

Constitutive secretion of MMP9 by early-passage cultured human endothelial cells

Jacky Arkell and Christopher J. Jackson*

Sutton Arthritis Research Laboratories, Royal North Shore Hospital, St. Leonards, NSW 2065, Australia

Matrix metalloproteinase-9 (MMP9) plays an important role during angiogenesis. It is an inducible enzyme which is known to be secreted from human endothelial cells in response to phorbol myristate acetate (PMA), but thought not to be constitutively expressed. We examined the secretion of MMP9 by primary culture (P0), passage 1 (P1) and passage 2 (P2) human umbilical vein endothelial cells (HUVE). Whereas there was no detectable MMP9 in P2 cells under basal conditions, P0 HUVE secreted MMP9, as detected by zymography and ELISA. RT-PCR and cycloheximide inhibition studies confirmed that MMP was synthesized by P0 HUVE. MMP9 secretion was passage-dependent, decreasing rapidly as the cells were passaged in culture and was not detected at P2. The decrease was largely due to the population doubling of cells as they are cultured. This is the first report to show that cultured HUVE constitutively express MMP9 and that this secretion is restricted to very early-passage cells. These findings may be relevant to the angiogenic potential of human endothelial cells as they age. Copyright © 2003 John Wiley & Sons, Ltd.

KEY WORDS — human endothelial cells; MMP9; matrix metalloproteinases; cell culture

INTRODUCTION

An essential step during angiogenesis is the secretion of matrix metalloproteinases (MMPs) by endothelial cells.¹ These enzymes are capable of degrading all the components of the extracellular matrix, thus allowing the cells to migrate and form new blood vessels. An important subclass of the MMPs is the gelatinases, MMP2 and MMP9. Both enzymes are expressed by endothelial cells and play an important role in endothelial invasion during angiogenesis as reflected by recent *in vivo* studies. MMP9 has been implicated in the 'switch' to the angiogenic phenotype during carcinogenesis.²

Whereas most cell types constitutively express MMP2, MMP9 is an inducible enzyme, being secreted from many cell types in response to cytokines/growth factors, such as tumour necrosis factor. There is limited information on the expression of MMP9 in endothelial cells. The tumour-promoting chemical,

phorbol myristate acetate (PMA) increases MMP9 synthesis in human microvascular and, to a lesser extent, macrovascular endothelial cells.^{3,4} Thrombospondin upregulates MMP9 in bovine aortic endothelial cells.⁵ There have been few reports on the effects of cytokines or angiogenic/growth factors on MMP9 secretion by human endothelial cells. Hanemaaijer *et al.*³ have shown that TNF- α can enhance the effect of PMA, but it does not stimulate endothelial MMP9 synthesis when used alone.

In addition to being non-responsive to cytokines/growth factors, previous reports have shown that HUVE do not constitutively secrete MMP9.^{3,4,6–8} However, in the current report, we show that primary culture (P0) human endothelial cells constitutively secrete MMP9. The secretion is passage-dependent with almost complete abrogation of enzyme secretion after the second passage.

MATERIALS AND METHODS

Cell culture

Human endothelial cells were obtained from human umbilical veins (HUVE), isolated as described by Jaffe *et al.*⁹ HUVE were grown and maintained in Biorich

*Correspondence to: Dr C. J. Jackson, Sutton Arthritis Research Laboratory, Royal North Shore Hospital, St. Leonards, NSW 2065, Australia. Tel: 612-99266043. Fax: 612-99266269. E-mail: cjackson@med.usyd.edu.au

medium (ICN Biomedicals, Costa Mesa, CA) containing 20% fetal calf serum plus $50 \mu\text{g ml}^{-1}$ endothelial cell growth supplement (Sigma, St Louis, MO) and $50 \mu\text{g ml}^{-1}$ heparin (Sigma, St Louis, MO). Cells had the characteristic cobblestone morphology and demonstrated positive staining for von Willebrand factor by immunohistochemistry (Dako Corporation, Carpinteria, CA).

