E1A stimulates FGF-2 release promoting differentiation of primary endothelial cells

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Abstract

Basic Fibroblast Growth Factor (FGF-2) is a growth and survival factor and represents one of the most potent differentiation agents of vascular system. In the present study we describe that adenoviral oncoprotein E1A regulates FGF-2 production and determines the acquisition of a pro-angiogenic phenotype in primary bovine aortic endothelial cells (BAEC). Following their transfection, wild type E1A proteins 12S and 13S (wtE1A) stimulated BAEC to differentiate on reconstituted basement membrane matrix (Matrigel). This outcome was paralleled by invasion and migration enhancement in wtE1A-transfected cells. This stimulating effect was absent with the E1A mutant dl646N. Accordingly, zymography and RT-PCR analyses showed that matrix metalloproteinase-9 protein- and mRNA-levels increased following wtE1A transfection. Interestingly, wtE1A-transfected BAEC showed FGF-2 mRNA- and protein-levels higher than controls. Further, FGF-2 neutralization reduced the amount of MMP-9 released in the supernatant of E1A-transfected cells and strongly inhibited BAEC differentiation, thus suggesting that wtE1A activates BAEC by a mechanism, at least partially, dependent on a FGF-2 autocrine/paracrine loop. *Cell Death and Differentiation* (2000) 7, 292–301.

Keywords: angiogenesis; E1A; FGF-2; matrix metalloproteinase; endothelial cell; differentiation

Abbreviations: BAEC, primary bovine aortic endothelial cells; TS, tubular structures; CM, conditioned medium; EC, endothelial cells; E1A, product of early region 1 of adenovirus genome; FGF-2, basic

fibroblast growth factor; MMP-9, matrix metalloproteinase 9; R, aminoacid residues; wtE1A, wild type E1A 12S and wild type E1A 13S; LMW, low molecular weight; HMW, high molecular weight; DMEM, Dulbecco's modified Eagle's medium

Introduction

Differentiation of vascular cells in new blood vessels, or angiogenesis, occurs in physiological conditions such as the menstrual cycle and pregnancy as well as during pathological processes including growth of primary tumors and metastasis, wound healing and ischemia. Angiogenesis proceeds through a series of steps schematically summarized as follows: (a) degradation of extracellular matrix, (b) endothelial cells (EC) migration, (c) invasion and proliferation and, finally, (d) re-establishment of cell-to-cell contacts and lumen formation.^{1–3}

This process is modulated by a balance of positive and negative stimuli provided by growth factors and by EC interaction with extracellular matrix components.^{4–8} Local production of angiogenic factors including vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs) and platelet-derived growth factors (PDGFs), have been shown to guide EC toward their organization in new vascular structures.^{9–13} The FGF-2 member of the FGF family is known as a potent growth, survival and differentiation factor; it exists in five distinct isoforms: the low molecular weight 18 kDa (LMW) and the high molecular weight 22, 22.5, 24 and 34 kDa (HMW).^{14–16} The LMW isoform is the most abundant secreted type of FGF-2 and acts through a specific tyrosine kinase receptor binding and internalization process.^{17–21} FGF-2 lacks a signal peptide and is exported outside the cell via a poorly characterized alternative pathway.^{22–24} FGF-2 has been shown to modulate several functions of vascular cells including survival, differentiation, migration and invasion^{25–28} but, despite the large interest, mechanisms controlling FGF-2 production need to be further clarified. Interestingly, several studies reported that the expression of some viral oncoproteins in cultured cells may stimulate their proliferation, partially mimicking growth factors activity.^{29–32} E1A, the product of the adenovirus early region 1, is multifunctional with respect to immortalizing functions, trans-activating properties of viral and cellular genes and induction of apoptosis. E1A splicing isoforms 12S and 13S, coding for the 243 aminoacid residues (R) and the 289 R proteins, respectively, contain the conserved regions named CR1 and CR2. The 46 R CR3 domain is present only in E1A 13S and it seems to be required for the transcription of early viral genes and for the transactivation of different viral and cellular promoters.^{33,34} E1A has been reported to interact with different classes of intracellular molecules interfering with their functions.^{35–40} Specifically, E1A isoforms are known to associate with cyclin A, with the product of the retinoblastoma susceptibility gene (pRb), with other members

