Temporal expression of the matrix metalloproteinase MMP-2 correlates with fibronectin immunoreactivity during the development of the vascular system in the chick embryo chorioallantoic membrane

DOMENICO RIBATTI[1], BEATRICE NICO[1], ANGELO VACCA[2], MONICA IURLARO[2], LUISA RONCALI[1]

[1] *Institute of Human Anatomy, Histology and Embryology, and* [2] *Department of Biomedical Sciences and Human Oncology, University of Bari Medical School, Bari, Italy*

(*Accepted 9 March 1999*)

ABSTRACT

In this study we have examined in the chick embryo chorioallantoic membrane (ChAM) the expression of the matrix metalloproteinase 2 (MMP-2) and have correlated this parameter with the expansion of the ChAM vasculature and with the expression of 3 extracellular matrix components (fibronectin, laminin and type IV collagen), which differentially modulate angiogenesis. In the early phases of ChAM development, between d 6 and d 8 of incubation, when the increase of the ChAM vasculature is maximal, higher values of MMP-2 and, respectively, of fibronectin immunoreactive area, are detectable. These results indicate that MMP-2 activity and fibronectin expression are 2 strictly related components of angiogenesis occurring in vivo.

Key words: Angiogenesis; metalloproteinase 2.

INTRODUCTION

The chick embryo chorioallantoic membrane (ChAM) is an extraembryonic membrane which serves as a gas exchange surface (Romanoff, 1960) and its function is supported by an extensive capillary network. ChAM is formed on d 4 of incubation, when undifferentiated blood vessels are scattered in its mesoderm. They grow very rapidly until d 8, thus originating a network of capillaries that migrate to occupy an area beneath the chorion and mediate gas exchange with the outer environment. Rapid capillary proliferation continues until d 11; thereafter, the endothelial cell mitotic index declines rapidly, and the vascular system attains its final arrangement on d 18, just before hatching (Ausprunk et al. 1974). Extensive angiogenesis with the formation of new vessels from preexisting ones takes place during the development of this vascular system, which is partly mediated by basic fibroblast growth factor (FGF2) (Ribatti et al. 1995, 1997*a*, 1998).

Angiogenesis is influenced by both growth factors and adhesion proteins associated with the extracellular matrix (ECM) and is characterised by vascular cell migration, proliferation and differentiation. An essential step in angiogenetic processes is the proteolysis of ECM components (Aznavoorian et al. 1993). The matrix metalloproteinases (MMPs) are a large family of enzymes with specific and selective activities in many components of the ECM (Matrisian, 1992). They have been classified into subgroups based on substrate preference. Gelatinase A (MMP-2, or 72 kDa gelatinase) is able to degrade several ECM proteins including denaturated collagens (gelatin), type IV collagen and fibronectin.

In this report we have examined the expression of MMP-2 in the developing ChAM and we have correlated its expression with a quantitative evaluation of the expansion of the vascular system and of the expression of 3 ECM components (fibronectin, laminin and type IV collagen), which differentially modulate the angiogenetic processes.

Correspondence to Dr Domenico Ribatti, Institute of Human Anatomy, Histology and Embryology, Piazza G. Cesare 11, Policlinico, I-70124 Bari, Italy. Fax: +39.80.5478309; e-mail: ribatti@anatomia.uniba.it

MATERIALS AND METHODS

Animals

Fertilised White Leghorn chicken eggs were incubated under conditions of constant humidity at a temperature of 37 °C. On d 3 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 ChAM from the shell. The window was sealed with a glass of the same dimensions and the eggs were returned to the incubator. At d 6, 8, 10, 12, 14, 16 and 18 of incubation 10 ChAMs from each stage and from different eggs were processed for morphometric measurements, immunohistochemistry and metalloproteinase activity.

