Int. J. Dev. Biol. 55: 99-102 (2011)
doi: 10.1387/ijdb.103138dr

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Tryptase and chymase are angiogenic *in vivo* in the chorioallantoic membrane assay

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ABSTRACT Human mast cells (MCs) are divided in two types depending on the expression of tryptase and chymase in their granules. Literature data indicate that both tryptase and chymase are angiogenic, but there is currently no evidence of their direct angiogenic activity *in vivo*. In this study, we have investigated the capacity of tryptase and chymase to promote vasoproliferation in chick embryo chorioallantoic membrane (CAM), a well established *in vivo* assay to study angiogenesis and anti-angiogenesis. The results showed that both tryptase and chymase stimulate angiogenesis and that the response is similar to that obtained with vascular endothelial growth factor (VEGF), a well-known angiogenic cytokine, and confirm the angiogenic activity of these two proteases stored in MC granules.

KEY WORDS: *angiogenesis, chorioallantoic membrane, chymase, mast cells, tryptase*

Human mast cells (MCs) are conventionally divided in two types depending on the expression of different proteases in their granules (Irani *et al*., 1986). MCs that contain tryptase only, are designed as MC_T of "immune associated" MCs. They are predominantly located in the respiratory and intestinal mucosa, where they co-localize around T lymphocytes. MCs that contain both tryptase and chymase, along with other proteases such as carboxypeptidase A and cathepsin G, are referred as MC_{TC}. They are predominantly found in the connective tissue areas, such as skin, hypodermis and intestine, breast parenchyma, myocardium, lymph node, conjunctiva, and synovium. A third type of MC, called MCc has been identified, which express chymase without tryptase and resides mainly in the submucosa and mucosa of the stomach, small intestinal submucosa, and colonic mucosa (Irani and Schwartz, 1994).

Tryptase and chymase are involved in angiogenesis after their release from activated MC granules. Their proteolytic activities degrade extracellular matrix components or release matrix-associated growth factors (Taipale *et al*., 1995), and they also act indirectly by activating latent matrix metalloproteinases (Gruber *et al*., 1989) and plasminogen activators, both key enzymes of proteolytic systems that contribute to the degradation of extracellular matrix components (Stack and Johnson, 1994). Extracellular matrix degradation is a critical step in the early stages of angiogenesis as well as during invasion and metastasis of tumor cells.

Blair *et al*. (1997), have demonstrated the angiogenic potential of tryptase *in vitro* and its important role in neovascularization. Tryptase added to microvascular endothelial cells cultured on Matrigel caused a pronounced increase in capillary growth, and this was suppressed by specific tryptase inhibitors. Moreover, tryptase directly induced endothelial cell proliferation in a dose-dependent fashion. Human chymase can induce angiogenesis by converting angiotensin I to angiotensin II, as was demonstrated in a hamster sponge model (Muramatsu *et al*., 2000).

The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane which serves as a gas exchange surface and its function is supported by a dense capillary network. Because of its extensive vascularization and easy accessibility, the CAM has been broadly used to study the morphofunctional aspects of the angiogenesis process *in vivo* and to investigate the efficacy and mechanisms of action of pro-angiogenic and anti-angiogenic natural and synthetic molecules (Ribatti, 2008).

In this study, we have investigated the capacity of exogenous tryptase and chymase to promote vasoproliferation in the CAM assay.

Abbreviations used in this paper: CAM, chorioallantoic membrane; MC, mast cell.

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Accepted: 22 June 2010. *Final author corrected PDF published online:* 21 January 2011

ISSN: Online 1696-3547, Print 0214-6282

Results and Discussion

At day 12 of incubation, vehicle alone did not induce any angiogenic response (mean number of vessels = 8±2) (not shown) (Fig. 1), whereas a significant angiogenic response was induced by vascular endothelial growth factor (VEGF) in form of numerous allantoic neovessels developing radially towards the implant in a 'spoked-wheel' pattern (mean number of vessels = 25±4) (not shown) (Fig. 1). When human recombinant tryptase or chymase were tested at a concentration of 200 ng, they induced a strong angiogenic response (mean number of vessels = 24±4 for tryptase and, respectively, 22±3 for chymase (Figs. 1 and 2 A,B). Lower doses did not have an angiogenic effect, while when both tryptase and chymase were tested at 300 ng/embryo they induce a comparable angiogenic effect.

