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ORIGINAL PAPER

Immunohistochemical and transcriptional expression of matrix metalloproteinases in full-term human umbilical cord and Human Umbilical Vein Endothelial Cells

Annamaria Mauro · Maria Buscemi · Aldo Gerbino

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Abstract Matrix metalloproteinases (MMPs) are extracellular zinc-dependent endopeptidases involved in the degradation and remodelling of extracellular matrix in physiological and pathological processes. MMPs also have a role on cell proliferation, migration, differentiation, angiogenesis and apoptosis. Umbilical cord is a special organ subjected to many changes during pre-natal life and whose cells can maintain a certain degree of plasticity also in post-natal period; for example recently they have been used as a source of stem cells. In this work we investigated the expression of MMPs in human umbilical cord and Human Umbilical Vein Endothelial Cells (HUVEC) though immunohistochemistry, RT–PCR and gelatin zymography. MMP-2 protein is expressed in the amniotic epithelium of human umbilical cord and in few sub-epithelial fibroblasts, while MMP-3 and MMP-10 only in the umbilical epithelium. MMP-8, MMP-9 and MMP-13 immunoreactivity is localised in the epithelium and in Wharton's jelly mesenchymal cells. Immunocytochemistry also revealed protein expression for MMP-2, 3, 8, 9 and 10 in cultured HUVEC. In agreement with immunohistochemical data, RT–PCR analysis performed on samples of whole umbilical cord confirmed the transcriptional expression for the genes encoding all the six matrix metalloproteinases investigated, while in HUVEC only the expression of MMP-2, 3, 9, 10 and 13 mRNAs was detected. Gelatin zymograpgy showed a clear MMP-2 and MMP-9 enzymatic activity in the conditioned medium of HUVEC at different culture passages, suggesting that HUVEC secrete gelatinases, that afterwards undergo extracellular activation, and this ability is not affected by passage number.

Keywords Matrix metalloproteinases · Human umbilical cord · HUVEC

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Introduction

Matrix metalloproteinases (MMPs) are a family of extracellular zinc-dependent neutral endopeptidases collectively capable of degrading essentially all ExtraCellular Matrix (ECM) components (Birkedal-Hansen et al. 1993). They play an important role in ECM remodelling in physiological processes, such as embryonal development, organ morphogenesis, tissue regeneration, wound healing, nerve growth, bone remodelling, angiogenesis and apoptosis. MMPs also play a role in pathological conditions involving accelerated turnover of ECM, for example rheumatoid arthritis, osteoarthritis, periodontitis, chronic ulcerations, cardiovascular disease, neurological disease, cirrhosis and metastasis (Mandal et al. 2003; Lipari et al. 2009).

They are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands (such as the FAS ligand) and chemokine inactivation. They are also thought to play a major role in processes such as cell proliferation, migration, differentiation, angiogenesis, apoptosis and host defence (Nagase and Woessner 1999).

The most commonly used classification of matrix metalloproteinases is based on substrate specificity and cellular localization. They can be divided into secreted MMPs and membrane-bound-MMPs (MT-MMPs). Secreted

MMPs have been divided into collagenases (MMPs-1, 8 and 13), gelatinases (MMPs-2 and 9), stromelysins (MMPs-3, 10 and 11) and matrilysins (MMP-7 and 26). All MMPs share a common structure with three conserved domains: (1) the pro-domain (an autoinhibitory domain whose function is to maintain the enzyme in the inactive form); (2) the catalytic domain and (3) the C-terminal hemopexin-like domain. MMP-2 and MMP-9 also have three repeats of fibronectin-type II domain inserted in the catalytic domain, whose function is to interact with collagens and gelatins (Nagase and Woessner 1999; Morgunova et al. 1999; Trexler et al. 2003).

The activity of MMPs is controlled at many different levels, including transcription, activation of zymogens and interaction with specific inhibitors, firsts of all the Tissue Inhibitors of MMPs (TIMPs) (Whatling et al. 2004). Transcription of many MMPs genes is inducible: the enhancers of MMPs genes transcription include growth factors, cytokines, chemical agents, physical stresses, oncogenic cellular transformation, etc…, while suppressive factors are Transforming Growth Factor-β (TGF-β), retinoic acids and glucocorticoids (Nagase and Woessner 1999; Han et al. 2008; Jackson et al. 2009; Viappiani et al. 2009). All MMPs are synthesized as pre-pro-enzyme and secreted as pro-enzyme (inactive form). Activation is extracellular and requires the disruption of the "cysteine switch" and the removal of the pro-peptide (Nagase and Woessner 1999; Viappiani et al. 2009).

The umbilical cord is the organ connecting the foetus to the placenta. Umbilical cord at term is made of three vessels (two arteries coiled around a vein) surrounded by the Wharton's jelly, a soft connective tissue composed of few mesenchymal stromal cells and abundant extracellular matrix (Fig. 1) (Hoyes 1969; Parry and Abramovich 1972; Meyer et al. 1983; Sobolewski et al. 1997; Can and Karahuseyinoglu 2007). Human umbilical cord is a special organ subjected to many changes during pre-natal life and whose cells can maintain a certain degree of plasticity also in post-natal period; for example they have wide differentiation potentiality (Karahuseyinoglu et al. 2007) and recently have been used as a source of stem cells (Conconi et al. 2006; Fu et al. 2004).