Morphometric measurements of the expansion of the ChAM vasculature

Morphometric measurements of the ChAM vasculature were made using a Zeiss stereomicroscope SR equipped with the MC 63 Camera System (Zeiss, Oberkochen, Germany) and a computerised image analysis system (AutoCad 12, Autodesk, Sausalito, CA, USA) according to a previously described method (Ribatti, 1995). Briefly, on transparent sheets covering the photographic reconstructions of the ChAMs from different specimens for each stage studied at $\times 30$ final magnification, the outlines of the vasculature were drawn and separately entered into an image processing system manually via a digitiser. Finally, the computer reconstructions were processed by a filling algorithm in order to recreate the areas.

Antibodies and immunohistochemistry

The murine monoclonal antibodies (MAbs) used in this study were: (1) MAb L8271 against laminin (Sigma, St Louis, MO); (2) MAb F 7387 against fibronectin (Sigma); (3) Mab 2D3/49 against type IV collagen (Biogenesis, Bournemouth, UK). A 3-layer biotin-avidin-peroxidase system was used as described previously (Ribatti et al. 1998). Briefly, 8 μm acetone-fixed cyostat sections were depleted of their endogenous peroxidase by 7.5% H_2O_2, and sequentially incubated with: (1) primary antibodies; (2) biotin-labelled swine antirabbit IgG (Vector, Burlingame, CA); (3) streptavidin-peroxidase conjugate (Vector). The reaction was performed in 0.05 M acetate buffer pH 5.1 with 0.02% 3-amino-9-ethylcarbazole grade II (Sigma Chemical., Co.), and 0.05% H_2O_2. Sections were then washed in the same buffer, counterstained in Gill's haematoxylin (grade II, Polyscience, Warrington, PA) and mounted in buffered glycerin. In negative controls, primary antibodies were replaced by an unrelated monoclonal IgG1 produced by the P3X63/Ag8 mouse secretory myeloma (Vacca et al. 1995). Antibodies were used as purified ascites fluid protein, 10 μg/ml in PBS and supplemented with 1% bovine serum albumin.

Quantitation of the area occupied by the extracellular matrix components immunoreactive to fibronectin, laminin and type IV collagen

A morphometric method of 'point counting' with slight modifications for measuring areas of tissue components was used (Elias & Hyde, 1983; Ribatti et al. 1997*b*). Briefly, $4–6 \times 250$ fields (area 12.5×10^{-2} mm² per field) per section, covering almost the whole section of each of 2 sections per sample, were examined with a Leitz Dialux photomicroscope (Leitz, Wetzlar, Germany) and photographed. Fibronectin, laminin and type IV collagen staining outlines, drawn on transparent sheets placed on each picture, were separately entered into a computer image analysis system (AutoCad 12) via a digitiser. Computer reconstructions were processed by a filling algorithm to calculate the area covered by each staining, which was also rated as a percentage of the field's reference area. Means $\pm$ 1 standard deviation (S.D.) were calculated per section, per sample, and per group of samples.

Metalloproteinase activity in the developing ChAM

Extracts of ChAM were examined by gelatin zymography in order to identify MMP-2 activity at d 6, 8, 10, 12, 14, 16 and 18 of incubation. Extracts were solubilised at 4 °C in 0.1 M phosphate, 0.2% Triton X-100, pH 7.3, and subsequently analysed by gelatin zymography, performed to visualise the MMP-2 activity present in the samples according to Albini et al. (1987). 5 μg of protein were applied to 7.5% SDS-PAGE gels copolymerised with type A gelatin from porcine skin (Sigma) at a final concentration of 0.6 mg/ml. After electrophoresis, gels were washed in 2.5% Triton X100 for 1 h to remove SDS, incubated for 18 h at 37 °C in collagenase buffer and stained in 0.1% Coomassie brilliant blue. The gelatinolytic activity was visualised as a transparent band against a blue background and quantified by computerised image analysis of the band.

