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Temporal relationship between MMP production and angiogenic process in HUVECs

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Abstract

Alterations in both cell–cell and cell–matrix interactions are associated with the activation of endothelial cells that initiate angiogenesis. Cell–matrix interactions are affected by changes in both cell surface receptors for matrix proteins and the composition of ECM. One of the molecular mechanisms involved in changes in these components is the action of neutral proteinases, particularly matrix metalloproteinases. To understand the involvement of MMPs in angiogenic processes, the *in vitro* model of human umbilical vein endothelial cells in culture was used. Zymography and ELISA showed that, as cell–cell contact and network-like structures were formed, there was down regulation of MMP-2 and MMP-9 associated with high levels of their endogenous inhibitors TIMP-1 and TIMP-2. On treatment with aspirin, which inhibited the cell–cell contact and network-like structure formation, there was no down regulation of MMPs and cells continued to produce MMP-2 and MMP-9. These results indicate a temporal relationship between MMP-2 and MMP-9 production by endothelial cells and the onset of angiogenic event.

Keywords: MMPs; Aspirin; TIMPs; Angiogenesis; HUVECs

1. Introduction

Endothelial cells undergo alterations in their phenotype associated with alterations in both cell–cell and cell–matrix interactions during angiogenesis (Folkman, 1995; Ferrara and Alitalo, 1999; Nelson et al., 2000; Hass and Madri, 1999). Cell–matrix interactions are affected by changes in both cell surface receptor for matrix proteins and the composition of the extracellular matrix (ECM). One of the molecular mechanisms involved in changes in the composition of ECM is the action of neutral proteinases, particularly matrix metalloproteinases (MMPs) (Davis and Senger, 2005). Matrix metalloproteinases are a group of over 20 enzymes that are characterized by their ability to degrade the extracellular matrix in a cation dependent manner at neutral pH (Chang and Werb, 2001). MMPs play a major role in growth, development and differentiation and are under tight regulation at transcription, post transcription and at protein levels via activators and inhibitors (for reviews see: Sternlicht and Werb, 2001; Mott and Werb, 2004). The MMPs are inhibited by their endogenous inhibitors, the TIMPs (TIMP-1, -2, -3, -4), each capable of inhibiting virtually all members of the MMP family (Henriet et al., 1999). The equilibrium between TIMPs and MMPs is important in localized proteolysis.

Cultures of endothelial cells, which are used as model cell systems to study angiogenesis, have been shown to express MMP-1, -2, -3, -9 and -14 and TIMP-1, -2 (Moses, 1997). The requirement of MMPs produced by endothelial cells (ECs) for angiogenesis is evidenced by the studies where naturally occurring (Moses et al., 1990; Fisher et al., 1994; Schnaper et al., 1993; Anand-Apte et al., 1997) or synthetic inhibitors (Montesano and Orci, 1985; Hass et al., 1998;

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Maekawa et al., 1999) of MMPs caused inhibition of angiogenesis or various events thereof. Additional evidence for the requirement of MMPs during angiogenesis comes from genetic studies in mice. With regard to the MMP knockout mice deficient in MMP-2, tumor induced angiogenesis was markedly reduced in dorsal sac assay with B16-BL6 melanoma cells and in MMP-9 deficient mice there was reduced bone growth plate angiogenesis (Itoh et al., 1998; Vu et al., 1998).

Although most reports suggest the requirement of MMPs for angiogenesis, it is not clear at which stage MMPs are involved; it is also not clear whether angiogenesis causes changes in MMP levels. Most of the studies on the involvement of MMPs during angiogenesis were either carried out in the presence of serum, growth factors or in the presence of matrix proteins, which are inducers of MMPs. The use of natural or synthetic inhibitors against MMP might not completely explain the role of MMPs as, apart from MMP inhibition, these inhibitors can also affect various other components of angiogenic process. This is evidenced by the report where TIMP-2 inhibited angiogenesis independent of MMP inhibition by irreversibly binding to growth factor receptors (Seo et al., 2003). Further, the involvement of MMPs at various stages of angiogenic process is not known. In this paper we describe the relationship between the production of MMPs, particularly gelatinases, their endogenous inhibitors and angiogenic process using human umbilical vein endothelial cells (HUVEC) as a model system. Results of experiments using aspirin as an inhibitor of angiogenesis indicate that there is a temporal relationship between production of gelatinases, their endogenous inhibitors and angiogenic process, and alterations in this relationship can inhibit angiogenesis.

