of cathepsin G and elastase, inhibited compound 48/80-induced angiogenesis to the same extent, suggesting the significant role of chymase, which was the primary enzyme inhibited by these inhibitors, in angiogenesis.

CI-21 was a chloromethylketone derivative that was highly specific for human and hamster chymase. Its inhibitory action on elastase, cathepsin G, tryptase or trypsin was much less potent (more than 1000 times), as compared with that on hamster chymase (Table 1). Therefore, the result of the present experiment shown in Fig. 4D strongly suggests that chymase, not tryptase, elastase or cathepsin G, should be responsible for the compound 48/80-induced angiogenesis.

The presence of chymase in the compound 48/80-treated sponge granulomas was confirmed by the enzymatic activity and the immunoreactivity. As shown in Fig. 6, the increase in chymase activity paralleled the progress of angiogenesis. Immunohistochemical studies also revealed that chymase-like immunoreactivity was also de-

tected in sponge granulomas, mainly in the granules of mast cells and in the matrix adjacent to mast cells. These results suggest a close relationship between mast cell-derived chymase and the angiogenesis in this hamster angiogenesis model. Probably, the active chymase, released from mast cells activated by compound 48/80 or various endogenous stimuli, may be retained on the cell surface of mast cells or extracellular matrix by binding to heparin-like molecules and may exert its angiogenic effects.

The possible role of mast cell chymase in the activation of matrix metalloproteinases and in subsequent angiogenesis has been recently reported in mice (Coussens et al., 1999). The substrate specificity of mouse chymase is different from that of hamster and human chymase. Therefore, the substrates of chymase responsible for neovascularization are still unknown in hamsters. It is probable that chymase may promote angiogenesis by degrading the matrix proteins, generating active cytokines from their latent forms or activating certain enzymes, including matrix metalloproteinases in hamsters (Vartio et al., 1981; Gruber et al., 1990; Saarinen et al., 1994; Taipale et al., 1995; Kido et al., 1998). It is also likely that the local generation of Angiotensin II is mediated by chymase in the sponge granulomas. Probably, these factors may synergistically promote the migration and the proliferation of vascular wall cells, resulting in the formation of new blood vessels.

As previously reported (Majima et al., 1997; Muramatsu et al., 2000), the hemoglobin contents in the sponge implants and surrounding granuloma tissues correlated with the extent of angiogenesis. We also confirmed here that the hemoglobin contents in sponge granulomas treated with compound 48/80 correlated significantly with the number and lumen area of newly formed microvessels (Fig. 3A and B). Furthermore, in our preliminary study, we investigated the expression of CD31 (P-selectin) as a marker of the endothelial cells in the sponge granulomas by reverse-transcriptional polymerase chain reaction (RT-PCR) and found that expression of CD31 was also correlated with the hemoglobin contents in the sponge granulomas (unpublished observations). Therefore, the hemoglobin content represented the extent of angiogenesis in sponge implants.

The sensitivity of mast cells to compound 48/80 varies between species (Barrett et al., 1983; Leung and Pearce, 1984). For example, guinea pig lung mast cells are resistant to the action of compound 48/80. We have confirmed that hamster peritoneal and cheek pouch mast cells are activated by treatment with compound 48/80 at physiological concentrations. Although we have not thoroughly investigated the specificity of compound 48/80 in hamsters, it did not show any cytotoxicity or other pharmacological effects when used with various hamster cell lines including HPD-NR (Syrian hamster pancreatic cancer cell line), HIT-T15 (cell line from Syrian hamster pancreatic β cells) and BHK-21 (cell line from Syrian hamster kidney) at the doses employed in the present study. Therefore, we believe that compound 48/80 is specific as a mast cell secretagogue in hamsters at the doses used in hamsters, as in rats.