RESULTS

Morphometric measurements of the ChAM vasculature

On d 6 of incubation, the vascularised area of the ChAM was 5.5 ± 2.2 cm^2; on d 8, 26.2 ± 3.8 cm^2; on d 10, 46.7 ± 4.4 cm^2; on d 12, 55.4 ± 7.2 cm^2; on d 14, 62.6 ± 6.1 cm^2; on d 16, 66.2 ± 8.1 cm^2; and on d 18, 68.2 ± 7.4 cm^2.

Immunohistochemistry and areas of staining of fibronectin, laminin and type IV collagen

The Table shows each separate area as an absolute value. The fibronectin area underwent increment between d 6 and d 8 of incubation and immunoreactivity was intense at the base of the chorion, in the extracellular matrix and along the surface of the primitive blood vessels (Fig. 1*A*); thereafter, it declined and was not detectable beginning from d 16. The laminin area underwent increment between d 6 and d 10 of incubation, when staining for laminin was more intense in the capillaries located beneath the chorion (Fig. 1*B*); thereafter, it did not alter. Type IV collagen was detectable starting from d 10, when staining was present in the chorion and along the abluminal surface of endothelial cells (Fig. 1*C*); thereafter, increased until d 16.

Table. *Area occupied by stromal components in ChAM during chick embryo development*

	Area occupied by		
D	Fibronectin	Laminin	Type IV collagen
6	1.3 ± 0.1*	0.5 ± 0.2	—
8	2.1 ± 0.6	1 ± 0.3	—
10	0.9 ± 0.3	1.4 ± 0.2	$0.8 + 0.3$
12	0.3 ± 0.2	1.5 ± 0.3	$1.8 + 0.5$
14	0.2 ± 0.1	1.6 ± 0.4	$2.6 + 0.7$
16	—	1.7 ± 0.3	$3.4 + 0.6$
18	—	1.7 ± 0.5	$3.6 + 0.5$

* Data are expressed as mean ± 1 S.D. The area is given as mm$^2 \times 10^{-2}$.

Metalloproteinase activity in the developing ChAM

Extracts of ChAM were examined by gelatin zymography in order to identify metalloproteinases present in the ChAM from embryos between d 6 and d 18 of incubation. A metalloproteinase of 72 kDa (MMP-2) was detected and the levels of this enzyme decreased progressively from d 6 to d 12 of incubation, and then did not appear to change significantly (Fig. 2).

DISCUSSION

This report shows that in the developing ChAM vasculature fibronectin expression, measured as fibronectin area, is highly correlated with angiogenesis, measured as vascularised area, and with metalloproteinase-2 activity, measured by gelatin zymography.

Fibronectin area underwent a significant increment between d 6 and d 8 of incubation; thereafter, it progressively declined and was not detectable as from d 16. A close relationship between fibronectin overexpression and angiogenesis has been demonstrated in vivo (Mayer et al. 1981; Sariola et al. 1984; Risau &