Human Umbilical Vein Endothelial Cells (HUVEC) are highly dynamic cells: they show angiogenic ability and actively participate to the regulation of vascular tone through the production of vasoactive substances such as eNOS, COX-1, COX-2 and $cPLA_2$ (Gifford et al. 2004), moreover they have shown to be endowed with an high degree of plasticity; they have the potentiality to differentiate into a wide range of different cell types, for example neuron, smooth muscle cells and bone (Su et al. 2006; Krenning et al. 2008; Aguilar-Vázquez et al. 2008).

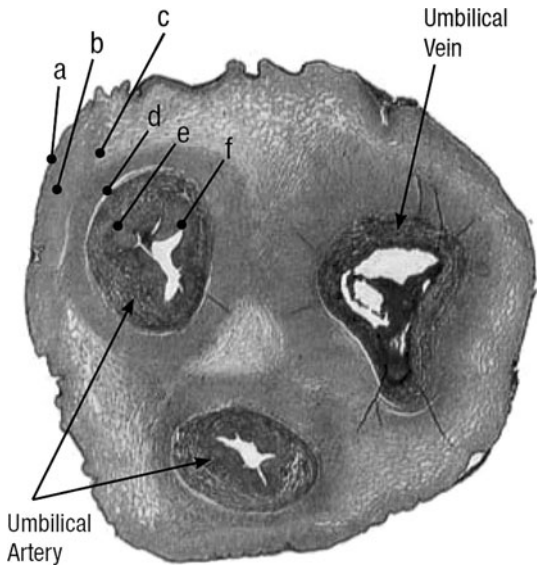


Fig. 1 Azan staining of human umbilical cord at term. *a* amniotic epithelium; *b* subamniotic Wharton's jelly; *c* inner Wharton's jelly; *d/e* circular and longitudinal vascular musculature; *f* vascular endothelium. Umbilical vein and arteries are indicated by the *arrows*

In this work we investigated the expression of MMPs in human umbilical cord and particularly in HUVEC, since both of them are highly dynamic territories where these enzymes, involved not only in ECM degradation but also in processes such us cell proliferation, differentiation, angiogenesis and surface molecule cleavage, could have an important functional significance. The matrix metalloproteinases we investigated in this study are the gelatinases MMP-2 and MMP-9, the collagenases MMP-8 and MMP-13 and the stromelysin MMP-3 and MMP-10.

Materials and methods

Samples

The analysis was performed on 17 full-term human umbilical cords obtained by caesarean section. The umbilical cords were provided by the Gynecologic and Obstetric clinic of the University Hospital "Paolo Giaccone" in Palermo. Informed consent was obtained from patients few days before surgery. The experimentation was approved by the ethics committee.

Immunohistochemistry

Human umbilical cords were collected in physiological solution, one section (about 1 cm thick) was dissected from each umbilical cord, formalin-fixed, dehydrated in a series of alcohols, cleared in xylene and paraffin-embedded. Sections of 7 μm were cut with a Leica microtome

RM2145, dried overnight at 37°C and then stored at room temperature until use; prior to immunostaining slides were de-waxed in xylene, re-hydrated in a series of alcohols and rinsed in distilled water for 5 min.

Immunohistochemistry was performed using the "DakoCytomation EnVision + System-HRP (AEC)" kit from Dako following manufacturer's instructions: the sections of umbilical cord were covered with the "Peroxidase block" reagent and incubated for 5 min at room temperature, then rinsed once in Phosphate Buffer Saline (PBS) pH 7.2, covered with the antibody solution and incubated at 4°C overnight. Mouse anti human MMP-2 monoclonal antibody (Chemicon) (1:800 dilution), mouse anti MMP-3 monoclonal antibody (Chemicon) (1:20), mouse anti human MMP-8 monoclonal antibody (Chemicon) (1:400), rabbit anti human MMP-9 polyclonal antibody (DAKO) (1:100), mouse anti human MMP-10 monoclonal antibody (Chemicon) (1:100) and mouse anti MMP-13 monoclonal antibody (Chemicon) (1:1000) were used. As negative controls for our antibodies we used mouse IgG1 antibody (Dako, clone DAK-G01) (1:100 dilution) and rabbit immunoglobulin fraction (Dako) (1:200 dilution). All antibodies were diluted in a 0.1% Bovine Serum Albumine (BSA) solution.

Samples were rinsed twice in PBS pH 7.2, incubated with "Peroxidase Labelled Polymer" reagent for 30 min, rinsed twice in PBS pH 7.2 and incubated with "Substrate-Chromogen" reagent. The staining reaction was stopped rinsing the slides in distilled water and staining time was the same for all the samples. Slides were coverslipped using the "DakoCytomation Faramount Aqueous Mounting Medium" (Dako). The specimens were observed with a Leica DM1000 light microscope.

Semi-quantitative evaluation of immunoreactivity

The intensity of immunostaining for the 6 Matrix Metalloproteinases in our specimens was evaluated by three independent observers. Score values were assigned according to the following criteria: (−) no staining, (+) light staining, (++) moderate staining, (+++) intense staining.