The importance of mast cells has been suggested in experiments demonstrating that activation of mast cells could induce microvessel formation in several in vivo angiogenesis models including the chick chorioallantoic membrane (Clinton et al., 1988) and the rat mesentery microcirculation model (Norrby et al., 1986). The hamster sponge-implant model is different from these angiogenesis models in that angiogenesis is accompanied by development of granulation tissue and is partly influenced by inflammatory and/or wound healing reactions. Therefore, it is probable that certain endogenous angiogenic/anti-angiogenic factors would synergistically act with mast cell-derived mediators. We confirmed in the previous study (Muramatsu et al., 2000) that the angiogenic effects of chymase was pronounced in the presence of other angiogenic mediators such as basic FGF, although chymase alone was a proangiogenic factor. Therefore, we used the sponge-implant model in the present study because the angiogenic action of chymase should be evaluated in the presence of endogenous factors.

In addition to chymase, mast cells release or produce several types of mediators with a variety of biological functions, such as histamine, proteoglycans, e.g., heparin and neutral proteases including tryptase, carboxypeptidase A and cathepsin G. Recent studies also suggest the presence of cytokines and growth factors in mast cells (Brown et al., 1987; Gordon and Galli, 1990; Reed et al., 1995; Grützkau et al., 1998). Activation of mast cells also induces the de novo synthesis and release of lipid-mediators, such as leukotrienes (leukotriene $C_4$ and leukotriene $B_4$), prostaglandins (prostaglandin $D_2$) and platelet-activating factor (PAF). Several of these factors have been proved to be angiogenic. The in vivo angiogenic effects of histamine were first reported in the cornea (Zauberman et al., 1969). Heparin is also angiogenic in vivo and protamine sulphate, a heparin inhibitor, blocks angiogenesis (Taylor and Folkman, 1982). Tryptase stimulates the proliferation of various types of cells including smooth muscle cells and fibroblasts (Ruoss et al., 1991; Brown et al., 1995). It also stimulates endothelial cells to form a tubular structure in vitro (Blair et al., 1997).

We also investigated in the present study the roles of some of these factors in the hamster sponge angiogenesis model. Our results suggest that histamine is, at least, in part implicated in compound 48/80-induced angiogenesis because pyrilamine (histamine H1 receptor antagonist) had only weak inhibitory effects. Further, pyrilamine showed only a slight inhibitory effect in antigen-induced angiogenesis. Hence, histamine released from mast cells seems to be only partially involved in the angiogenic process in hamsters.

Compound 48/80-induced angiogenesis is probably not mediated by mast cell-derived tryptase, because leupeptin, a potent inhibitor of tryptase, did not suppress angiogene-

sis at a dose of 100 μg/site. In addition, leupeptin did not inhibit the development of granulomas, suggesting that tryptase is unlikely to be involved in the proliferation of fibroblasts. Although the reason for the discrepancy between the in vitro mitogenic/angiogenic activity in cultured cells and the present in vivo study is not known, it is likely that tryptase activity in vivo is regulated by the intrinsic inhibitors.

Mast cell is known as a major participant in certain inflammatory diseases including allergic reactions and asthma. The present results suggest that repetitive antigen challenge could induce angiogenesis via chymase-dependent mechanisms in these human diseases. These findings suggest the therapeutic potential of specific chymase inhibitors in allergic diseases.

In conclusion, we demonstrated in the present study that mast cell activation by compound 48/80 or antigen induced angiogenesis in the hamster sponge angiogenesis model. Our results also showed that the angiogenic response was inhibited by treatment with chymase inhibitors, suggesting that mast cell-derived chymase is an important mediator of mast cell-dependent angiogenesis in hamsters.

## References


Andrade, S.P., Fan, T.-P.D., Lewis, G.P., 1987. Quantitative in-vivo studies on angiogenesis in a rat sponge model. Br. J. Pathol. 68, 755–766.