Experimental protocols

Endothelial cells were seeded at confluence, at a density of 40 000 cells per well into 96-well plates (Nunc, Kamstrup, Denmark) in growth medium. After 5 days, the monolayers of endothelial cells were washed twice in Hank's balanced salt solution (HBSS) and pre-incubated in basal medium (Biorich plus 2% normal pooled serum—pre-stripped of MMP2 and 9 using a gelatin sepharose (Pharmacia, Uppsala Sweden) column) for the specified time period. Cells were then incubated in fresh basal medium and the supernatants collected after 24 h and stored at -20°C until use. To ensure that the results were standardized between wells, cell numbers were measured using Celltiter 96 Aqueous One solution reagent (Promega Corp, Madison, WI). Samples were equally loaded according to cell numbers.

To test the effect of trypsin/EDTA (Figure 8), endothelial cells were trypsinized and seeded at confluence (40 000 cells per well) into triplicate wells in a 96-well plate. The remaining cells were transferred to a new flask. After 3 h, when the cells had adhered to the flask they were trypsinized and seeded into triplicate wells of the 96-well plate. Remaining cells were again replated for a further 3 h and the procedure repeated. The cells were maintained for 5 days in growth medium before incubation in basal medium and supernatants collected as above.

To examine the effect of cell doubling (Figure 9), endothelial cells were plated at 5000, 10 000, 20 000 and 40 000 cells per well into a 96-well plate. After 5 days, all cells had reached confluence (40 000 cells) and the cells were incubated in basal medium and supernatants collected as above.

Zymography

Conditioned medium and a proMMP9 standard (Oncogene Research Products—CN Biosciences, Darmstadt Germany) were applied to 10% acrylamide gels incorporated with gelatin (1 mg ml^{-1}). Samples were mixed with an equal volume of $2 \times$ sample buffer (5% SDS,

10% glycerol, 0.13 M Tris-HCl, pH 6.8 and 0.1% bromophenol blue). Following electrophoresis, the gels were renatured in 0.25% Triton X-100 for 1 h at room temperature. The gels were then incubated at 37°C overnight in developing buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl_2). The gels were stained for 10 min with 0.2% Coomassie blue R-250 in 50% ethanol and 10% acetic acid. MMP2 and 9 activity appears as clear bands against the blue background of the stained gel.

ELISA

Conditioned medium was assayed for MMP9 by enzyme-linked immunosorbent assay (ELISA) kit (Oncogene Research Products—CN Biosciences, Darmstadt Germany) according to the manufacturer's instructions. The limit of detection was 625 pg ml^{-1} .

Immunohistochemistry

Cells were seeded at confluence onto 35-mm tissue culture dishes and fixed with cold (-20°C) ethanol. Peroxidase staining was carried out using the ABC kit from Vector Laboratories (Burlingame, CA) as per the manufacturer's instructions. Mouse monoclonal antibody against MMP9 was obtained from Oncogene Research Products (CN Biosciences, Darmstadt Germany).

RNA extraction and RT-PCR

RNA was extracted from HUVE using Tri-reagent (Molecular Research Center Inc, Cincinnati, OH) as per the manufacturer's instructions. The RNA pellet was finally washed in 70% cold ethanol, air-dried and redissolved in $10 \mu\text{l}$ of diethyl-pyrocabonate (DEPC)-treated water. The RNA concentration was determined spectrophotometrically. RNA ($2 \mu\text{g}$) was used as a template for RT-PCR using the Access RT-PCR System kit (Promega Corp, Madison, WI). The primers for MMP9 were as follows: sense oligonucleotide (5'-CACTGTCCACCCCTCAGAGC-3') and antisense oligonucleotide (5'-GCCACTTGTCGGC-GATAAGG-3'). The single strand cDNA generated was amplified by PCR with 40 cycles of a 45-s denaturation step at 94°C , a 1-min annealing step at 59°C and a 3-min extension step at 70°C . The amplified PCR products were electrophoresed on 0.7% agarose gel containing ethidium bromide. DNA step ladder (1 kb, Promega Corp, Madison, WI) was used as markers.