Histological examination of the sponges treated with tryptase and chymase showed numerous blood vessels inside the sponge trabeculae (Fig. 2C). Moreover, at the boundary between the sponge and the CAM mesenchyme, numerous host capillaries were recognizable, piercing the sponge at some points. In contrast, no blood vessels were recognizable among the sponge trabeculae in sponges treated with PBS (Fig. 2D).

In this study, for the first time we have demonstrated an angiogenic activity of human recombinant tryptase and chymase *in vivo* in the CAM assay, comparable to the angiogenic response induced by a well-known angiogenic cytokine, namely VEGF.

There is increasing evidence to support the view that angiogenesis and inflammation are mutually dependent (Ribatti and Crivellato, 2009a). During inflammatory reactions, immune cells, including macrophages, neutrophils, lymphocytes and mast cells, synthesize and secrete pro-angiogenic factors that promote neovascularization. On the other hand, the newly formed vascular supply contributes to the perpetuation of inflammation by promoting the migration of inflammatory cells to the site of inflammation. Tumor cells are surrounded by an infiltrate of inflammatory cells, which communicate via a complex network of intercellular signaling pathways, mediated by surface adhesion molecules, cytokines and their receptors. Accordingly, immune cells cooperate and synergize with stromal cells as well as malignant cells in stimulating endothelial cell proliferation and blood vessel formation (Ribatti *et al.*, 2006b).

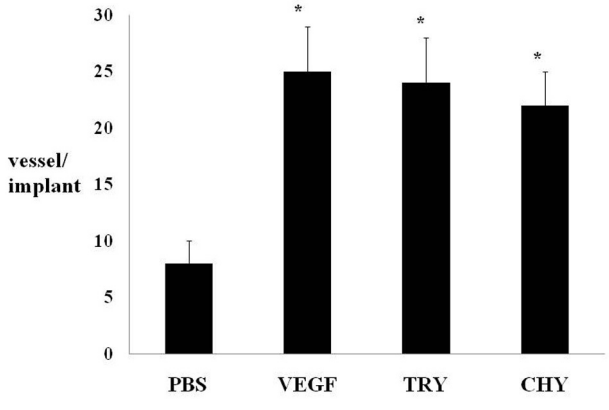

Fig 1. Tryptase (TRY) and chymase (CHY) trigger an angiogenic response in the chorioallantoic membrane (CAM) assay. *This response is comparable to that induced by vascular endothelial growth factor (VEGF). * p < 0.001 vs PBS.*

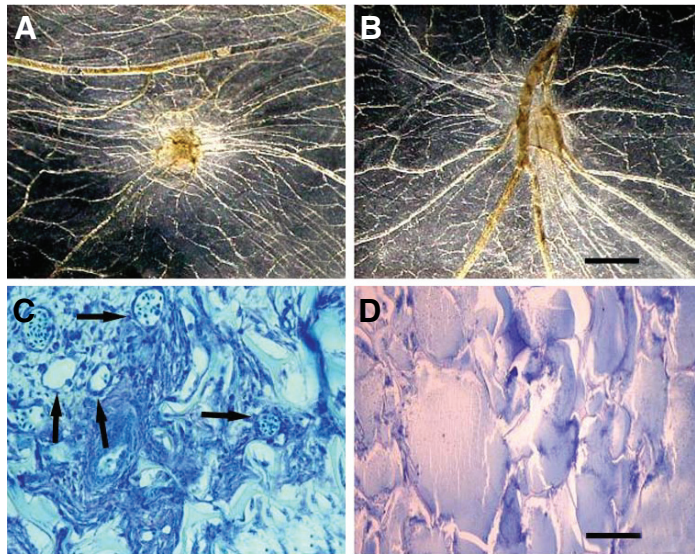

Fig. 2. Tryptase and chymase are angiogenic *in vivo* in the CAM assay. *In* **(A,B)** *macroscopic pictures of CAMs at day 12 of incubation, treated with tryptase* **(A)** *and chymase* **(B)** *respectively. Note the presence of numerous blood vessels converging toward the implant.* **(C,D)** *Histological pictures of CAMs treated with tryptase and the vehicle alone, respectively. Note in* **(C)** *numerous microvessels inside the sponge (arrows), while in the control specimen* **(D)***, no blood vessels are recognizable. Scale bars:* (A,B) *25 μm;* (C,D) *100 μm.*

For almost two decades MCs, strategically located in proximity of blood vessels, have been associated with tumor angiogenesis. A positive correlation between MC density and microvascular density has been established in esophageal squamous cell carcinoma (Tomita *et al.*, 2001), primary colorectal cancer (Acikalin *et al.*, 2005; Gulubova and Vlaykova, 2009), hepatocellular carcinoma (Peng *et al.*, 2005); pancreatic adenocarcinoma (Esposito *et al.*, 2004), renal cell carcinoma (Tuna *et al.*, 2006), non-small cell lung cancer (Imada *et al.*, 2000; Ibaraki *et al.*, 2005).