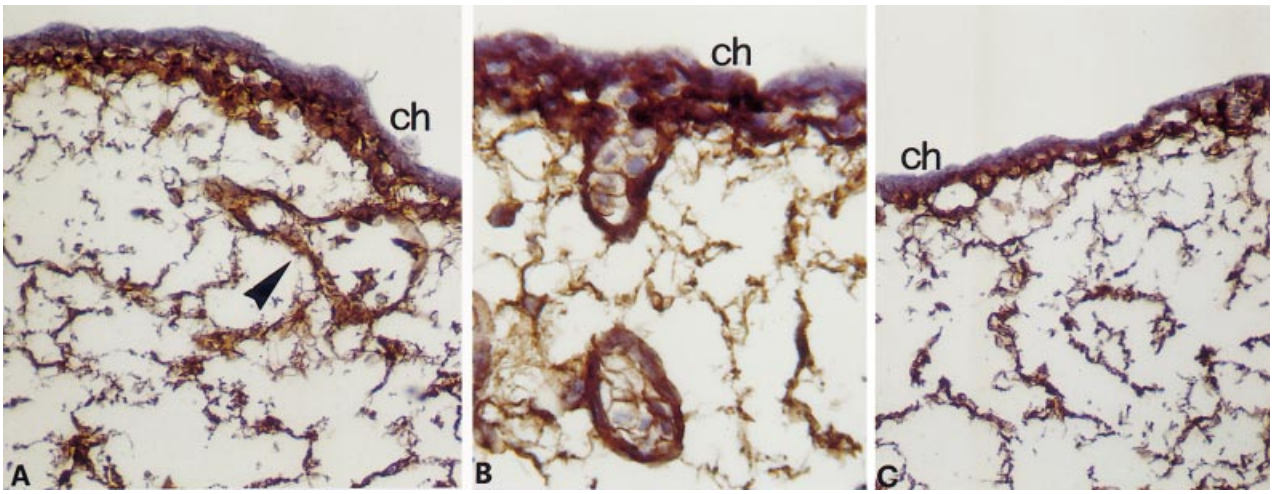

Fig. 1. ECM immunohistochemistry. (*A*) Fibronectin immunoreactivity at d 6 of incubation is intense in the chorion (ch), in the extracellular matrix and along the surface of the primitive blood vessels (arrowhead). (*B*) Laminin and (*C*) type IV collagen immunoreactivity at d 10 of incubation are detectable in the chorion (ch) and in the capillaries located beneath the chorion. *A*,*C*, $\times 250$; *B*, $\times 400$.