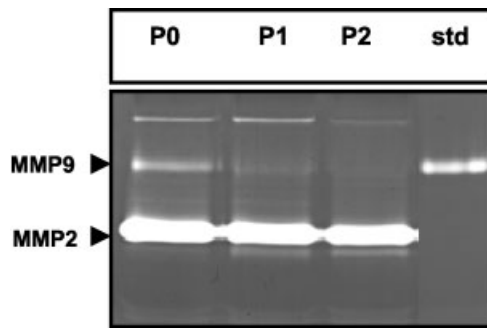


Figure 1. Passage-dependent secretion of MMP9. HUVE from different passages (P0, P1 and P2) were pre-incubated in basal media for 6 h followed by incubation in fresh basal media for 20 h. Conditioned medium was analysed by gelatin zymography as described in the Methods section. Lane 4 (std) shows proMMP9 standard. There was a rapid passage-dependent decrease in MMP9 secretion. Similar results were obtained in five separate HUVE experiments

RESULTS

To compare MMP2 and 9 secretion of P0, P1 or P2 HUVE, cells were incubated for 20 h in basal medium and the conditioned media collected and assessed for gelatinolytic activity using zymography. The results are shown in Figure 1. No MMP9 was detected in the conditioned medium of P2 cells. In contrast, the conditioned medium of P0 HUVE contained substantial quantities of MMP9 under basal conditions. There was a rapid passage-dependent decrease in MMP9 secretion, with less enzyme secreted by P1 cells. Similar results were obtained with HUVE from all five separate isolations tested. In contrast to MMP9, equal amounts of pro-MMP2 were secreted by P0, P1 and P2 cells (Figure 1). MMP9 was detected in the conditioned medium of P0 HUVE as early as 4 h, the levels of which progressively increased up to 24 h (Figure 2). MMP9 secreted by the cells was in the latent form only (pro-MMP9) and was quantitated using ELISA (Figure 3). The concentration of MMP9 in the conditioned media of three different P0 HUVE cell lines ranged from 0.8 to 1.6 ng ml⁻¹. No MMP9 was detected in the conditioned medium of P2 cells treated identically, using ELISA (data not shown).

Immunohistochemistry was used to detect the expression of MMP9 in P0 HUVE. The cells were incubated for 20 h in basal medium prior to processing. There was strong cytoplasmic staining for MMP9 in P0 cells compared to IgG control (Figure 4A and B). Since it was possible that these cells had accumulated MMP9 from another source, we next examined whether the enzyme was being synthe-

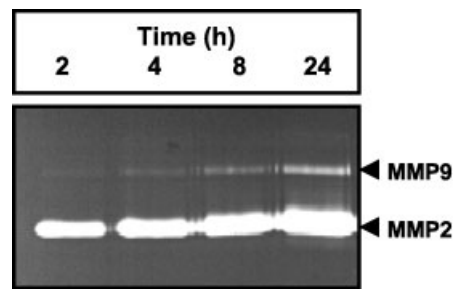


Figure 2. Time response of MMP9 secretion by passage 0 HUVE. P0 HUVE were pre-incubated in basal media for 6 h followed by incubation in fresh basal media. Conditioned medium was collected at the indicated timepoints (2,4,8 and 24 h) and analysed for MMP9 activity by gelatin zymography. MMP9 was detected as early as 4 h and progressively increased over 24 h