HUVEC isolation and culture

Human Umbilical Vein Endothelial cells (HUVEC) have been isolated from full-term human umbilical cords obtained from caesarean section immediately after the partum, and stored in physiologic solution until isolation procedure. Isolation of cells was performed within 3 h of tissue collection. The method for HUVEC isolation is based on the separation of endothelial cells from vein vessel wall using collagenase digestion of the sub-endothelial basement membrane, according to previously published methods (Kumar et al. 2004; La Rocca et al. 2009) with slight modifications in the composition of culture and isolation media. Briefly, umbilical cord vein was cannulated and perfused with a heparin solution (50 U/ml), followed by Dulbecco Phosphate Buffer Saline (DPBS) without Ca2+ and Mg2+ (SIGMA) in order to remove blood residue from vessel. The vein was perfused with collagenase solution (Type II, GIBCO) 1 mg/ml and, after clamping of the ends, the vein was filled with collagenase solution and incubated for 15 min at 37°C. Collagenase solution containing HUVEC was collected in a sterile falcon tube, and the vein was thoroughly flushed with isolation medium: M-199 supplemented with antibiotic/antimycotic solution (SIGMA) 2X, in order to detach endothelial cells from vessel wall. After centrifugation, cells were resuspended in complete culture medium: M-199 supplemented with 10% FBS (SIGMA), antibiotic/antimycotic solution (SIGMA) 1X, 0.1 mM L-glutamine, 20 μg/ml Endothelial Cell Growth Factor (ECGF, Roche), 50 μg/ml heparin sodium salt (SIGMA). Cells were seeded in gelatin-coated culture flasks at a concentration of 30,000/cm^2. Primary HUVEC were used for experiments at passages 2–3.

Immunocytochemistry

HUVEC grown in chamber-slides (BD Falcon), were washed with PBS and fixed with 4% paraphormaldeyde for 15 min at room temperature, then washed again with PBS. Slides were exposed to 0.3% H_2O_2 in H_2O for 10 min and then washed with PBS. Slides were treated with 1% BSA in PBS for 3 min, then incubated with the primary antibody at 4°C overnight. The following part of the procedure was the same of the one previous described for immunohistochemistry.

Total RNA extraction

Samples of human umbilical cord were frozen in liquid nitrogen immediately after caesarean section and stored at −80°C until use. Cultured HUVEC were subjected to RNA extraction immediately after the removal of culture medium. Total RNA extraction was accomplished using the "illustra RNAspin Mini Kit" (Amersham Biosciences) following manufacturer's instructions. RNA yield was evaluated spectrophotometrically (A260/A280) and RNA aliquots were stored at −80°C until use.

Reverse-transcription (RT)-polymerase chain reaction (PCR)

For RT reaction 2 μg RNA was used. To avoid DNA contamination of RNA samples DNAse digestion was performed using "AMPD1 kit" (SIGMA). RT reaction was

performed using the "Enhanced avian HS RT–PCR kit" (SIGMA) following manufacturer's instructions. Briefly: 1 µl random nonamers and 1 µl anchored oligo (dT)23 were added to the DNAse digestion product and incubated at 70°C for 10 min to denature the sample. Then 2 µl 10× Buffer, 1 µl deoxynucleotide mix, 1 µl RNAse inhibitor, 1 µl Enhanced AMV Reverse Transcriptase enzyme and DEPC water were added to the sample. RT reaction was performed in 20 µl total volume at 42°C for 50 min, followed by a step at 95°C for 5 min to inactivate the enzyme.

PCR reaction was performed as follow. 2 µl of DNA (RT reaction product) was mixed with 18 µl distilled H_2O, 2.5 µl gold buffer 10X, 0.75 mM $MgCl_2$, deoxy-nucleotides mix (dNTPs) 4 µM, primers mix 0.2 µM, Ampli Taq Gold enzyme 1 U. The reaction was cycled for 9 min at 95°C, then 40 cycles of 45 s 95°C, 1 min 60°C, and a final extension step of 10 min at 60°C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as internal positive control. PCR products were visualized on 2% agarose gels, stained with sybr green (SIGMA). Primers sequences are shown in Table 1.

Gelatin Zymography

HUVEC culture medium has been collected from different passages (P1–P3), then dialyzed against Hepes 10 mM solution, lyophilized and resuspended in distilled H_2O. Samples were separated on a 7.5% SDS–Polyacrylamide gel containing 0.1% gelatine. For each sample 40 ug of total protein was loaded. Human serum protein was used as internal positive control. Following constant voltage electrophoresis, gels were washed in renaturation buffer (2.5% Triton X-100 in 50 mM Tris–HCl pH 7.5) for 1 h in an orbital shaker. Then the zymograms were incubated for 18 h at 37°C in incubation buffer (0.15 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 in 50 mM Tris–HCl pH 7.5). Gels were then stained with Coomassie Brillant Blue and destained with 7% methanol and 5% acetic acid solution. Areas of enzymatic activity appeared as clear bands over the dark background.

Semi-quantitative evaluation of lytic activity

Following zymography, the degree of gelatin digestion was quantified using a scanner equipped with a transparency adapter interfaced to an IBM PC. Gels were scanned using 1D ScanEX software, version 3.1 for Windows (Scanalytics), in a grey scale mode. The image was digitally inverted, so that the integration of bands was reported as positive values. The pixel density was determined after background subtraction and used to calculate the integrated density of a selected band. Values of integrated density were reported in volume units of pixel intensity per mm^2. The integrated density of each band is reported as the mean of three different measurements of the same band for each sample run in triplicate. Moreover, for each experiment, the intensity of each lytic band was measured three times in order to exclude software-driven measurement errors. Statistical analysis of data was performed by the chi-square test, and P-value less than 0.05 was taken to be significant.