of this family, and with the 300-kDa (p300) and 400-kDa (p400) transcriptional adapters.^{41–47} These proteins are usually inactivated or deregulated as a consequence of E1A interaction, leading to different biological effects including induction of DNA synthesis, mitosis and apoptosis.^{48,49} Since forced expression of E1A has been shown to induce growth factors release,^{50–52} we investigated whether its overexpression in bovine aortic endothelial cells (BAEC) affected their differentiation properties. Our results show that E1A 12S and 13S activate a process characterized by

enhanced differentiation into tubular structures, increased motility, invasivity and gelatin-degrading activity. Such effects were associated with increased FGF-2 production.

Results

E1A enhances BAEC differentiation on Matrigel

BAEC plated on reconstituted basement membrane matrix (Matrigel) stop their proliferation and differentiate into tubular

Figure 1 Effect of E1A on BAEC TS formation. (A) Micrographs of transfected BAEC plated on Matrigel. Mock, E1A 12S, E1A 13S and E1A dl646N-transfected BAEC were plated on Matrigel 48 h after transfection and cultured in DMEM containing 10% FCS as described in Materials and Methods. Pictures taken 3 h after plating show TS development in E1A 12S-transfected cells (panel c) and in E1A 13S-transfected cells (panel d). E1A dl646N (panel e) and mock transfected cells (panel b) did not show any TS formation at the same time point. Mock transfected cells, in the same conditions (panel a) developed TS 8 h after plating. Magnification 100 $\times$. (B) Quantification of TS formed by E1A-transfected BAEC after plating on Matrigel. Cells were seeded on Matrigel 48 h after transfection, in the presence of DMEM with 10% FCS and TS were counted by phase contrast microscopy using a 100 $\times$ magnification. TS formation, measured 3 h after plating, revealed a significant increase for E1A 12S and E1A 13S compared to mock and E1A dl646N-transfected cells ($P < 0.05$). Differences between E1A dl646N and mock transfected cells were not statistically significant. Results represent the average of four independent experiments and are expressed as mean $\pm$ S.E.

structures (TS),⁵³ partially recapitulating the process of new blood vessel formation occurring *in vivo*.⁵⁴ In order to investigate the effects of E1A on TS organization on Matrigel, BAEC were transfected with various expression vectors coding for E1A 13S, E1A 12S and the CR1 domain-deleted protein E1A dl646N. Transfection efficiency reached approximately 30% and transgene expression was confirmed by Western blot analysis 48 h after transfection (not shown). Differentiation assays on Matrigel were carried out 48 h after transfection and were performed in medium containing 10% fetal calf serum (FCS). Preliminary signs of TS formation occurred in E1A 12S and E1A 13S-transfected cells as soon as 3 h after plating (Figure 1A, panel c and d). Conversely, E1A dl646N and mock transfected cells were not organized at 3 h (Figure 1A, panels e and b) and formed TS 8 h after plating (Figure 1A, panel a). As shown in Figure 1B, TS quantification 3 h after plating revealed 4–6-fold increase for E1A 12S and E1A 13S-transfected cells, respectively, as compared to mock transfected cells cultured in the same conditions. These experiments indicated that wtE1A specifically accelerated TS formation on Matrigel *in vitro*.

E1A stimulates EC invasion and migration

Since EC organization in new vessels depends on their ability to migrate and invade extracellular matrix,⁵⁵ E1A-transfected BAEC invasion and migration were evaluated in a modified Boyden chamber assay, 48 h after transfection. Invasion assays were performed on Matrigel-coated filters and revealed that E1A 12S and E1A 13S-transfected cells invaded 51% and 40% more than control, respectively (34 cells/field in mock transfected cells on average) (Figure 2A). In contrast, the dl646N mutant did not stimulate BAEC invasion. Migration assays on uncoated filters (Figure 2B) showed that migration of E1A 12S and 13S-transfected cells was 3–3.5-fold higher than E1A dl646N or mock transfectants (45 cells/field in mock experiments on average). These results indicated that typical features of the angiogenic phenotype such as invasion and migration were specifically stimulated by wtE1A.

