

# Mast cells and their secretory granules are angiogenic in the chick embryo chorioallantoic membrane

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## Summary

**Background** Many data suggest that the density of mast cells is highly correlated with the extent of both normal and pathological angiogenesis.

**Objective** In this study we have compared in an *in vivo* assay, the chick embryo chorioallantoic membrane, the angiogenic potential of mast cell suspensions isolated from rats, degranulated mast cells and their secretory granules.

**Methods** Gelatin sponges adsorbed with cell suspensions of rat mast cells, degranulated mast cells and their secretory granules were implanted on the top of the chorioallantoic membrane at day 8 of incubation. At day 12 the angiogenic response was evaluated macroscopically, microscopically and by a morphometric method of 'point counting'.

**Results** Isolated mast cells and their secretory granules, but not degranulated mast cells, induced an angiogenic response in the chorioallantoic membrane. The addition of anti-fibroblast growth factor-2 or antivascular endothelial growth factor antibodies reduced the angiogenic response of both mast cells and their secretory granules by 50% and 30%, respectively.

**Conclusion** These data support the evidence that the angiogenic properties of mast cells depend on the angiogenic molecules contained in their secretory granules and indicate that fibroblast growth factor-2 and vascular endothelial growth factor are the angiogenic cytokines primarily and perhaps synergistically responsible for this vasoproliferative activity.

**Keywords:** angiogenesis, chorioallantoic membrane, fibroblast growth factor-2, mast cell, vascular endothelial growth factor

*Clinical and Experimental Allergy*, Vol. 31, pp. 602–608. Submitted 6 March 2000; revised 25 May 2000; accepted 19 June 2000.

## Introduction

Angiogenesis, a biological process by which new capillaries are formed, is essential in many physiological (embryo development, ovulation and wound repair) and pathological conditions, such as arthritis, diabetic retinopathy, and tumours [1]. Inflammatory cells surround the newly formed

blood vessels. They consist of fibroblasts, macrophages, mast cells (MCs) and leucocytes which contribute to induction of the angiogenic response by secreting cytokines and proteolytic enzymes that mobilize angiogenic factors stored in the extracellular matrix [2].

Many data suggest that the density of MCs is highly correlated with the extent of both normal and pathological angiogenesis, such as vessel formation which occurs in chronic inflammatory diseases and tumours (for review see [3, 4]). MCs release a variety of factors known to enhance

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angiogenic phenotypes, namely heparin [5], histamine [6], tryptase [7, 8] and several polypeptide growth factors, including fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), transforming growth factor  $\beta$  (TGF- $\beta$ ), tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin-8 (IL-8) [9–14].

The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane which serves as a gas exchange surface [15] and its function is supported by an extensive capillary network. It has been widely utilized as a target for angiogenic and antiangiogenic compounds (see [16] for a review). In this study we have compared the angiogenic potential of cell suspensions of MCs isolated from rats, degranulated MCs and their secretory granules adsorbed on gelatin sponges and implanted on top of the developing CAM.

## Materials and methods

### *Mast cell collection and stimulation by compound 48/80*

MCs were isolated from Wistar rats (Charles River, Como, Italy) as previously described [17]. Briefly, animals were anaesthetized with ether and killed by cervical dislocation. Their abdomens were massaged for 30 s and the peritoneal cavity was then exposed through a ventral incision. After intraperitoneal injection of 10–20 mL of buffered saline solution (BSA: containing 154 mM NaCl, 2.7 mM KCl, 0.68 mM CaCl<sub>2</sub>, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 1 g/L bovine serum albumin), adjusted to pH 7.2, cells were sedimented by centrifugation at 150 *g* for 10 min. The supernatant was removed and cells were resuspended in buffered medium. MCs were purified according to Lagunoff and Rickard [18]; their purity in the final preparation, as determined by staining with toluidine blue, was more than 90% (it could reach about 95–97% in some experiments). The contaminating cell population observed at the electron microscopy was constituted by middle-sized or large non-granular lymphocytes and, to a lesser degree, by macrophages.