Results

MMPs protein expression in full-term human umbilical cord

Immunohistochemistry showed an intense expression of MMP-2 protein in the umbilical cord epithelial cells and in Wharton's jelly fibroblasts (Fig. 2a). The distribution of

Table 1 Name, nucleotide sequence and length of the amplification product for the primers used in this work

Primer name	Primer sequence	Amplification product
GAPDH human forward	GAG TCA ACG GAT TTG GTC GT	238 base pairs
GAPDH human reverse	TTG ATT TTG GAG GGA TCT CG	
MMP-2 human forward	TGA TGG TGT CTG CTG GAA AG	280 base pairs
MMP-2 human reverse	GAC ACG TGA AAA GTG CCT TG	
MMP-3 human forward	GCA GTT TGC TCA GCC TAT CC	214 base pairs
MMP-3 human reverse	GAG TGT CGG AGT CCA GCT TC	
MMP-8 human forward	ATC TCA CAG GGA GAG GCA GA	372 base pairs
MMP-8 human reverse	GCT TGG TCC AGT AGG TTG GA	
MMP-9 human forward	CAT TTC GAC GAT GAC GAG TTG	554 base pairs
MMP-9 human reverse	AAG CCC CAC TTC TTG TCG CT	
MMP-10 human forward	GGC TCT TTC ACT CAG CCA AC	241 base pairs
MMP-10 human reverse	TCT CCC CTC AGA GTG CTG AT	
MMP-13 human forward	GAC CCT GGA GCA CTC ATG TT	192 base pairs
MMP-13 human reverse	TCC TCG GAG ACT GGT AAT GG	

GAPDH glyceraldehyde 3-phosphate dehydrogenase; *MMP* matrix metallo proteinase; *G* guanine; *C* cytosine; *A* adenine; *T* timine

positive cells in Wharton's jelly did not appear homogenous; the amount of fibroblasts expressing MMP-2 being higher in the region immediately below the umbilical epithelium and decreasing in the inner jelly towards blood vessels.

MMP-3 protein expression was localized exclusively in the umbilical cord epithelial cells (Fig. 2b) while all the other umbilical cord regions were completely negative for this enzyme. In the epithelium we observed a cytoplasmic immunoreactivity in which the nucleus appeared clear while the remaining part of the cell was filled with dark brown staining granules. MMP-8 protein expression has been identified in the amniotic epithelium (Fig. 2c), in the stromal cells of Wharton's jelly (Fig. 2d) and in some cells of the blood vessel musculature. Immunoreactivity is intense on the epithelium, while in Wharton's jelly and in vessel musculature MMP-8 protein expression appears moderate.

MMP-9 immunostaining is intense with well defined edges. Protein expression was localized in the umbilical cord epithelial cells and in Wharton's jelly fibroblasts and mesenchymal cells (Fig. 2e). We also detected a staining of the musculature around blood vessels which appears uniform and diffuse so that we believe it is a poorly specific labelling (data not shown). MMP-10 protein expression was clearly identified only in epithelial cells (Fig. 2f); all the other umbilical cord regions did not show an evident immunostaining for this protein, we could detect just a faint reactivity in some of Wharton's jelly cells.

MMP-13 immunoreactivity was localized in the umbilical cord epithelium, in Wharton's jelly fibroblasts and mesenchymal cells (Fig. 2g), and showed a diffuse but intense staining also in the cells of the muscular layers around blood vessels (Fig. 2h). In the epithelial cells the immunostaining was cytoplasmic, intense and well-defined, thus making evident the boundary between the cells and between the nucleus and the cytoplasm.

MMPs gene expression in full-term human umbilical cord

RT–PCR analysis performed on the whole umbilical cord was in agreement with immunohistochemical data showing transcriptional expression of the genes encoding for all the six matrix metalloproteinases investigated. Figure 3 shows DNA bands corresponding to the specific amplification products for the six genes.

MMPs protein expression in HUVEC

Immunocytochemistry showed MMP-2 protein expression in HUVEC. All the cultured cells were positive for this enzyme. Immunostaining was cytoplasmic and intense on a very clear background (Fig. 4a), moreover the single dark brown staining granules could be distinguished around the nucleus and in cell processes. MMP-3 immunoreactivity is evident in all the cultured HUVEC; the reactivity is clearly cytoplasmic, mainly located around the nucleus and also evident in the cytoplasmic processes of the cell (Fig. 4b, c). The immunostaining appeared as single granules filling the cytoplasm of the cells.

A strong cytoplasmic MMP-8 protein expression was identified in all the cultured cells (Fig. 4d). By immunohistochemistry we detected MMP-9 protein expression in HUVEC, even if the immunoreactivity appeared moderate compared to the other MMPs tested (Fig. 4e). MMP-10 immunoreactivity was identified in all the cultured cells, it was an intense reactivity, localized into the cytoplasm, and appeared as condensed in dark brown granules (Fig. 3f). Immunocytochemistry did not detect any protein expression for MMP-13 in HUVEC (data not shown).