E1A increases FGF-2 mRNA and stimulates secretion of the 18 kDa isoform from transfected BAEC

In order to investigate whether angiogenic factors were involved in the observed modulation of EC functions, FGF-2 and VEGF release was evaluated. ELISA assays revealed a significant increase of FGF-2 levels in CMs collected from E1A 12S and E1A 13S-transfected cells compared to the CMs from mock and E1A dl646N-transfected cells. Figure 3A shows FGF-2 levels in CMs collected after 24 h incubation in serum free condition, 60 h from transfection. FGF-2 levels were 11 ± 5 and 17 ± 1 pg/ 10^5 cells/24 h in CMs from E1A 12S and E1A 13S-transfected cells, respectively, while they were markedly lower in CMs from E1A dl646N (1 ± 0.5 pg/ 10^5 cells/24 h) and mock transfected cells (0.22 ± 0.1 pg/ 10^5 cells/24 h). Conversely, ELISA assay for VEGF indicated that this factor was not induced by E1A (data not shown). Western blot analysis on CMs from E1A-transfected cells

showed that E1A 12S and E1A 13S induced the secretion of the 18 kDa FGF-2 LMW isoform (Figure 3B), while the high molecular weight (HMW) isoforms were not detected. Conversely, both LMW and HMW isoforms were present in whole cell extracts from transfected cells but they did not appear modulated (Figure 3C). Northern blot analysis indicated that in the presence of wtE1A the FGF-2 steady state mRNA level increased 3–5-fold compared to mock or dl646N-transfected cells (Figure 3D). Taken together these results indicated that wtE1A expression in BAEC modulated FGF-2 mRNA and protein production.

E1A increases MMP-9 mRNA level and protein secretion

A correlation between FGF-2 levels and production of matrix-digesting enzymes has been described in EC.⁵⁶ Therefore, it was determined whether E1A expression increased matrix

Figure 2 E1A effect on BAEC invasion/migration. (A) Quantitative evaluation of BAEC invasion through Matrigel-coated filters 48 h after E1A transfection. wtE1A-transfected cells showed invasiveness significantly higher ($P < 0.05$) than dl646N and mock transfected cells. Results represent the average of three different experiments performed in triplicate and are expressed as mean $\pm$ S.E. (B) Quantitative evaluation of BAEC migration through uncoated filters 48 h after transfection. wtE1A-transfected cells showed migration significantly higher than dl646N and mock transfected cells ($P < 0.05$). Migratory ability of E1A dl646N-transfected cells was comparable to control. Results represent the average of three different experiments in triplicate and are expressed as mean $\pm$ S.E.

Figure 3 E1A induces FGF-2 production. **(A)** ELISA measurement of FGF-2 in E1A-transfected cells CMs. The graph shows the production of FGF-2, evaluated by ELISA, detected in E1A-transfected BAEC CM. Cells were cultured in serum-free medium for 24 h and CMs were collected 60 h after transfection. CMs were concentrated tenfold and assayed for the presence of FGF-2 by ELISA. Data were normalized for cell number and show a significant increase of FGF-2 production in samples from E1A 12S and E1A 13S-transfected cells, as compared to samples obtained from mock and E1A dl646N-transfected cells ($P < 0.05$). Results represent the mean $\pm$ S.E. of three independent experiments. **(B)** Western blot analysis of FGF-2 released in CMs collected from E1A-transfected cells. E1A-transfected cells were cultured in serum-free medium for 24 h, and CMs were collected 60 h after transfection. CMs were concentrated and immediately assayed for FGF-2. Samples were normalized for cell number. The 18 kDa FGF-2 isoform was found in E1A 12S and E1A 13S-transfected cells CMs while it was absent in CMs from mock and E1A dl646N-transfected cells. The positive control corresponding to recombinant human 18 kDa FGF-2 (rhFGF-2) is also shown. **(C)** Western blot analysis of FGF-2 in whole cell extracts from E1A-transfected BAEC. Each lane was loaded with lysate from 10^5 cells, 48 h from transfection. The HMW isoforms of 22 and 24 kDa as well as the LMW isoform of 18 kDa were found in all the samples and appeared not modulated. **(D)** Northern blot analysis of FGF-2 in total RNA from E1A-transfected cells. Twenty micrograms of RNA were loaded in each lane. A single FGF-2 mRNA band (6.7 Kb) was revealed in all samples and it appeared markedly increased in E1A 12S and E1A 13S-transfected cells if compared to E1A dl646N and mock transfected cells. Ribosomal RNAs 28S and 18S are also shown