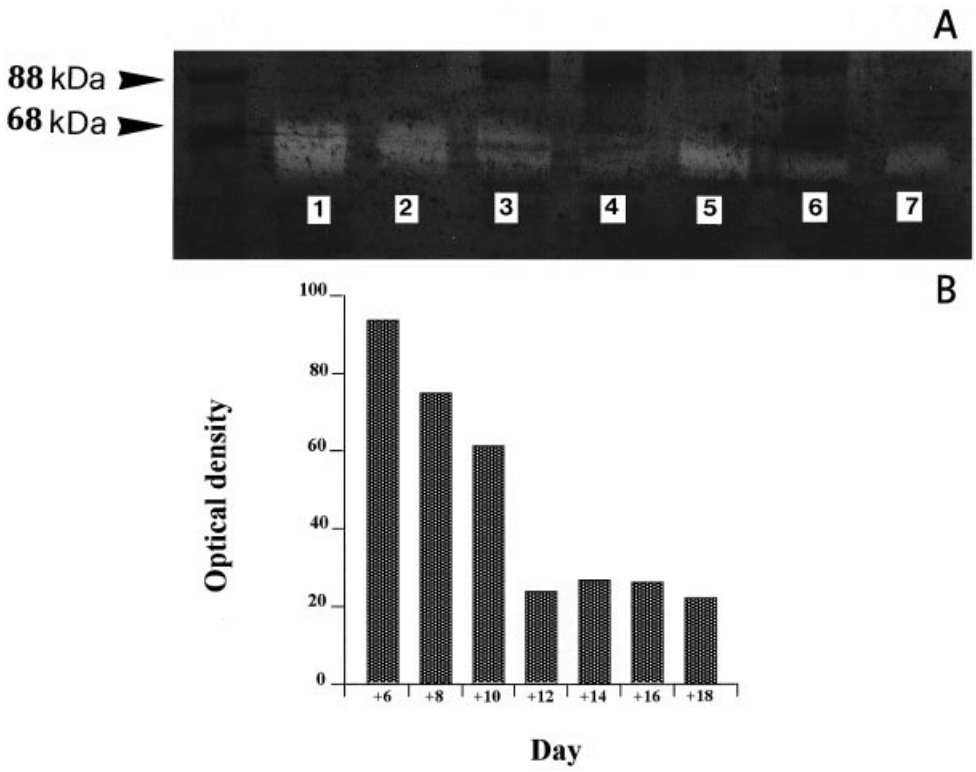

Fig. 2. Metalloproteinase activity as detected by gelatin zymography at d 6, 8, 10, 12, 14, 16 and 18 of incubation. Equal amounts of protein extract from each of the indicated d were analysed by SDS-PAGE zymography as described in Materials and Methods. (*A*) Note the white bands against a dark background with an apparent Mr equal to 62000 corresponding to activated MMP-2 (lane 1 = 6 d; lane 2 = 8 d; lane 3 = 10 d; lane 4 = 12 d; lane 5 = 14 d; lane 6 = 16 d; lane 7 = 18 d. (*B*) Quantitation of MMP-2 activity by computerised image analysis of the band. Values are the mean ±S.D. for n = 10 experiments.

Lemmon, 1988). Thus in the developing wing bud, capillaries grow within a fibronectin-rich matrix; during brain development capillary sprouts invading the neuroectoderm, migrate in a fibronectin-rich matrix (Risau & Lemmon, 1988) and capillaries which invade the developing chick lung express fibronectin (Chen et al. 1986). Moreover, in ChAM fibronectin overexpression in extracellular matrix parallels the vasoproliferative processes induced by an angiogenic stimulus (Ribatti et al. 1997*b*).

Laminin expression, in contrast, gradually increased from d 5 to d 18. Laminin immunoreactivity is present during all stages of vessel formation in ChAM development, consistent with its role both in the early formation and in later differentiation of the subendothelial basement membrane (Risau & Lemmon, 1988; Ribatti et al. 1996).

Type IV collagen immunoreactivity was not evident in the ChAM until d 10 of development. Type IV collagen appears in the later stages of ChAM vascular development concomitantly with the terminal differentiation of endothelial cells and maturation of basement membrane. The appearance of type IV collagen results in a progressive decrease of the proliferative rate of the microvascular endothelial cells and correlates with the formation of a lumen, a gradual reduction in endothelial migration, the establishment of cell polarity and the acquisition of a differentiated endothelial phenotype (Form et al. 1986; Nicosia & Madri, 1987).

The maximal increment in the expansion of the ChAM surface area takes place between d 6 and d 8 of incubation, when the area increases 4.76 fold. Our quantitative data are in accord with those of other authors (Burton & Palmer, 1989; De Fouw et al. 1989). This expansion is sustained by the formation of new vessels by an angiogenetic process. Schlatter et al. (1997), by means of morphometric analysis, have shown that in this early phase of ChAM angiogenesis, from d 5 to d 7, the principal mechanism of capillary growth is sprouting. On d 7, the extension of the capillary network over the surface of the egg has nearly reached its maximum (Burton & Palmer, 1992) while, between d 9 and d 13 of incubation, there is a vascular histodifferentiation and, between d 14 and d 18, maturation of the ChAM microvasculature was completed (Shumko et al. 1988).

An essential first step of angiogenesis is the secretion

of matrix metalloproteinases (MMP) (Mignatti & Rifkin, 1996), allowing the endothelial cells to migrate through the connective tissue stroma. Microvascular endothelial cells produce MMPs in vivo and in vitro and their production is increased in reponse to angiogenic agents (Pepper et al. 1996). Here we have shown that the proteolytic capacity of ChAM tissues, in term of MMP-2 secretion, decreased progressively from d 6 to d 12, and then did not appear to change significantly over time. This proteolytic activity parallels an increase of the levels of fibronectin present in ChAM extracellular matrix, which undergoes increment between d 6 and d 8 of incubation and thereafter declines. MMP-2 degrades types IV, V, VII and X collagens as well as fibronectin (Mignatti & Rifkin, 1996).

Other proteolytic systems are involved in the ChAM angiogenetic process, such as the plasminogen activator-plasmin complex (Pepper et al. 1996). Iruela-Arispe et al. (1995) have determined the levels of active plasmin during ChAM development and have observed a peak of this enzyme between d 7 and d 15 of development.