Tryptase and chymase are involved in angiogenesis after their release from activated mast cells granules (Ribatti *et al.*, 2009 b). Tryptase-positive mast cells increase in number and vascularization increases in a linear fashion in solid tumors, such as human malignant melanoma (Ribatti *et al.*, 2003 a, b), endometrial carcinoma (Benítez-Bribiesca *et al.* 2001, Ribatti *et al.* 2005), breast cancer (Ribatti *et al.*, 2007a; Ranieri *et al.*, 2009), uterine leiomyomas (Ribatti *et al.*, 2007b), colorectal cancer (Gulubova and Vlaykova, 2009).

As concerns hematological tumors, in benign lymphadenopathies and B cell non-Hodgkin's lymphomas, angiogenesis correlates with tryptase-positive mast cell counts, and both increase in step with the increase with malignancy grades (Ribatti *et al.*, 2000). In the bone marrow of patients with inactive and active multiple myeloma as well as those with monoclonal gammopathies of undetermined significance, angiogenesis highly correlates with tryptase-positive mast cells (Ribatti *et al.*, 1999). A similar pattern of correlation between bone marrow microvessel count, total and tryptase-positive mast cell density and tumor progression has been found in patients with myelodysplastic syndrome (Ribatti *et*

al., 2002) and B cell chronic lymphocytic leukemia (Ribatti *et al.*, 2003 c). In B cell chronic lymphocytic leukemia, the density of tryptase-positive mast cell in the bone marrow has been shown to predict the outcome of the disease (Molica *et al.*, 2003).

Other authors have parallely investigated the presence of MC_T, MC_C and MC_{TC} and have demonstrated a correlation between their number and microvascular density in uterine cervix cancer (Cabanillas-Saez *et al.*, 2002; Wilk *et al.*, 2010), gastric cancer (Ribatti *et al.*, 2010) and in non small cell lung cancer (Carlini *et al.*, 2010).

The complex interplay between MCs and angiogenesis have led to consider the therapeutic use of angiogenesis inhibitors, which specifically target the angiogenic activity of tryptase and chymase. In this context, tryptase inhibitors such as gabexate mesilate and nafamostat mesilate (Erba *et al.*, 2001; Mori *et al.*, 2003; Uchima *et al.*, 2007) might be proposed as antiangiogenic agents in combination with chemotherapy in the treatment of cancer.

Materials and Methods

Fertilized White Leghorn chicken eggs (20 per group) were incubated at 37°C at constant humidity. On day 3, a square window was opened in the shell, and 2 to 3 ml of albumen were removed to allow detachment of the developing CAM from the shell. The window was sealed with a glass, and the eggs were returned to the incubator. On day 8, eggs were treated with 1 mm^3 sterilized gelatin sponges (Gelfoam Upjohn Company, Kalamazoo, MI, USA) placed on top of the growing CAM, as previously described (Ribatti *et al.*, 2006a) and loaded with: 2 μl of PBS as negative control; 2 μl of PBS containing 50 ng of recombinant VEGF-A alone as positive control (R & D Systems, Abington, UK) or 2 of PBS containing human recombinant tryptase or chymase administered at a concentration of 50, 100, 200, 300 ng (Sigma-Aldrich, St Louis, MO, USA). CAMs were examined daily until day 12 and photographed *in ovo* with a stereomicroscope equipped with a camera and image analyzer system (Olympus Italia, Rozzano, Italy). At day 12 the angiogenic response was evaluated by the image analyzer system as the number of vessels centering the sponges within the focal plane of the CAM. Means ± 1 Standard Deviation (SD) were evaluated for all the parameters and the statistical significance of the differences between the counts was determined by Student's *t*-test for unpaired data.

CAMs were also processed for light microscopy. Briefly, the embryos and their membranes were fixed *in ovo* in Bouin's fluid. The sponges and the underlying and immediately adjacent CAM portions were removed and processed for embedding in paraffin. Eight μm serial sections were cut along a plane parallel to the CAM surface, stained with 0.5% aqueous toluidine blue (Merck, Darmtadt, Germany) and observed under a light microscope.

Acknowledgements

Supported in part by MIUR (PRIN 2007), Rome, and Fondazione Cassa di Risparmio di Puglia, Bari, Italy.

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