MMPs gene expression in HUVEC

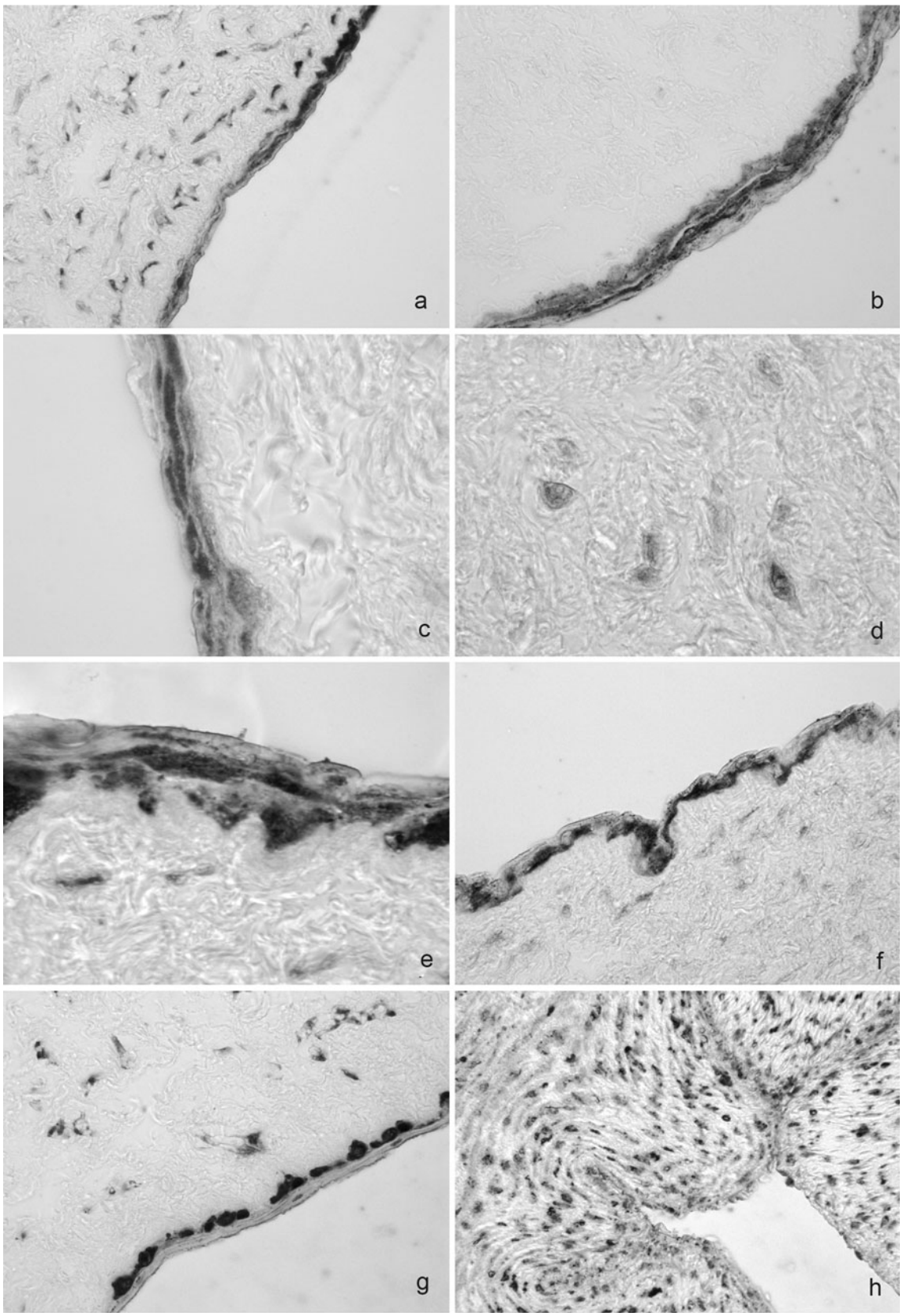
The results of RT–PCR analysis performed on cultured HUVEC confirmed immunocytochemical findings and revealed the transcriptional expression of the genes encoding for MMP-2, MMP-3, MMP-9, MMP-10 and MMP-13, while MMP-8 gene expression was not detected; Fig. 5 shows DNA bands corresponding to the specific amplification products for all the genes investigated but MMP-8.

Gelatinolytic activity of secreted gelatinases

Immunocytochemistry provided information only on the presence of MMPs proteins, without distinguish between the inactive pro-enzyme and the cleaved, activated enzyme.

We decided to investigate the presence of MMPs enzymatic activity and, since MMPs are secreted proteins whose activation takes place in the extracellular environment, we searched for this activity in the conditioned medium of HUVEC. We tested the enzymatic activity of MMP-2 and MMP-9 secreted by HUVEC through gelatin zymograpgy assay. Moreover, the analysis has been performed on the conditioned medium harvested from HUVEC at different culture passages to determine if cellular senescence affects either the ability of HUVEC to synthesize and secrete MMP-2 and MMP-9 or the extracellular activation of these enzymes.