Although a requirement for MMP activity has been demonstrated in several in vivo assays for studying angiogenesis, there is as yet no definitive evidence that the plasminogen activator-plasmin complex is required for angiogenesis in the in vivo settings (Pepper et al. 1996). Karelina et al. (1995) have shown in fetal skin that MMP-2 and MMP-9 are the principal degrading enzymes found in both early microvessels developing from undifferentiated mesoderm and in microvessels involved in elongation and sprout formation from preexisting vessels.

The importance of MMP-2 in angiogenetic processes in ChAM vasculature has been further shown by Brooks et al. (1996), who have demonstrated a colocalisation of MMP-2 and integrin $a_v\beta_3$ on the surface of ChAM newforming vessels.

In summary, our data show the relationship occurring between MMP-2 activity and fibronectin expression during the early phases of angiogenetic processes taking place in ChAM, when the expansion of its vasculature is maximal. Moreover, the evaluation of these 2 parameters might be useful in studying the response of ChAM vasculature to an exogenous angiogenic stimuli.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST, 60%), Rome, Italy to DR, and by a grant from Associazione Italiana per la Ricerca sul Cancro (AIRC), Milan, Italy to DR and AV.

REFERENCES

ALBINI A, IWAMOTO Y, KLEINMAN HR, MARTIN GR, AARONSON SA, KOZLOWSKI JM et al. (1987) A rapid *in vivo* assay for quantitating the invasive potential of tumor cells. *Cancer Research* **47**, 3239–3245.

AUSPRUNK D, KNIGHTON D, FOLKMAN J (1974) Differentiation of vascular endothelium in the chick chorioallantois: a structural and autoradiographic study. *Developmental Biology* **38**, 237–249.

AZNAVOORIAN S, MURHY AN, STETLER-STEVENSON WG, LIOTTA L (1993) Molecular aspects of tumor cell invasion and metastasis. *Cancer* **71**, 1368–1383.

BROOKS PC, SROMBLAD S, SANDERS LC, von SCHALSCHA TL, AIMES RT, STETLER-STEVENSON WG et al. (1996) Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interactions with integrin $a_v\beta_3$. *Cell* **85**, 683–693.

BURTON GJ, PALMER ME (1989) The chorioallantoic capillary plexus of the chicken egg: a microvascular corrosion casting study. *Scanning Microscopy* **3**, 549–558.

BURTON GJ, PALMER ME (1992) Development of the chick chorioallantoic capillary plexus under normoxic and normobaric hypoxic and hyperoxic conditions: a morphometric study. *Journal of Experimental Zoology* **262**, 291–298.

CHEN WT, CHEN JM, MUELLER SC (1986) Coupled expression and colocalization of 140 k cell adhesion molecules, fibronectin, and laminin during morphogenesis and cytodifferentiation of chick lung cells. *Journal of Cell Biology* **103**, 1073–1090.

DE FOUW DO, RIZZO VJ, STEINFELD R, FEINBERG RN (1989) Mapping of the microcirculation in the chick chorioallantoic membrane during normal angiogenesis. *Microvascular Research* **38**, 136–147.

ELIAS H, HYDE DM, eds (1983) *A Guide to Practical Stereology*, pp. 25–44. Basel: Karger.

FORM DM, PRATT BM, MADRI JA (1986) Endothelial cell proliferation during angiogenesis. *In vitro* modulation by basement membrane components. *Laboratory Investigation* **55**, 521–530.

IRUELA-ARISPE ML, LANE TE, REDMOND D, REILLY M, BOLENDER RP, KAVANAGH TJ et al. (1995) Expression of SPARC during development of the chicken chorioallantoic membrane: evidence for regulated proteolysis *in vivo*. *Molecular Biology of the Cell* **6**, 327–343.

KARELINA TV, GOLDBERG GI, EISEN AZ (1995) Matrix metalloproteinases in blood vessels development in human fetal skin and in cutaneous tumors. *Journal of Investigative Dermatology* **105**, 411–417.