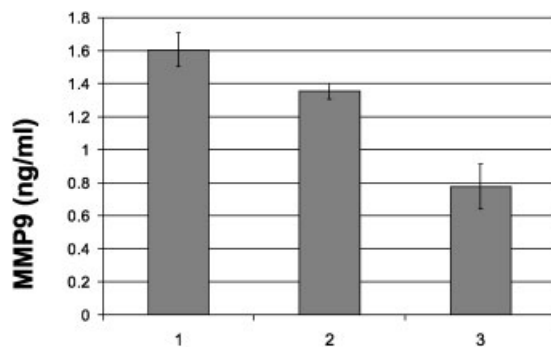


Figure 3. MMP9 secretion by P0 HUVEs determined by ELISA. P0 HUVE from three separate isolations (1,2 and 3) were pre-incubated in basal media for 6 h followed by incubation in fresh basal media for 20 h. MMP9 was measured using ELISA, and expressed as ng ml⁻¹ (mean \pm SD)

sized and/or secreted by P0 HUVE. First, P0 HUVE were incubated with the protein trafficking inhibitor Brefeldin A (1–10 μ g ml⁻¹). The inhibitor completely prevented the secretion of MMP9 (Figure 5), indicating that an intracellular transport mechanism is required for the secretion of MMP9. Second, HUVE were incubated in the presence or absence of the protein synthesis inhibitor, cycloheximide (0.1–10 μ M), for 24 h. The addition of cycloheximide inhibited MMP9 secretion in a dose-responsive manner (Figure 6). Finally, we used RT-PCR to detect whether P0 HUVE expressed mRNA for MMP9. The cells were incubated for 20 h in basal medium and RNA was isolated as described in the Methods section. RT-PCR products were obtained as a single band of 243 bp, the expected size of the MMP9 mRNA fragment (Figure 7). Together, these results indicate that P0 HUVE synthesize and secrete MMP9.

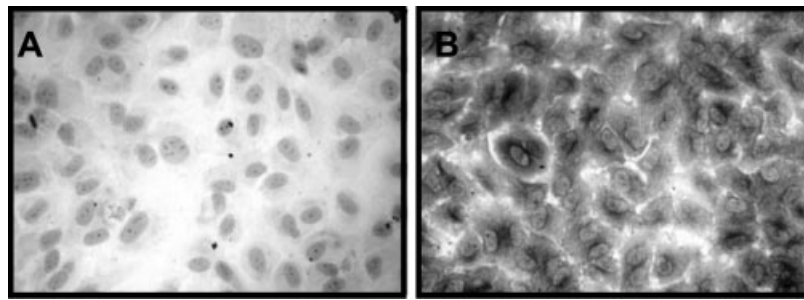


Figure 4. Immunolocalization of MMP9 in early passage HUVE. P0 HUVE were pre-incubated in basal medium for 6 h followed by 24 h incubation in fresh basal medium. Cells were challenged with an irrelevant IgG (A) or antibody to MMP9 (B) and processed for immunohistochemistry, as described in the Methods section. Strong cytoplasmic staining was detected for MMP9. Magnification $\times 100$

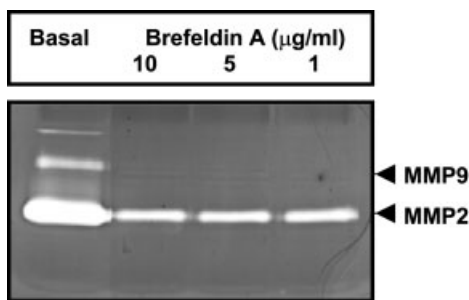


Figure 5. Brefeldin A inhibits the basal secretion of MMP9. P0 HUVE were pre-incubated in basal medium for 6 h followed by incubation in fresh basal media containing Brefeldin A (1 – $10 \mu\text{g ml}^{-1}$) for 20 h. Conditioned medium was collected and analysed for MMP9 activity by gelatin zymography

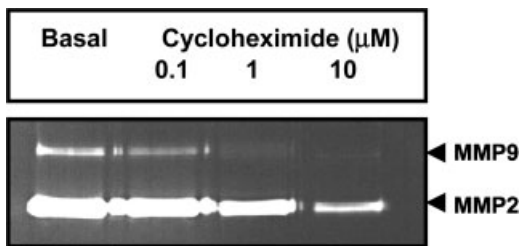


Figure 6. Cycloheximide inhibits the basal secretion of MMP9. P0 HUVE were pre-incubated in basal medium for 6 h followed by incubation in fresh basal medium containing cycloheximide (0.1 – $10 \mu\text{M}$) for 24 h. Conditioned medium was collected and analysed for MMP9 activity by gelatin zymography