metalloproteinases production. CMs were collected after 24 h incubation in serum-free medium, 60 h after transfection and gelatin-degrading proteinase activity was assayed. The zymographic analysis showed the presence of matrix metalloproteinase 9 (MMP-9) in CMs collected from E1A 13S and E1A 12S-transfected cells while MMP-9 was absent in both E1A dl646N and mock transfected cells (Figure 4A). Densitometric analysis also indicated that wtE1A transfection

increased MMP-2 levels approximately 30% as compared to both E1A dl646N and mock transfected cells (not shown). To further investigate E1A regulation of MMP-9 production, RT-PCR was performed to evaluate the mRNA expression (Figure 4B). Bovine MMP-9 mRNA was induced in BAEC transfected with E1A 12S and E1A 13S whereas MMP-9 mRNA was barely detectable in E1A dl646N and absent in mock transfected cells. Finally, the effect of anti-FGF-2

Figure 4 E1A effect on BAEC MMP-9 production. **(A)** Zymography assay. E1A-transfected cells were cultured in serum free medium for 24 h and CMs were collected 60 h after transfection. CMs were then immediately assayed for the presence of matrix metalloproteinases by zymography. Samples were normalized for cell number. MMP-2 gelatin degrading activity was found in all samples tested while MMP-9 gelatin degrading activity was present only in samples collected from E1A 13S and E1A 12S-transfected cells as well as in samples from HT1080 cells, used as positive control. A representative example of four different experiments is shown. **(B)** RT-PCR analysis. MMP-9 mRNA levels were evaluated by RT-PCR analysis using total RNA extracted from BAEC 48 h after E1A transfection. MMP-9 mRNA was induced by transfection with E1A 12S and E1A 13S while it was barely detectable in E1A dl646N and in mock transfected cells. The amount of loaded mRNA was similar in all lanes as indicated by β -actin co-amplification. A similar result was obtained in three independent experiments. **(C)** Zymography analysis. The picture shows a zymography performed as in **(A)** analysing CMs from E1A 13S and mock transfected cells exposed to control antibodies (IgG) or neutralizing anti-FGF-2 antibodies during the 24 h incubation in serum free medium. A reduction of about 50% of MMP-9 produced by E1A 13S-transfected cells was detected in the presence of anti-FGF-2 antibodies

neutralizing antibodies or control antibodies was analyzed in a series of zymographic assays. Figure 4C shows that wtE1A-transfected cells, in the presence of neutralizing anti-FGF-2 antibodies, produced markedly less MMP-9 (about 50% less) as compared to wtE1A-transfected cells in the presence of control antibodies. These results indicated that E1A expression increased MMP-9 protein and mRNA levels. This process might be at least in part dependent on biologically active FGF-2.