Degranulated MCs and their granules were obtained as follows:  $1\text{--}4 \times 10^6$  MCs were incubated with 1 mL of BSA. After pre-incubation at 37 °C for 15 min, they were stimulated by the addition of 5  $\mu\text{g/mL}$  of compound 48/80 (Sigma Chemical Co., St Louis, MO, USA), and incubated for another 15 min to ensure their complete degranulation. The cells were then sedimented by centrifugation at 150 *g* for 10 min. The supernatant was removed and centrifuged at 8000 *g* for 20 min to sediment the exocytosed granules [19]. The sediment was washed twice by resuspension in PBS and recentrifuged at 8000 *g* for 20 min. The pellet was washed twice with PBS to remove compound 48/80.

### *CAM assay*

Fertilized White Leghorn chicken eggs (20 for each experimental series) were incubated under conditions of constant humidity at a temperature of 37 °C. On the third day of incubation, a square window was opened in the egg shell after removal of 2–3 mL of albumen so as to detach the developing CAM from the shell. The window was sealed with a glass of the same dimension, and the eggs were returned to the incubator.

Gelatin sponges (1 mm<sup>3</sup>; Gelfoam, Upjohn Company, Kalamazoo, USA) were placed on the CAM at day 8 and adsorbed with a 3- $\mu\text{L}$  suspension of MCs or degranulated MCs in PBS (18 000 cells per sponge) [20]. In a second set of experiments, the suspension contained MC granules in PBS. In some experiments, cell suspensions and MC granules were mixed with 400 ng/embryo of anti-FGF2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, catalogue SC-79-G) before implantation or with 400 ng/embryo of anti-VEGF antibody against N-terminal peptide 1–140 (Santa Cruz, catalogue SC-507) or with a mixture of the two antibodies. Sponges containing irrelevant rabbit IgG or vehicle alone (PBS) were used as negative controls, while sponges containing 1  $\mu\text{g}$  of recombinant FGF-2 (R & D Systems, Abingdon, UK) were used as positive controls.

At day 12, CAMs were photographed *in ovo*. All CAMs were then processed for light and electron microscopy. Briefly, the embryos and their membranes were fixed *in ovo* in 3% phosphate-buffered glutaraldehyde. The sponges and the underlying and immediately adjacent portions of CAM were dehydrated in serial alcohols, post-fixed in 1% phosphate-buffered OsO<sub>4</sub>, and embedded in Epon 812. One micrometer semithin and ultrathin sections were cut on a LKB V ultramicrotome according to a plane parallel to the surface of CAM. The semithin sections were stained with a 0.5% aqueous solution of Toluidine blue (Merck, Darmstadt, Germany) and observed under a Leitz-Dialux 20 light microscope (Leitz, Wetzlar, Germany). The ultrathin sections were stained with uranyl acetate followed by lead citrate and examined under a 9-A Zeiss electron microscope.

The angiogenic response was assessed histologically by a planimetric method of 'point counting' [20]. Briefly, every third section in 30 serial slides from each specimen was analysed under a 144-point mesh inserted into the eyepiece of the photomicroscope. The total number of intersection points occupied by transversally cut vessels (3–10  $\mu\text{m}$  diameter) was counted at  $\times 250$  both inside the sponge and at its boundary with the surrounding CAM mesenchyme in six randomly chosen fields per each section. Mean values  $\pm$  SD were determined for each analysis. Vascular density was defined as the final mean number of the occupied