2. Materials and methods

2.1. Materials

MCDB 131 medium, Penicillin, Antibiotics, Gelatin, Ortho-dianisidine, DEPC, DMSO, MTT, Diamino benzidine, Tris, Glycine, Bovine serum albumin, Monoclonal antibodies to MMP-2, MMP-9, E-Selectin, von Willebrand factor/Factor VIII antigen, TIMP-1 and TIMP-2 and HRP-conjugated secondary antibody were purchased from M/s Sigma Aldrich Co., USA. NC membrane was from BIORAD. ELISA and tissue culture plates were from NUNC Denmark. Perfect RNA Mini isolation kits and C-Master RT Plus PCR kits were purchased from Eppendorf India.

2.2. Isolation and culture of HUVECs

Endothelial cells were isolated by collagenase perfusion of the umbilical vein (Jaffe et al., 1973). The yield and viability of isolated HUVECs were determined by Trypan blue exclusion; 13×10^5 cells per well in serum free MCDB 131 medium were seeded in NUNC multiwell plates, allowed to attach for 4–5 h, unattached cells were removed, fresh medium was added and maintained in culture overnight before starting the treatment. The cells were treated with 0.1 mM aspirin in MCDB 131.

2.3. MTT assay

The viability of HUVECs in culture on treatment with aspirin was assessed by performing MTT assay (Sladowski et al., 1993). Briefly HUVECs were maintained in MCDB 131 medium containing 0.1 mM aspirin. Cells supplemented with MCDB 131 medium alone served as control. At intervals of 24 h the medium was removed and the cells were washed with Krebs Hanseleit buffer. The cells were then incubated with 0.5 mg/ml MTT in Krebs Hanseleit buffer for 4 h at 37 °C in a CO_2 incubator, after which the solution was removed and the formazan dye formed in the cell was solubilized in DMSO. The absorbance at 570 nm was measured using a multiwell scanning spectrophotometer and the viability of the aspirin treated cells expressed as a percentage of the untreated control.

2.4. Zymography

The activity of MMPs secreted by HUVECs into the medium maintained in culture was determined by gelatin zymography (Ambili and Sudhakaran, 1998). Zymogram gels consisted of 7.5% polyacrylamide gel polymerized together with gelatin (1 mg/ml). After electrophoresis, the gels were washed with 2.5% Triton X-100 and incubated with substrate buffer (50 mM Tris, 5 mM $CaCl_2$, pH 7.5) at 37 °C for 24 h. The gels were stained with Coomassie brilliant blue R 250 and destained with water. Gelatinolytic activities appearing as clear zone were quantitated using Quantity One – 4.5.0 – Software (BioRad).

2.5. Reverse zymography

In order to study the activity of TIMP proteins, reverse zymographic analysis was performed (Oliver et al., 1997). Aliquots of freeze-dried medium equivalent to the same amount of cell protein were applied to 12% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS, 0.5 mg/ml gelatin and MMPs from HeLa cells conditioned medium. After electrophoresis, gels were washed three times with 2.5% (v/v) Triton X-100 at intervals of 1 h, followed by incubating gels overnight at 37 °C in substrate buffer (50 mM Tris–HCl, 5 mM $CaCl_2$, pH 8.0). Subsequently, gels were stained for 30 min with 0.1% (w/v) Coomassie Blue G-250, and destained with a solution of 30% (v/v) methanol and 10% (v/v) glacial acetic acid. TIMPs in the samples inhibit MMP action, and were visualized as dark bands on a clear background. The intensity of bands was quantitated using Quantity One – 4.5.0 – Software (BioRad).

2.6. ELISA

Amount of E-Selectin, von Willebrand Factor (vWF), gelatinases, TIMP-1 and 2 were quantitated by ELISA (Engvall and Perlman, 1971). Cell culture medium pre-coated onto ELISA plates served as the antigen. O-dianisidine was used as the substrate. The concentrations of these antigens were estimated by measuring the absorbance of the colored HRP product spectrophotometrically at 405 nm.

2.7. Immunoblot analysis

The freeze-dried culture medium was subjected to SDS-PAGE on 7.5% acrylamide gels (Towbin et al., 1979). Proteins were transferred to the NC membrane using a semidry blotting apparatus and were probed with specific antibody and developed using HRP-conjugated secondary antibody.

2.8. Microphotography

In order to study angiogenesis, the morphology of the HUVECs in culture was examined using an Olympus Phase contrast microscope fitted with SC35 Type 12 TTL Auto-exposure 35 mm SLR Camera. The morphology of the cells was monitored and photographed at regular intervals.

2.9. RT PCR of VEGF and VEGF R2

Total RNA was isolated from HUVECs using Perfect RNA Mini isolation kits procured from Eppendrof India as per manufacturer's instruction. The primer pairs for human VEGF, VEGF R2 and GAPDH were as