E1A induction of BAEC differentiation depends on secreted FGF-2

In these experiments it was examined whether the enhanced BAEC organization on Matrigel following E1A 13S-transfection was FGF-2 dependent. Neutralizing anti-FGF-2 monoclonal antibodies were added to wtE1A-transfected cells plated on Matrigel. Figure 5 shows pictures taken 24 h after plating on Matrigel in the presence of medium supplemented with 1% serum, a condition *per se* not efficiently supporting EC differentiation in our experimental conditions. TS formation in wtE1A-

transfected cells was evident in the presence of control antibodies (Figure 5b) while it was completely inhibited by addition of neutralizing anti-FGF-2 antibodies (Figure 5e). Mock transfected cells differentiated poorly in 1% FCS alone (not shown) or in the presence of control antibodies (Figure 5d). TS formation in 1% serum and 10 ng/ml recombinant FGF-2 is shown for mock transfected cells (Figure 5c) and it was completely inhibited in the presence of neutralizing anti-FGF-2 antibodies (Figure 5f). As control, TS formation in 10% serum for mock transfected cells is also shown (Figure 5a). These results supported the hypothesis that wtE1A-induced EC differentiation depends on biologically active FGF-2.

Discussion

A large number of secretory, membrane-bound and intracellular molecules regulate EC response to a variety of angiogenic stimuli.⁵⁷ Interestingly, several oncogenes were found to stimulate angiogenic factors production from transformed cells^{58,59} or to directly activate EC,^{60,61} indicating that multiple signals may lead to their differentia-

Figure 5 Neutralizing anti-FGF-2 antibodies inhibit E1A-induced BAEC differentiation on Matrigel. Transfected BAEC were plated on Matrigel 48 h after transfection, cultured in DMEM 1% FCS and pictures were taken 24 h after plating. A network of TS was evident in E1A 13S-transfected cells plated with control antibodies (panel b), while it was abolished in the presence of neutralizing anti-FGF-2 antibodies (panel e). Mock transfected cells differentiated poorly in the presence of control antibodies (panel d). Mock transfected cells in the presence of 10 ng/ml recombinant FGF-2 (panel c) or 10 ng/ml recombinant FGF-2 and neutralizing anti-FGF-2 antibodies (panel f) are shown, indicating that the specific antibodies inhibit TS induced by recombinant FGF-2. Mock transfected cells cultured in 10% FCS, as positive control of TS formation, are also shown (panel a). Pictures were taken at 50 $\times$ magnification. Similar results were obtained in three independent experiments

tion. To investigate further the mechanisms of EC activation, adenovirus wtE1A proteins and the non-functional mutant dl646N were transiently transfected in primary BAEC. Wild type E1A-transfected EC differentiated in TS markedly faster than controls (3 vs 8 h shown in Figure 1). Interestingly, wtE1A overexpression increased FGF-2 and MMP-9 mRNA and protein levels (Figures 3 and 4) and significantly stimulated BAEC migratory and invasive capacity (Figure 2). Therefore we speculated that activation of an autocrine/paracrine growth factor-dependent loop might underlie the observed biological effects, as a consequence of FGF-2 induction. FGF-2 is known to stimulate secretion of matrix degrading proteins in both endothelial and smooth muscle cells^{62,63} and this may explain the MMP-9 increase observed in wtE1A-transfected cells. In fact, anti-FGF-2 neutralizing antibodies reduced the amount of MMP-9 released in the CM of transfected BAEC (Figure 4C) however they did not influence the migration/invasion capacity of transfected cells (not shown). This result is in agreement with the evidence that the intracellular FGF-2, not sensitive to neutralizing antibodies present in culture medium, plays a role in the regulation of the migration/invasion process.^{64,65} E1A overexpression increased the steady-state level of FGF-2 mRNA (Figure 3D) thus indicating the possible activation of either RNA transcription and/or stabilization. Accordingly, Western blot analysis indicated that the secretory 18 kDa FGF-2 isoform is increased in the CMs of wtE1A-transfected cells (Figure 3B). In the same conditions, the intracellular content of LMW or HMW FGF-2 isoforms was not significantly

changed (Figure 3C), thus indicating that, besides the increased mRNA levels, the release or mobilization of the 18 kDa protein might be specifically stimulated by E1A. In agreement with this hypothesis, Barillari *et al.* recently described that a different viral protein, the HIV product TAT, facilitates the mobilization of membrane-bound FGF-2 in endothelial cells.⁶⁶ While the molecular mechanisms remain to be elucidated, taken together, our observations suggest that forced expression of E1A modulates FGF-2 expression at multiple levels.