MATRISIAN LM (1992) The matrix-degrading metalloproteinases. *BioEssay* **14**, 455–463.

MAYER BW, HAY ED, HYNES RO (1981) Immunohistochemical localization of fibronectin in embryonic chick trunk and *area vasculosa*. *Developmental Biology* **82**, 267–286.

MIGNATTI P, RIFKIN DB (1993) Biology and biochemistry of proteinases in tumor invasion. *Physiological Reviews* **73**, 161–195.

NICOSIA RF, MADRI JA (1987) The microvascular extracellular matrix. Developmental changes during angiogenesis in the aortic ring plasma clot model. *American Journal of Pathology* **128**, 78–90.

PEPPER MS, MONTESANO R, MANDRIOTA SJ, ORCI L, VASSALLI JD (1996) Angiogenesis: a paradigm for balanced extracellular proteolysis during cell migration and morphogenesis. *Enzyme Protein* **49**, 138–162.

RIBATTI D (1995) A morphometric study of the expansion of the chick area vasculosa in shell-less culture. *Journal of Anatomy* **186**, 639–644.

RIBATTI D, URBINATI C, NICO B, RUSNATI M, RONCALI L, PRESTA M (1995) Endogenous basic fibroblast growth factor is implicated in the vascularization of the chick embryo chorioallantoic membrane. *Developmental Biology* **170**, 39–49.

RIBATTI D, VACCA A, NICO B, FANELLI M, RONCALI L, DAMMACCO F (1996) Angiogenesis spectrum in the stroma of B-cell non-Hodgkin's lymphomas. An immunohistochemical and ultrastructural study. *European Journal of Haematology* **56**, 43–53.

RIBATTI D, NICO B, BERTOSSI M, RONCALI L, PRESTA M (1997*a*) Basic fibroblast growth factor-induced angiogenesis in the chick embryo chorioallantoic membrane: an electron microscopic study. *Microvascular Research* **53**, 187–190.

RIBATTI D, VACCA A, COSTANTINO F, MINISCHETTI M, LOCCI P, BECCHETTI E et al. (1997*a*) Exogenous heparin induces fibronectin overexpression parallel to angiogenesis in the extracellular matrix of the chick embryo chorioallantoic membrane. *Tissue & Cell* **29**, 131–136.

RIBATTI D, BERTOSSI M, NICO B, VACCA A, RIA R, RIVA A et al. (1998) Role of basic fibroblast growth factor in the formation of the capillary plexus in the chick embryo chorioallantoic membrane. An in situ hybridization, immunohistochemical and ultrastructural study. *Journal of Submicroscopic Cytology and Pathology* **30**, 127–136.

RISAU W, LEMMON V (1988) Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis. *Developmental Biology* **125**, 441–450.

ROMANOFF AL (1960) The extraembryonic membranes. In *The Avian Embryo*: *Structural and Functional Development*, pp. 1039–1141. New York: Macmillan.

SARIOLA HM, KUUSELA P, EKBLOM P (1984) Cellular origin of fibronectin in interspecies hybrid kidneys. *Journal of Cell Biology* **99**, 2099–2107.

SCHLATTER P, KONIG MF, KARLSSON LM, BURRI PH (1997) Quantitative study of intussusceptive capillary growth in the chorioallantoic membrane (CAM) of the chicken embryo. *Microvascular Research* **54**, 65–73.

SHUMKO JZ, DEFOUW DO, FEINBERG RN (1988) Vascular histodifferentiation in the chick chorioallantoic membrane: a morphometric study. *Anatomical Record* **220**, 179–189.

VACCA A, DI LORETO M, RIBATTI D, DI STEFANO R, GADALETA-CALDAROLA G, IODICE G et al. (1995) Bone marrow of patients with active multple myeloma: angiogenesis and plasma cell adhesion molecules LFA-1, VLA-4, LAM-1 and CD-44. *American Journal of Hematology* **50**, 9–14.