Balcells, E., Meng, Q.C., Johnson, W.H. Jr., Oparil, S., Dell'Italia, L.J., 1997. Angiotensin II formation from ACE and chymase in human and animal heart: method and species considerations. Am. J. Physiol. 273, H1769–H1774.

Barrett, K.E., Ennis, M., Pearce, F.L., 1983. Mast cells isolated from guinea-pig lung: characterization and studies on histamine secretion. Agents Actions 13, 122–126.

Blair, R.J., Meng, H., Marchese, M.J., Ren, S., Schwartz, L.B., Tonnesen, M.G., Gruber, B.L., 1997. Human mast cells stimulate vascular tube formation. Tryptase is a novel, potent angiogenic factor. J. Clin. Invest. 99, 2691–2700.

Brown, M.A., Pierce, J.H., Watson, C.J., Falco, J., Ihle, J.N., Paul, W.E., 1987. B cell stimulatory factor-1/interleukin-4 mRNA is expressed by normal and transformed mast cells. Cell 50, 809–818.

Brown, J.K., Tyler, C.L., Jones, C.A., Ruoss, S.J., Hartmann, T., Caughey, G.H., 1995. Tryptase, the dominant secretory granular protein in human mast cells, is a potent mitogen for cultured dog tracheal smooth muscle cells. Am. J. Respir. Cell Mol. Biol. 13, 227–236.

Chandrasekharan, U.M., Sanker, S., Glynias, M.J., Karnik, S.S., Husain, A., 1996. Angiotensin II-forming activity in a reconstructed ancestral chymase. Science 271, 502–505.

Clinton, M., Long, W.F., Williamson, F.B., Duncan, J.I., Thompson, W.D., 1988. Effect of the mast cell activator compound 48/80 and heparin on angiogenesis in the chick chorioallantoic membrane. Int. J. Microcirc.: Clin. Exp. 7, 315–326.

Coussens, L.M., Raymond, W.W., Bergers, G., Laig-Webster, M., Behrendtsen, O., Werb, Z., Caughey, G.H., Hanahan, D., 1999. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. Genes Dev. 13, 1382–1397.

Fan, T.-P.D., Brem, S., 1992. The search for anti-cancer drugs. In: Waring, M.J., Ponder, B.A.J. (Eds.), Cancer Biology and Medicine Series vol. 3 Kluwer Academic Publishing, Dordrecht, pp. 185–229.

Folkman, J., Shing, Y., 1992. Angiogenesis. J. Biol. Chem. 267, 10931–10934.

Goldstein, S.M., Kaempfer, C.E., Proud, D., Schwartz, L.B., Irani, A.-M., Wintroub, U., 1987. Detection and partial characterization of a human mast cell carboxypeptidase. J. Immunol. 139, 2724–2729.

Gordon, J.R., Galli, S.J., 1990. Mast cells as a source of both preformed and immunologically inducible TNF-α/cachectin. Nature 346, 274–276.

Gruber, B.L., Marchse, M.J., Carsons, S.E., Schecter, N.M., 1990. Human mast cell chymase degrades basement membrane components laminin, fibronectin and type IV collagen. Clin. Res. 38, 578A.

Grützkau, A., Krüger-Krasagakes, S., Baumeister, H., Schwarz, C., Kögel, H., Welker, P., Lippert, U., Henz, B.M., Möller, A., 1998. Synthesis, storage, and release of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) by human mast cells: implications for the biological significance of VEGF206. Mol. Biol. Cell 9, 875–884.

Hogaboam, C.M., Donigi-Gale, D., Shoupe, T.S., Bissonnette, E.Y., Befus, A.D., Wallace, J.L., 1992. Platelet-activating factor synthesis by peritoneal mast cells and its inhibition by two quinoline-based compounds. Br. J. Pharmacol. 105, 87–92.

Hopsu, V.K., Glenner, G.G., 1963. A histochemical enzyme kinetic system applied to the trypsin-like amidase and esterase activity in human mast cells. J. Cell Biol. 17, 503–520.