We examined the reason for the rapid decrease of MMP9 secretion as HUVE are passaged in culture. Two marked changes that occur when cells are passaged in culture are treatment with trypsin–EDTA and cell doubling. To investigate the effect of trypsin/EDTA treatment on MMP9 secretion, P0 HUVE were sequentially treated with trypsin/EDTA in rapid

succession, as described in the Methods section. This protocol allowed the cells to undergo trypsin/EDTA treatment equivalent to 1, 2 and 3 passages without undergoing replication. The initial trypsin/EDTA treatment partially inhibited MMP9 secretion, however, further trypsin/EDTA treatment did not significantly ($p=0.37$, for three different HUVE lines) reduce MMP9 secretion (Figure 8).

To investigate whether the passage-dependent decrease in MMP9 secretion was due to cell doubling, HUVE were plated at varying densities in a 96-well plate, as described in the Methods section. This protocol allowed the comparison of cells that had undergone the equivalent of 3, 2, 1 and 0 population doublings. There was a dose-dependent decrease in MMP9 secretion as the number of population doublings increased

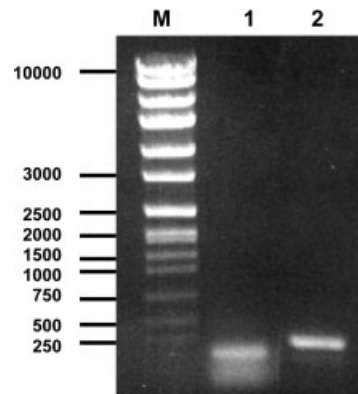


Figure 7. RT-PCR analysis of MMP9 mRNA expression in P0 HUVE. P0 HUVE were incubated in basal medium for 6 h followed by incubation in fresh basal medium. Total RNA was extracted from the cells and subjected to RT-PCR as per conditions in the Methods section: P0 HUVE express MMP9 transcript (lane 1). Lane M represents the DNA ladder (1 kb) and lane 2 shows the kit internal control

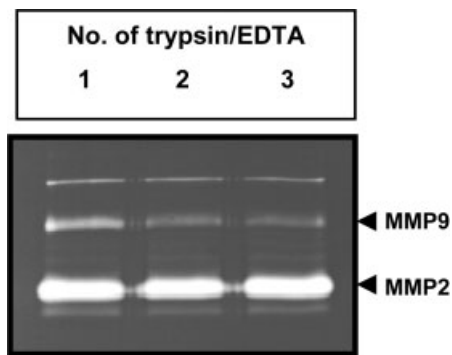


Figure 8. Effect of trypsin/EDTA treatment on the basal secretion of MMP9. Confluent P0 HUVE were trypsinized and seeded at confluence (40 000 cells per well) into triplicate wells in a 96-well plate. The remaining cells were transferred to a new flask and after 3 h, when the cells had adhered, were trypsinized and seeded into triplicate wells of the 96-well plate. This procedure was repeated and the cells were maintained for 5 days in growth medium before incubation in basal medium for 24 h. Conditioned medium was analysed by gelatin zymography. Initial trypsin/EDTA treatment (1) partially inhibited MMP9 secretion. Further trypsin/EDTA treatments (2, 3) did not significantly ($p=0.37$, for three different HUVE lines) reduce MMP9 secretion

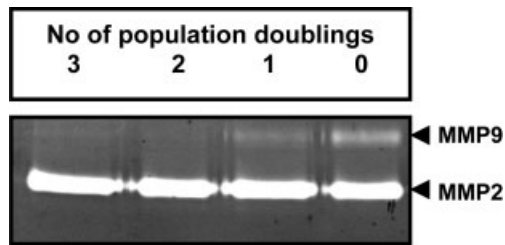


Figure 9. Effect of cell doubling on the basal secretion of MMP9. P0 HUVE were seeded at varying densities to represent passages 0, 1, 2 or 3, as described in the Methods section. Cells were incubated for 24 h in basal medium and conditioned medium was analysed by gelatin zymography. There was a dose-dependent decrease in MMP9 secretion as the number of population doublings increased. No MMP9 was detected in the conditioned medium of cells that had undergone three population doublings

(Figure 9), with no detectable MMP9 in the conditioned medium of cells that had undergone three population doublings. These results indicated that cell doubling is largely responsible for the passage-dependent decrease in MMP9 secretion by HUVE.