Zymography showed that the level of MMP-2 enzymatic activity did not vary significantly from passage 1 to passage 3 ($P > 0.05$). The same pattern was showed by MMP-9 ($P > 0.05$) (Fig. 6). In all the samples (P1, P2, P3) gelatinolytic activity of MMP-2 was higher than MMP-9 and the difference was statistically significant ($P < 0.05$).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◀ **Fig. 2** Immunohistochemistry on human umbilical cord sections. **a** MMP-2 in the umbilical epithelium and in the cells of the Wharton's jelly (40X); **b** MMP-3 in the epithelium (40X); **c** MMP-8 in the epithelium (40X); **d** faint MMP-8 expression in Wharton's jelly cells (100X); **e** MMP-9 in the epithelium and in the cells of the Wharton's jelly (100X); **f** MMP-10 in the amniotic epithelium (40X); **g** MMP-13 in the epithelium and few fibroblasts (40X); **h** MMP-13 in cells of the vessel musculature (40X)

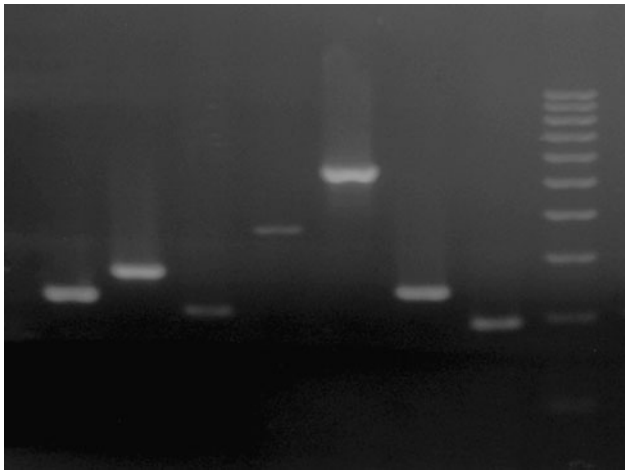

Fig. 3 Representative picture of RT–PCR analysis performed on human umbilical cord. Amplification products for; lane 1: GAPDH (232 bp); lane 2: MMP-2 (280 bp); lane 3: MMP-3 (214 bp); lane 4: MMP-8 (372 bp); lane 5: MMP-9 (554 bp); lane 6: MMP-10 (241 bp); lane 7: MMP-13 (192 bp); lane 8: 100 bp DNA ladder

Chart 1 represents a semi-quantitative analysis of MMP-9 and MMP-2 gelatinolytic activity. Data represented are means of three replicated experiments, with the indication of the standard deviation.

Discussion

We investigated, through immunohistochemistry, the expression and localization of six matrix metalloproteinases in the different regions of full-term human umbilical cord (Table 2).

We detected the expression of gelatinases (gelatinase-A and B; MMP-2 and MMP-9) in the cells of umbilical epithelium and Wharton's jelly. These data are in agreement with previous studies reported in literature; Sobolewski et al. (2001) found the expression of MMP-2 and 9 in Wharton's jelly cells and they made the hypothesis that an alteration in the expression, or activity, of these enzymes could be related to the development of Oedema/proteinuria/hypertension (EPH) gestosis.

We also detected the presence of stromelysins (stromelysin-1 and -2; MMP-3 and MMP-10) only in epithelial cells of umbilical cord. The expression of MMP-3 and MMP-10 in epithelial cells both in normal and pathological tissue remodelling has been reported by many authors and some of them related stromelysins expression to processes such as wound healing, cell migration and tumor development (Daniels et al. 2003; Krampert et al. 2004; McCawley et al. 2008).

Our experiments showed expression of collagenases (collagenase-1 and 2; MMP-8 and MMP-13) in umbilical cord epithelium, in Wharton's jelly cells and in blood vessel musculature.

After we detected protein expression and spatial distribution of these six matrix metalloproteinases in human umbilical cord, we wanted to investigate transcriptional expression of the genes encoding for these enzymes, thus we performed RT–PCR analysis on total RNA extracted from human umbilical cords. The analysis confirmed the transcriptional expression of the genes encoding for all the six matrix metalloproteinases tested.

In the continuation of our study we focused our attention on the expression of these six proteases in HUVEC isolated from umbilical cord. These cells have shown to be endowed with an high degree of plasticity; they have the potentiality to differentiate into different cell types, for example neuron-like cells (Su et al. 2006), vascular smooth muscle cells (Krenning et al. 2008) and osteogenic ligneage (Aguilar-Vázquez et al. 2008). The presence of HUVEC in co-culture with other cell type can affect the differentiation and/or the proliferation rates of both cell types (Hofmann et al. 2008; Lai et al. 2009). They also have a role in the stimulation/regulation of the activity of the hematopoietic progenitor cells (Yamaguchi et al. 1998). HUVEC are also known to actively participate in angiogenesis and vascular tone modulation through the production of vasoactive substances; under this point of view they can be considered one of the umbilical cord cell types more dynamic and for this reason we thought it was interesting to deepen our research investigating the expression of MMPs in these cells.

Immunocytochemistry showed the expression of MMP-2, 3, 8, 9 and10 in all cultured HUVEC, while MMP-13 protein was not detected. The results of RT–PCR analysis performed on HUVEC revealed the transcriptional expression of the genes encoding for MMP-2, 3, 9, 10 and 13, while MMP-8 gene is not expressed.

In HUVEC we detected MMP-13 mRNA but we did not find the protein. This could be due to a low MMP-13 protein synthesis or to the possibility that the protein, after being synthesized, is secreted into the extracellular environment where it exerts its function.