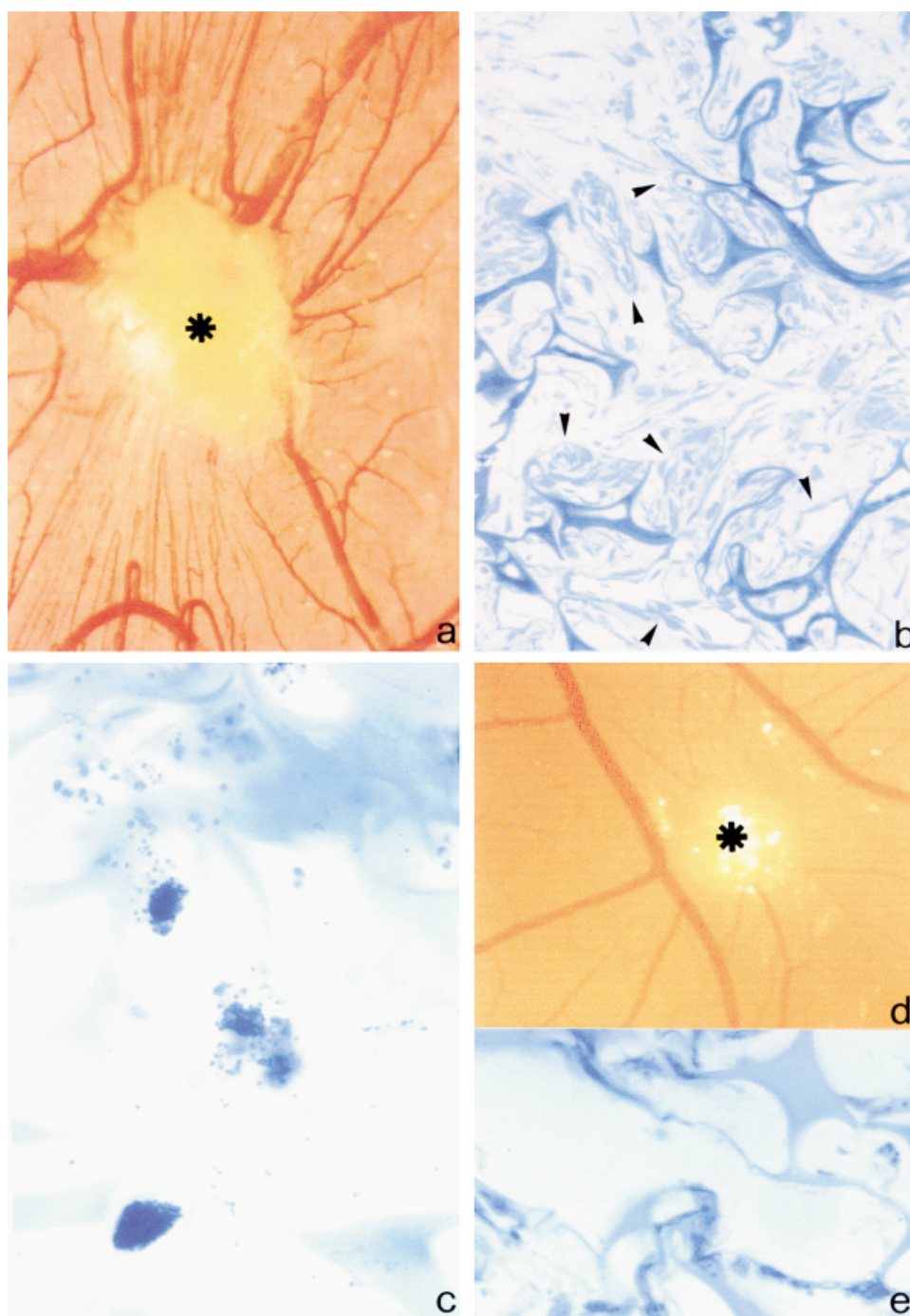


**Fig. 1.** Effect of mast cells (a–c) and vehicle alone (PBS) (d–e) on CAM vascularization. Cells were delivered on the top of the CAM at day 8 using a gelatin sponge implant. Macroscopic observation of the membranes was performed at day 12. In (a) a gelatin sponge is surrounded by allantoic vessels that develop radially towards the implant (asterisk) in a ‘spoked-wheel’ pattern. In (b–c) histological analysis showing among the sponge trabeculae in (b) a highly vascularized tissue consisting of newly formed blood vessels (arrowheads) and infiltrating fibroblasts within a network of collagen fibres and in (c) metachromatic mast cells and their secretory granules. In (d) no vascular reaction is detectable around the sponge (asterisk) and in (e) no blood vessels are recognizable among the sponge tabeculae. Original magnification: a, d  $\times 50$ ; b, c, e,  $\times 250$ .