DISCUSSION

MMP9 plays an important role during physiological and pathological angiogenesis.^{2,10} *In vivo*, MMP9 is

expressed by endothelial cells and upregulated in the endometrium during the menstrual cycle and the synovium in rheumatoid arthritis.^{11,12} MMP9 is an inducible enzyme and is thought not to be secreted by cultured human endothelial cells under basal conditions.^{3,4,6-8} The tumour-promoting chemical, PMA, upregulates MMP9 in HUVE. Surprisingly there are no physiological angiogenic factors/cytokines that have been found to upregulate MMP9 in human endothelial cells. Here we show that cultured HUVE are capable of secreting MMP9 in the absence of any exogenous stimulus. This secretion is restricted to very early-passage cells, decreases rapidly as the cells are passaged in culture and is not detected by P2. RT-PCR and inhibition studies with cycloheximide confirmed that MMP9 was synthesized by P0 HUVE and not simply released from cellular storage reservoirs. The reason why MMP9 has not been detected in previous studies, is likely due to the fact that P2 or later passage cells were used.^{3,4,6-8}

Previous studies have noted variable changes in the expression of MMPs as human endothelial cells are passaged in culture. Commercially obtained late-passage (P28) cultured human dermal microvascular endothelial cells express less interstitial collagenase than younger cells.¹³ In contrast, P11 human dermal microvascular endothelial cells express increased levels of MMP1, MMP3 and MMP2 but decreased TIMP-1 compared to P5 cells.⁷ It is notable that the cells used in both these studies had undergone multiple passages. The striking feature of our study is the rapidity of the loss of MMP9 expression after only two passages. Rapid loss of MMP9 secretion has been observed in human epithelial ovarian carcinoma cells as they age in culture.¹⁴ Similar to our results, this study observed total abrogation of MMP9 secretion by passage 3 and no change in MMP2 secretion.

The inability of later passage cells to secrete MMP9 may simply be an artefact of using human endothelial cells in culture. If so, experiments using endothelial cells that have undergone multiple passages should be interpreted with care. However, our finding that the amount of MMP2 secretion remains unaltered up to P2 suggests that decreased MMP9 secretion is specific and not due to a generic reduction in all MMPs. It is feasible that the passage-dependent decrease in MMP9 is physiologically relevant. *In vivo*, normal endothelial cells have a very slow turnover rate of up to 30 years, especially in skin and brain blood vessels.¹⁵ These cells would undergo an average of only two population doublings in a human lifetime, approximately equivalent to cultured P2 HUVE. Thus, our cell culture findings may reflect a valid

physiological change, being the loss of MMP9 secretion from blood vessels in the elderly. Xia *et al.*¹⁶ have shown that keratinocytes from elderly donors have depressed MMP9 secretion and migratory activity when exposed to hypoxia, as compared to cells from young donors. Since MMP9 plays an important role in wound healing, these findings may help explain the impairment of wound healing in elderly patients.¹⁷ This is supported by our finding that the passage-dependent decrease in MMP9 secretion is, at least, partly due to cell doubling, which may reflect alterations in proliferation, ageing and/or de-differentiation of the cells.

This is the first report to demonstrate that HUVE constitutively secrete MMP9 and that this secretion occurs at very early passage, being rapidly lost by passaging the cells. These findings may be relevant to the angiogenic potential of human endothelial cells as they age.