E1A is known to mimic growth factors in different cell types stimulating proliferation in the absence of serum or inducing protection from apoptosis under certain conditions.^{50,52,67} Remarkably, the E1A functional homolog SV40 large T antigen has been shown to modulate intracellular signaling by directly interacting with the universal tyrosine kinase substrate protein *shc*,³² thus reproducing a growth factor-dependent effect targeting proliferating stimuli to the nucleus. E1A and large T antigen are known to recognize common intracellular targets.^{68–71} Therefore, although direct evidences are lacking, we speculated that E1A, similarly to large T antigen, might interact with some signaling-intervening molecules, to achieve EC activation and FGF-2 production. Further experiments are now in progress to elucidate this point.

To assess the biological relevance of FGF-2 production, transfected cells were plated on Matrigel in the presence of anti-FGF-2 antibodies and complete inhibition of TS formation was achieved (Figure 5). Thus, we concluded

that release of FGF-2 plays a relevant role in the EC differentiation induced by wtE1A, while an additional intracrine effect of FGF-2 can not be excluded. Other studies have shown that E1A-immortalized or transformed endothelial cells still required exogenous FGF-2 to proliferate and/or differentiate.^{72,73} Results of those studies are not comparable with the current study since: (a) cells were from microvascular endothelium; (b) cells were evaluated only after a long period of selection at the end of which, at least in one case,⁷² E1A expression could not be detectable any further; (c) all assays were performed on immortalized or transformed cell lines; and (d) the production of endogenous FGF-2 was not tested.

A large body of literature describes the role that growth factors and growth factor-receptors play in endothelial cell growth and differentiation. The present work may expand the knowledge about the role of oncogenes in this field. Unraveling the role of oncogenes in angiogenesis may contribute to design more efficient anti-angiogenic and anti-tumor therapeutic strategies.

Materials and Methods

Cell isolation and culture

Thoracic aortas of young calves were removed aseptically at the abattoir and stored on ice in phosphate buffered saline (PBS; Gibco BRL, Paisley, Scotland) with penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml). The aortas were then washed with Hanks' balanced salt solution (Hyclone Inc., Logan, UT, USA), opened longitudinally and incubated in Hanks' solution containing collagenase II (Sigma Chemical Co, St Louis, MO, USA) at 37°C. After 30 min BAEC were removed by scraper and resuspended in fresh Hanks' solution, then resuspended in Dulbecco's modified Eagle's medium (DMEM; Hyclone Inc.) containing 20% fetal calf serum (FCS; Hyclone Inc.), and finally plated. BAEC were grown in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.01 mg/ml glutamine and maintained at 5% CO₂ in an incubator at 37°C. All experiments were performed with BAEC at passage between 2 and 6. Purity of the cell population was assessed as previously described.⁷⁴ Cells were incubated in fresh culture medium containing acetylated low density lipoprotein labeled with 10 µg/ml of 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (Biomedical Technologies Inc., Stoughton, MA, USA). After 4 h cells were washed and positive cells were counted by fluorescence microscopy. Purity was always ≥95%.

Plasmids and transfections

Expression vectors for E1A 12S and E1A 13S and the E1A mutant dl646N, previously described,^{75–78} were kindly provided by Dr. A Felsani (ITBM, CNR, Italy). Both pUC 19 and/or pcDNA3 were used as control plasmids (Clontech Laboratories Inc., Palo Alto, CA, USA; Invitrogen Corporation, Carlsbad, CA, USA). EC transfection was carried out on 80% confluent cells in 60-mm plastic dishes using Dotap (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's instructions. Cells (approximately 8 × 10⁵ for each dish) were incubated with 30 µg Dotap/10 µg DNA in serum-free medium overnight. Then the medium was replaced with fresh culture medium. Transfection efficiency was evaluated by X-Gal staining for each experiment and was always ≥30%.