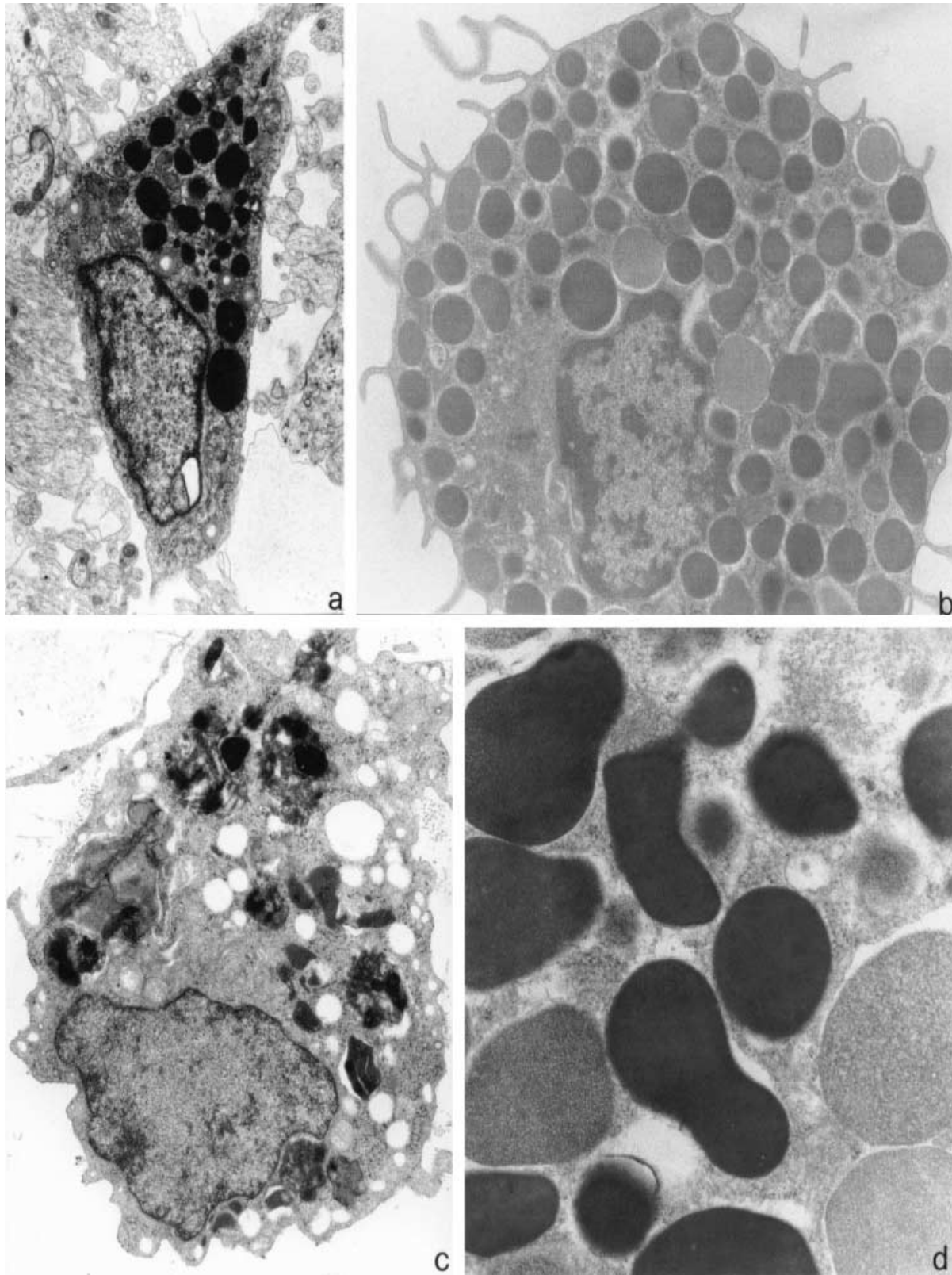


**Table 1** Angiogenic activity of MCs, degranulated MCs and their secretory granules in the chick embryo CAM

| Treatment                                   | N° of intersection points<br>(Mean $\pm$ SD) | Microvessel density<br>(%) |
|---------------------------------------------|----------------------------------------------|----------------------------|
| PBS                                         | 0 $\pm$ 1                                    | 0                          |
| Non immune rabbit IgG                       | 0 $\pm$ 1                                    | 0                          |
| MCs                                         | 35 $\pm$ 3                                   | 24.3                       |
| MCs + anti-FGF-2 ab                         | 18 $\pm$ 2*                                  | 12.5                       |
| MCs + anti-VEGF ab                          | 24 $\pm$ 3*                                  | 16.6                       |
| MCs + anti-FGF-2 + anti-VEGF ab             | 11 $\pm$ 4*                                  | 7.6                        |
| Degranulated MCs                            | 5 $\pm$ 2                                    | 3.5                        |
| MCs granules                                | 33 $\pm$ 4                                   | 22.9                       |
| MCs granules + anti-FGF-2 ab                | 16 $\pm$ 1**                                 | 11.1                       |
| MCs granules + anti-VEGF ab                 | 23 $\pm$ 2**                                 | 16.0                       |
| MCs granules + anti-FGF-2 +<br>anti-VEGF ab | 10 $\pm$ 2**                                 | 6.9                        |
| FGF2                                        | 30 $\pm$ 2                                   | 20.8                       |

\*Statistically different from MCs ( $P < 0.01$ )\*\*Statistically different from MCs granules ( $P < 0.01$ )

points. Statistics were obtained with Student's *t*-test for unpaired data.

## Results

### *Mast cells and their granules induce an angiogenic response in the chick embryo CAM*

Macroscopic observation of the membrane at day 12 showed that the sponges adsorbed with MCs or their granules were surrounded by allantoic vessels that developed radially towards the implant in a 'spoked-wheel' pattern (Fig. 1a). Microscopically, a highly vascularized tissue was observed among the trabeculae of these sponges that consisted of newly formed blood vessels, mainly 3–10  $\mu$ m diameter capillaries growing perpendicularly to the plane of the CAM, within an abundant network of collagen fibres (Fig. 1b). Metachromatic MCs and their secretory granules were also recognizable among the trabeculae (Fig. 1c).