The expression of MMP-8 protein and mRNA seems conflicting; we evidenced the presence of MMP-8 protein through immunohistochemistry but we did not detect the mRNA through RT–PCR; it is possible that the level of

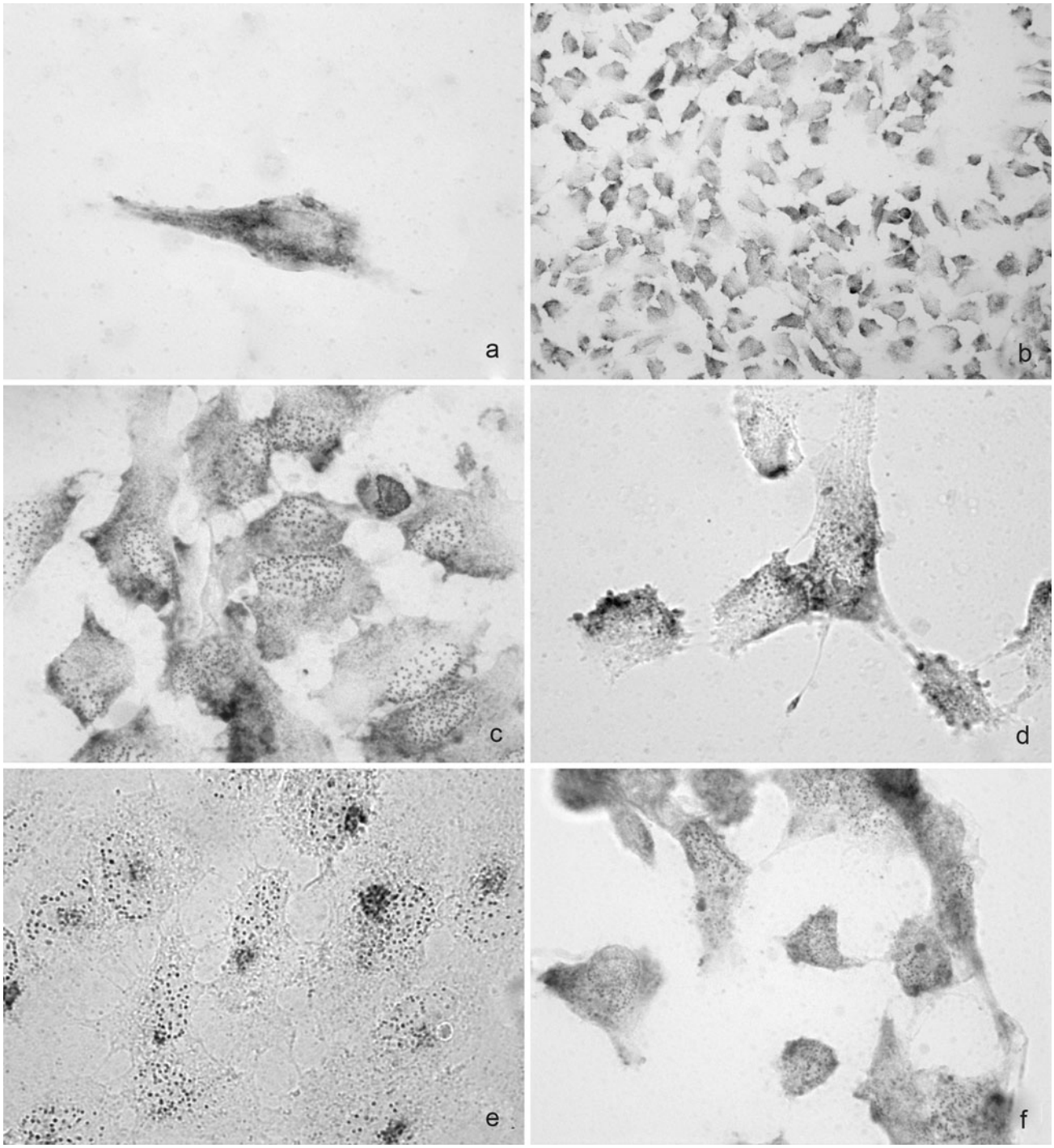

Fig. 4 Immunohistochemistry on cultured HUVEC. **a** cytoplasmic immunoreactivity for MMP-2 in HUVEC (100X); **b** MMP-3 expression in all the cultured cells (20X); **c** MMP-3 expression in the cytoplasm and in the processes of HUVEC, the single staining granules can be distinguished (100X); **d** MMP-8 staining in cytoplasm and cell processes (100X); **e** MMP-9 expression in the cytoplasm of all cultured cells (100X); **f** MMP-10 expression in HUVEC (100X)

expression of MMP-8 mRNA is below the sensitivity of the technique we used.

The fact we detected the expression of almost all MMPs in cultured HUVEC while none of them in the endothelium of whole umbilical cord could be explained considering that blood vessel endothelium is a very delicate structure that can be easily damaged during fixation/embedding procedures needed for immunohistochemistry.