ACKNOWLEDGEMENTS

We wish to thank Professor Ross Davey, Dr Minh Nguyen, Sheridan Henness, Dr Ian Kelso and the maternity staff at Royal North Shore Hospital for their assistance. This work was supported by the Arthritis Foundation of Australia, Lincoln Centre, Rebecca Cooper Foundation and the Northern Sydney Area Health Service.

REFERENCES

- Moses MA. The regulation of neovascularization of matrix metalloproteinases and their inhibitors. *Stem Cells* 1997; **15**: 180–189.
- Bergers G, Brekken R, McMahon G, *et al.* Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nature Cell Biol* 2000; **2**: 737–744.
- Hanemaaijer R, Koolwijk P, le Clercq L, de Vree WJ, van Hinsbergh HV. Regulation of matrix metalloproteinase expression in human vein and microvascular endothelial cells. Effects of tumour necrosis factor alpha, interleukin 1 and phorbol ester. *Biochem J* 1993; **296**: 803–809.
- Jackson CJ, Nguyen M. Human microvascular endothelial cells differ from macrovascular endothelial cells in their expression of matrix metalloproteinases. *Int J Biochem Cell Biol* 1997; **29**: 1167–1177.
- Qian X, Wang TN, Rothman VL, Nicosia RF, Tuszynski GP. Thrombospondin-1 modulates angiogenesis *in vitro* by up-regulation of matrix metalloproteinase-9 in endothelial cells. *Exp Cell Res* 1997; **235**: 403–412.
- Cornelius LA, Nehring LC, Roby JD, Parks WC, Welgus HG. Human dermal microvascular endothelial cells produce matrix metalloproteinases in response to angiogenic factors and migration. *J Invest Dermatol* 1995; **105**: 170–176.
- Kraling BM, Wiederschain DG, Boehm T, Rehn M, Mulliken JB, Moses MA. The role of matrix metalloproteinase activity in the maturation of human capillary endothelial cells *in vitro*. *J Cell Sci* 1999; **112**: 1599–1609.
- Nguyen M, Arkell J, Jackson CJ. Active and tissue inhibitor of matrix metalloproteinase-free gelatinase B accumulates within human microvascular endothelial vesicles. *J Biol Chem* 1998; **273**: 5400–5404.
- Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 1973; **52**: 2745–2756.
- Nguyen M, Arkell J, Jackson CJ. Human endothelial gelatinases and angiogenesis. *Int J Biochem Cell Biol* 2001; **33**: 960–970.
- Skinner JL, Riley SC, Gebbie AE, Glasier AF, Critchley HO. Regulation of matrix metalloproteinase-9 in endometrium during the menstrual cycle and following administration of intrauterine levonorgestrel. *Hum Reprod* 1999; **14**: 793–799.
- Ahrens D, Koch AE, Pope RM, Stein-Picarella M, Niedbala MJ. Expression of matrix metalloproteinase 9 (96-kd gelatinase B) in human rheumatoid arthritis. *Arthritis Rheum* 1996; **39**: 1576–1587.
- Reed MJ, Corsa AC, Kudravi SA, McCormick RS, Arthur WT. A deficit in collagenase activity contributes to impaired migration of aged microvascular endothelial cells. *J Cell Biochem* 2000; **77**: 116–126.
- Fishman DA, Bafetti LM, Banionis S, Kearns AS, Chilukuri K, Stack MS. Production of extracellular matrix-degrading proteinases by primary cultures of human epithelial ovarian carcinoma cells. *Cancer* 1997; **80**: 1457–1463.
- Denekamp J. Vascular endothelium as the vulnerable element in tumours. *Acta Radiol Oncol* 1984; **23**: 217–225.
- Xia YP, Zhao Y, Tyrone JW, Chen A, Mustoe TA. Differential activation of migration by hypoxia in keratinocytes isolated from donors of increasing age: implication for chronic wounds in the elderly. *J Invest Dermatol* 2001; **116**: 50–56.
- Agren MS. Gelatinase activity during wound healing. *Br J Dermatol* 1994; **131**: 634–640.