A higher microvessel density was detectable within the sponges treated with MCs and their granules than in those treated with exogenous FGF-2 when the angiogenic response was quantified by a morphometric method of 'point counting' (Table 1). When degranulated MCs were adsorbed to gelatin sponges, no vascular reaction was detectable at day 12, similarly to the sponges treated with PBS only (Fig. 1d) or with non-immune rabbit serum, and no blood vessels were recognizable among the trabeculae (Fig. 1e).

At the ultrastructural level, typical MCs with cytoplas-

mic matrix filled with numerous electron dense secretory granules comparable to the isolated MCs (Fig. 2a,b), partly degranulated MCs and isolated granules (Fig. 2c,d) were recognizable.

### *The angiogenic activity of mast cells and their granules is partly dependent on FGF-2 or VEGF*

To assess whether the angiogenic response elicited by MCs and their granules was due in part to FGF-2 or VEGF present in the granules and in part to increased mobilization of endogenous FGF-2 stored in the CAM extracellular matrix [21], MCs or their granules were added to the CAM in the presence of anti-FGF-2 or anti-VEGF antibodies, both of which abolish the angiogenic activity exerted by recombinant FGF-2 or VEGF exogenously added to the CAM vasculature ([22]; Ribatti, unpublished observations). Anti-FGF-2 antibodies reduced the angiogenic response of both MCs and their secretory granules by 50%, and anti-VEGF antibodies by 30%. When anti-FGF-2 and anti-VEGF antibodies were added together, they acted in a synergistic way, reducing the angiogenic response by 70%.

## Discussion

The demonstration that isolated MCs and their secretory granules, but not degranulated MCs, induce an angiogenic response in the CAM vasculature provides further evidence for the view that the angiogenic potential of MCs is

**Fig. 2.** Electron micrographs of a mast cell (a), a degranulated mast cell (c), cytoplasmic mast cell secretory granules (d) among the sponge trabeculae, compared to an isolated rat peritoneal mast cell (b). Original magnification: a, c,  $\times 6500$ ; b,  $\times 9000$ ; d,  $\times 18000$ .

determined by molecules contained in their secretory granules.