Hu, D.E., Hiley, C.R., Smither, R.L., Gresham, G.A., Fan, T.-P.D., 1996. Correlation of $^{133}$Xe clearance, blood flow and histology in the rat sponge model for angiogenesis. Further studies with angiogenic modifiers. Lab. Invest. 72, 601–610.

Jakobsson, A., Sorbo, J., Norrby, K., 1990. Protamine and mast-cell-mediated angiogenesis in the rat. J. Exp. Pathol. 71, 209–217.

Kido, H., Nakano, A., Okishima, N., Wakabayashi, H., Kishi, F., Nakaya, Y., Yoshizumi, M., Tamaki, T., 1998. Human chymase, an enzyme forming novel bioactive 31-amino acid length endothelins. Biol. Chem. 379, 885–891.

Lagunoff, D., Pritzl, P., 1976. Characterization of rat mast cell granule proteins. Arch. Biochem. Biophys. 173, 554–563.

Le Trong, H., Neurath, H., Woodbury, R.G., 1987. Substrate specificity of the chymotrypsin-like protease in secretory granules isolated from rat mast cells. Proc. Natl. Acad. Sci. U. S. A. 84, 364–367.

Leung, K.B., Pearce, F.L., 1984. A comparison oh histamine secretion from peritoneal mast cells of the rat and hamster. Br. J. Pharmacol. 81, 693–701.

Lewis, R.A., Soter, N.A., Diamond, P.T., Austen, K.F., Oates, J.A., Roberts, L.J., 1982. Prostaglandin $D_2$ generation after activation of rat and human mast cells with anti-IgE. J. Immunol. 129, 1627–1631.

Majima, M., Isono, M., Ikeda, Y., Hayashi, I., Hatanaka, K., Harada, Y., Katsumata, O., Yamashita, S., Katori, M., Yamamoto, S., 1997. Significant roles of inducible cyclooxygenase (COX)-2 in angiogenesis in rat sponge implants. Jpn. J. Pharmacol. 75, 105–114.

Meininger, C.J., Zetter, B.R., 1992. Mast cells and angiogenesis. Semin. Cancer Biol. 3, 73–79.

Metcalfe, D.D., Lewis, R.A., Silbert, J.E., Rosenberg, R.D., Wasserman, S.I., Austen, K.F., 1979. Isolation and characterization of heparin from human lung. J. Clin. Invest. 64, 1537–1543.

Muramatsu, M., Katada, J., Hayashi, I., Majima, M., 2000. Chymase as a proangiogenic factor; a possible involvement of chymase-angiotensin-dependent pathway in the hamster sponge angiogenesis model. J. Biol. Chem. 275, 5545–5552.

Norrby, K., Sorbo, J., 1995. Heparin enhances angiogenesis by a systemic mode of action. Int. J. Exp. Pathol. 73, 147–155.

Norrby, K., Woolley, D., 1993. Role of mast cells in mitogenesis and angiogenesis in normal tissue and tumour tissue. Adv. Biosci. 89, 71–116.

Norrby, K., Jakobsson, A., Sorbo, J., 1986. Mast cell-mediated angiogenesis: a novel experimental model using the rat mesentery. Virchows Arch. B 52, 195–206.

Okunishi, H., Miyazaki, M., Okamura, Y., Toda, N., 1987. Different distribution of two types of angiotensin II-generating enzymes in the aortic wall. Biochem. Biophys. Res. Commun. 149, 1186–1192.

Okunishi, H., Oka, Y., Shiota, N., Kawamoto, T., Song, K., Miyazaki, M., 1993. Marked species-difference in the vascular angiotensin II-forming pathways: humans versus rodents. Jpn. J. Pharmacol. 62, 207–210.

Rakusan, K., Sarkar, K., Turek, Z., Wicker, P., 1990. Mast cells in the rat heart during normal growth and cardiac hypertrophy. Circ. Res. 66, 511–516.