Differentiation assay

Two hundred microliters of Matrigel (Collaborative Research, Inc., Waltham, MA, USA) (12 mg/ml) were applied onto 24-well culture plates and incubated at 37°C for 60 min. Cells, harvested by trypsin treatment 48 h after transfection, were seeded (8 × 10⁴ transfected cells in 1 ml fresh culture medium) on Matrigel-coated wells. The phenotypic changes of EC and the appearance of TS were monitored 0, 2, 3, 4, 5, 6, 8 and 24 h after plating by phase contrast microscopy. TS were counted manually under a 100 × magnification.

Goat neutralizing anti-FGF-2 antibody (R&D System Europe Ltd; Abington, UK) was added at 500 ng/ml after plating cells on Matrigel, to block FGF-2. Normal goat serum (Zymed Laboratories Inc., South San Francisco, CA, USA) was used as control. Recombinant FGF-2 (Gibco) was used at 10 ng/ml.

Migration/invasion assays

Migration/invasion assays were performed 48 h after transfection by the modified Boyden chamber assay.⁷⁹ For migration assay, uncoated polycarbonate filters (12 µm pore diameter; Nucleopore Costar Scientific Corporation, Cambridge, MA, USA) were used. For invasion assay, filters were coated with 50 µl of Matrigel (0.3 mg/ml). Boyden chambers were assembled by adding DMEM 1% FCS to the lower chamber as chemoattractant. BAEC (2 × 10⁵) in DMEM containing 0.1% bovine serum albumin (BSA) were added to the upper chamber in a final volume of 850 µl. Boyden chambers were then incubated for 5 h at 37°C in a 5% CO₂ atmosphere and cells were counted at the optical microscope. Ten fields in each filter were evaluated at 400 × magnification and all experiments were run in triplicate.

ELISA test

ELISA Kits (Quantikine, R&D Import Export, Minneapolis, MN, USA) were used to measure FGF-2 and VEGF levels in cell culture supernatant, 48 h after transfection, according to the instructions. Supernatants were concentrated ten times by concentrators (Centricon tubes, Amicon Inc., Beverly, MA, USA) with a 1 × 10⁴ Dalton molecular weight cut-off.

Western blotting

BAEC were washed 36 h after transfection and incubated for 24 h with serum-free medium. Conditioned media were collected and tenfold concentrated in Centricon tubes. Total cell lysates were prepared 48 h after transfection by lysing and scraping cells off the culture plate with 10 mM Tris-HCl, pH 6.8, 0.4 mM EDTA, 2% SDS, leupeptin, aprotinin and antipain (10 µg/ml each); 1 mM phenylmethylsulfonyl fluoride and the following phosphatase inhibitors: 10 mM sodium fluoride, 0.4 mM sodium orthovanadate and 10 mM pyrophosphate. Both conditioned media and cell lysates were electrophoresed in SDS-15% polyacrylamide minigel under reducing conditions. Gels were then blotted to a polyvinylidene difluoride membrane (Immobilon P; Millipore Corp., Waters Chromatography, Bedford, MA, USA) for 1.5 h at 250 mA. The membrane was then incubated with 5% skim milk (Carnation; Nestlé Food Co., Glendale, CA, USA) in PBS with 0.1% Tween (J.T. Baker, Milano, Italy) for 1 h. FGF-2 was detected by incubating the membrane with 0.2 µg/ml of antibody anti-human FGF-2 (anti FGF-2; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Human recombinant FGF-2 (Life Technologies, Milano, Italy), loaded in the same minigel, was used as positive control. After incubation with horseradish peroxidase conjugated donkey anti-rabbit IgG (1:10000) for 1 h (Amersham Life

Science, Buckinghamshire, UK), immune complexes were detected with the chemiluminescence ECL detection system (Amersham Life Science) by exposure to autoradiographic films (Sigma Aldrich s.r.l., Milano, Italy).