The antibodies used for immunohistochemistry were not able to distinguish between the active and the inactive form

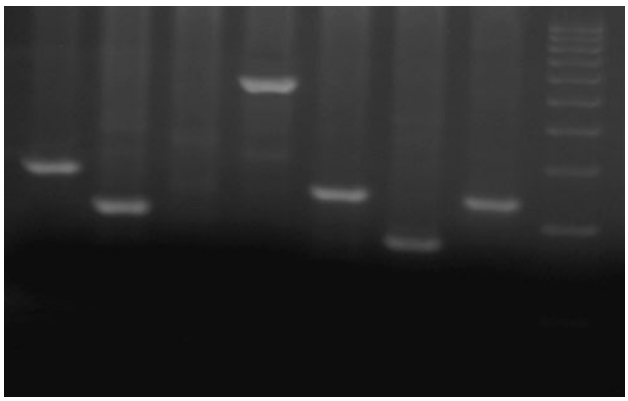

Fig. 5 Representative picture of RT–PCR analysis performed on cultured HUVEC. Lane 1: MMP-2 amplification product (280 bp); lane 2: MMP-3 amplification product (214 bp); lane 3: no amplification product for MMP-8; lane 4: MMP-9 amplification product (554 bp); lane 5: MMP-10 amplification product (241 bp); lane 6: MMP-13 amplification product (192 bp); lane 7: GAPDH amplification product (232 bp); lane 8: 100 bp DNA ladder

of the metalloproteinases investigated, so this experiment could give information only on the presence of proteins and not on their enzymatic activity. On the contrary, gelatin zymography allows to study the entity of MMP-2 and MMP-9 gelatin degrading activity. The result of the assay, performed on HUVEC conditioned medium, showed HUVEC ability to produce and secrete sensible amount of pro-gelatinases and that these pro-enzymes are efficiently activated in the extracellular environment. Moreover, zymography has been performed on the conditioned medium harvested from HUVEC at different culture passages to determine if cellular senescence affects either the ability of HUVEC to synthesize and secrete MMP-2 and MMP-9 or the extracellular activation of these enzymes. No significant difference has been found between the gelatinolytic activity of MMP-2 and MMP-9 in HUVEC conditioned medium from passages 1–3, thus senescence seems not to influence this aspect. The choice to use very close passage number (passages 1–3) was forced by the fact that HUVEC are primary cells with a very fast senescence process that, among the others typical features

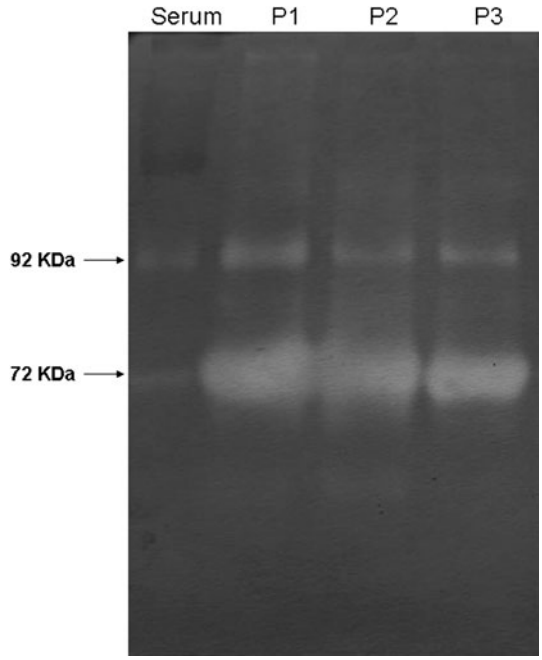

Fig. 6 Representative picture of gelatin zymography assay performed on HUVEC conditioned medium at different culture passage. Bands corresponding to MMP-9 (92 KDa) and MMP-2 (72 KDA) are indicated by the arrows and visible in all samples. Lane 1: human serum; lane 2: passage 1 (P1); lane 3: passage 2 (P2); lane 4: passage 3 (P3)

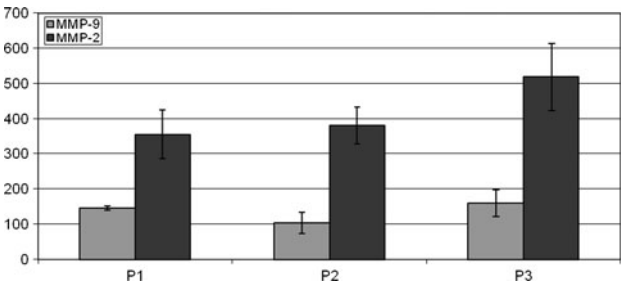

Chart 1 Semi-quantitative analysis of MMP-9 and MMP-2 gelatinolytic activity in HUVEC conditioned medium at different passage number (P1-P3). *Bars* represents the mean standard deviation

Table 2 Summary of immunohistochemical results in the different regions of human umbilical cord

	Epithelium	Wharton's jelly	Vessel musculature	Endothelium
MMP-2 (gelatinase-A)	+++	+++	−	−
MMP-9 (gelatinase-B)	+++	+++	−	−
MMP-8 (collagenase-2)	+++	+	+	−
MMP-13 (collagenase-3)	+++	++	++	−
MMP-3 (stromelysin-1)	+++	−	−	−
MMP-10 (stromelysin-2)	++	−	−	−

Score values (from − to +++) have been assigned by three independent operators

of senescence, implies growth delay and survival decrease, thus even cells at passage 4 appear unhealthy and not suitable for experiments.

This work makes light on the pattern of expression and distribution of several types of matrix metalloproteinases (gelatinases, collagenases and stromelysins) in the different regions of full-term human umbilical cord and in cultured HUVEC. The expression of the six matrix metalloproteinases we investigated is evident in almost all the regions of human umbilical cord at term. The meaning of the wide distribution of these enzymes in this organ has to be linked to their role in the degradation and remodelling of extracellular matrix and in other physiological processes such as cell proliferation, differentiation and apoptosis. These data could be useful in the comprehension of MMPs distribution and activity in this and other tissues in physiological and pathological conditions. It would be interesting to enrich this work with the study of all the other types of MMPs.

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