Razin, E., Mencia-Huerta, J.M., Lewis, R.A., Corey, E.J., Austen, K.F., 1982. Generation of leukotriene C-4 from subclass of mast cells differentiated in vitro from mouse bone marrow. Proc. Natl. Acad. Sci. U. S. A. 79, 4665–4667.

Reed, J.A., Albino, A.P., McNutt, N.S., 1995. Human cutaneous mast cells express basic fibroblast growth factor. Lab. Invest. 72, 215–222.

Rhodin, J.A.G., Fujita, H., 1989. Capillary growth in the mesentery of normal young rats. Intravital video and electron microscope analyses. J. Submicrosc. Cytol. Pathol. 21, 1–34.

Ruoss, S.J., Hartmann, T., Caughey, G.H., 1991. Mast cell tryptase is a mitogen for cultured fibroblasts. J. Clin. Invest. 88, 493–499.

Saarinen, J., Kalkkinen, N., Welgus, H.G., Kovanen, P.T., 1994. Activation of human interstitial procollagenase through direct cleavage of the $Leu^{83}$–$Thr^{84}$ bond by mast cell chymase. J. Biol. Chem. 269, 18134–18140.

Sayama, S., Iozzo, R.V., Lazarus, G.S., Schechter, N.M., 1987. Human skin chymotrypsin-like proteinase chymase. Subcellular localization to mast cell granules and interaction with heparin and other glycosaminoglycans. J. Biol. Chem. 262, 6808–6815.

Schechter, N.M., Fräki, J.E., Geesin, J.C., Lazarus, G.S., 1983. Human skin chymotryptic proteinase. Isolation and relation to cathepsin G and rat mast cell proteinase I. J. Biol. Chem. 258, 2973–2978.

Schechter, N.M., Irani, A.-M.A., Sprows, J.L., Abernethy, J., Wintroub, B., Schwartz, L.B., 1990. Identification of a cathepsin G-like proteinase with $MC_{TC}$ type of human mast cell. J. Immunol. 145, 2652–2661.

Schwartz, L.B., 1994. Tryptase: a mast cell serine protease. Methods Enzymol. 244, 88–100.

Schwartz, L.B., Lewis, R.A., Austen, K.F., 1981. Tryptase from human pulmonary mast cells: purification and characterization. J. Biol. Chem. 256, 11939–11943.

Sorbo, J., Norrby, K., 1992. Mast cell histamine expands the microvasculature spatially. Agents Actions 32, 387–389.

Taipale, J., Lohi, J., Saarinen, J., Kovanen, P.T., Keski-Oja, J., 1995. Human mast cell chymase and leukocyte elastase release latent transforming growth factor-β1 from the extracellular matrix of cultured human epithelial and endothelial cells. J. Biol. Chem. 270, 4689–4696.

Taylor, S., Folkman, J., 1982. Protamine is an inhibitor of angiogenesis. Nature 297, 307–312.

Unger, E.F., Sheffield, C.D., Epstein, S.E., 1991. Heparin promotes the formation of extracardiac to coronary anastomoses in a canine model. Am. J. Physiol. 260, H1625–H1634, (Heart Circ. Physiol. 29).

Vartio, T., Seppä, H., Vaheri, A., 1981. Susceptibility of soluble and matrix fibronectins to degradation by tissue proteinases, mast cell chymase and cathepsin G. J. Biol. Chem. 256, 471–477.

Wintroub, B.U., Kaempfer, C.E., Schechter, N.M., Proud, D., 1986. A human lung mast cell chymotrypsin-like enzyme. Identification and partial characterization. J. Clin. Invest. 77, 196–201.

Zauberman, H., Michaelsson, I.C., Bergmann, F., Maurice, D.M., 1969. Stimulation of neovascularization of the cornea by biogenic amines. Exp. Eye Res. 8, 77–83.