Northern blotting

Total RNA was isolated using TRIZOL Reagent (Life Technologies). Samples were processed following the manufacturer's protocol. Gels were blotted to a neutral nylon transfer membrane (Schleicher & Schuell, Keene, NH, USA). Northern-blot was prehybridized in hybridization buffer containing 50% formamide (Sigma Aldrich), 10% dextran sulfate (Sigma Aldrich), 1% SDS (Sigma Aldrich), 1.2 M NaCl (Sigma Aldrich), sonicated salmon sperm (Stratagene, La Jolla, CA, USA), at 42°C for 4 h. Hybridization was performed overnight in prehybridization buffer containing 10^6 c.p.m./ml of 32 P-labeled full-length cDNA probe encoding human FGF-2 (kindly provided by Professor M Presta). Filter was first washed in $2 \times$ SSC 0.1% SDS at room temperature, then in $0.5 \times$ SSC 0.1% SDS at 50°C, $0.2 \times$ SSC 0.1% SDS at 50°C, $0.1 \times$ SSC 0.1% SDS at 65°C. Filters were exposed on Kodak Biomax MS Films.

Zymography

Cells were incubated for 24 h in serum-free medium and collected 60 h after transfection. Gelatin-degrading proteinases secreted into the culture medium by EC were analyzed by non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 9% gels containing 0.1% (wt/vol) porcine gelatin (Sigma Chemical Co). After electrophoresis, gelatinases were renatured by incubation at room temperature for 1 h in 2.5% (vol/vol) Triton X-100 and then at 37°C for 18 h in 50 mM Tris-HCl (pH 7.5) containing 0.2 M NaCl, 5 mM CaCl_2 and 0.02% Brij 35 (wt/vol; Sigma Chemical Co). Gels were stained for 2 h with 0.5% Coomassie brilliant blue G-250 (Sigma Chemical Co) and destained for 1 h in 30% methanol, 10% acetic acid. Active metalloproteinases (MMPs) degraded gelatin giving white bands against the blue background. Computer assisted densitometric analysis was performed using Gel Doc 1000 apparatus and Molecular Analyst Software (Bio-Rad, Hercules, CA, USA). Goat neutralizing anti-FGF-2 antibody (R&D System Europe Ltd) or normal goat serum (Zymed) were added at 500 ng/ml, during the 24 h incubation period in serum free medium.

RNA isolation, reverse transcription (RT), PCR and Northern analyses

Total RNA was isolated from 10^6 cells 48 h after transfection using the High pure RNA isolation Kit (Boehringer Mannheim) and was quantified by measuring absorbance at 260/280 nm. RT-PCR was conducted with RT-PCR kit (Stratagene, La Jolla, CA, USA) to detect MMP-9 mRNA. Primers specific for the bovine MMP-9 gene sequence (GENE BANK accession number X78324) were designed. The forward primer sequence consisted of 5' ACC ACT ACC ACC ACC GAA CC 3' while the reverse primer sequence was 5' CGC CCA GAG AAG AAG AAA AT 3; they were synthesized to amplify the MMP-9 gene from nucleotide 1378 to nucleotide 1756. The synthesis of the first-strand cDNA with reverse transcriptase was performed using oligo dt primers. The amplification of the first-strand cDNA using the polymerase chain reaction was carried out with 1 unit Taq DNA polymerase (Perkin Helmer, Foster City, CA, USA) and PCR buffer (Perkin Helmer) containing 1.5 mM MgCl_2 . Each sample was amplified in a thermal cycler by 40 repeat cycles at 94°C for 2 min, 55°C for 1 min and 72°C for 1 min.

Computer assisted densitometric analysis was performed using Gel Doc 1000 apparatus and Molecular Analyst Software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

The STATVIEW SE+GRAPHICS software (ABACUS CONCEPT, Berkley, CA, USA) was used to perform statistical analyses and Anova tests. A *P* value equal or lower than 0.05 was considered significant.

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