MCs contain, in secretory granules, heparin that *in vitro* stimulates endothelial cell proliferation and migration and is angiogenic *in vivo* [5,23]. Histamine and tryptase, other MC-derived factors, also stimulate angiogenesis [6–8]. In addition, MCs produce a variety of multifunctional cytokines and growth factors, such as FGF-2, VEGF, TGF- $\beta$ , TNF- $\alpha$  and IL-8 [9–14], which may contribute to the angiogenic process.

Heparin, histamine, FGF-2 and VEGF are angiogenic when they are exogenously applied to the CAM vasculature. Heparin induces a strong vasoproliferative response when injected into the allantoic sac [5], and its inhibition by protamine sulphate retards CAM angiogenesis [24]. Histamine application enhances CAM microvascular proliferation [25,26]. FGF-2 and VEGF, among the best characterized and potent angiogenic molecules, are angiogenic in the CAM assay [22,27]. Moreover, endogenous FGF2 plays a rate-limiting role in CAM vascularization [22]. The Mwt 16 000 FGF2-like molecule we observed in the CAM [22] was identified as FGF-2 on the basis of its immune reactivity, affinity for heparin, and its ability to induce a biological response in cultured endothelial cells. In a parallel series of experiments, neutralization with anti-FGF-2 antibody adsorbed on methylcellulose discs exerted a potent antiangiogenic effect when applied on the surface of the CAM [22,28]. Histamine and heparin may work synergistically with angiogenic factors such as FGF-2 and VEGF in the secretory granules, to accelerate CAM angiogenesis. Moreover, metallo- and serine proteases also contained in the secretory granules may mobilize endogenous angiogenic factors, such as FGF-2, stored in the CAM extracellular matrix [29].

Indirect evidence of a substantial role played by MCs in the development of the CAM vascular system stems from the observations that infusion into the chick allantoic cavity of compound 48/80 causes MC degranulation and subsequent enhancement of CAM angiogenesis [30] and that a 40-fold increase in MC number precedes angiogenesis induced by tumour implants on the CAM [31].

The contaminating cells, such as lymphocytes and macrophages may also contribute to the vasoproliferative processes through angiogenic factors, such as FGF-2, VEGF, TGF- $\beta$  and TNF- $\alpha$ , stored in their cytoplasm [32–34].

#### *Neovascularization induced by degranulation is predominantly mediated by FGF-2 and VEGF*

Application of anti-FGF-2 or anti-VEGF antibodies reduced the angiogenic response of both MCs and their secretory granules by 50% and 30%, respectively. When the two antibodies were added together, they acted synergistically,

reducing the angiogenic response by 70%. This finding illustrates the principal role played by these two cytokines in the induction of an angiogenic response in the CAM vasculature. Qu et al. [35] demonstrated that FGF-2 immune reactivity was localized in the cytoplasmic granules of MCs in several human tissues and that immunogold particle localization for FGF2 was also present in the extruded granules of MCs. Yamada et al. [10] demonstrated that in rheumatoid arthritis synovial membrane MCs are the major source of VEGF and Boesiger et al. [11] showed that mouse or human MCs produce and secrete VEGF upon immunologically specific activation. Moreover, the partial inhibition of CAM angiogenesis exerted by the antibodies against FGF-2 or VEGF suggests that these two cytokines may act synergistically in the induction of the CAM vasoproliferative response, as demonstrated in other systems [36,37] and in the CAM assay (Ribatti, unpublished observations).

In conclusion, our results corroborate the view that MCs modulate angiogenesis in the CAM during development of its vasculature system. They also indicate that FGF-2 and VEGF are the angiogenic molecules in MC granules primarily and perhaps synergistically responsible for this vasoproliferative activity.

#### Acknowledgements

This work was supported in part by a grant from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST, local funds), Rome, Italy, to DR, from Associazione Italiana per la Lotta al Neuroblastoma, Genoa, Italy to DR, and by a grant from Associazione Italiana per la Ricerca sul Cancro (AIRC), Milan, Italy, to DR and AV.

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