Hypoxia of endothelial cells leads to MMP-2-dependent survival and death

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Hypoxia of endothelial cells leads to MMP-2-dependent survival and death. Am J Physiol Cell Physiol 289: C1321–C1331, 2005; doi:10.1152/ajpcell.00079.2005.—Exposure of endothelial cells (ECs) to hypoxia has separately been shown to induce their angiogenesis or death. Matrix metalloproteinase (MMP-2) is associated with EC angiogenesis, although recent studies also implicate this molecule in EC death. We studied the effect of hypoxia in the absence or presence of TNF-α (characteristic of the inflammatory microenvironment accompanying hypoxia) on MMP-2 expression and its role in angiogenesis (proliferation, migration, and tube formation) and in the death of primary human umbilical vein endothelial cells (HUVECs). Hypoxia alone (24–48 h in 0.3% O2 in the hypoxic chamber) and furthermore, when combined with TNF-α, significantly enhanced MMP-2 expression and activity. Hypoxia also led to a reduction in membrane type 1 MMP (MT1-MMP) and tissue inhibitor of metalloproteinase-2 mRNA and protein while enhancing the expression of αβ3 integrin and the cytoskeletal protein phosphopaxillin. Moreover, hypoxia led to colocalization of αβ3 and MMP-2, but not MT1-MMP, with phosphopaxillin in ECs. These results suggest MT1-MMP-independent activation of MMP-2 during hypoxia and support interactions between the ECM, integrins, and the cytoskeleton in hypoxia-induced MMP-2-related functions. Hypoxia enhanced EC migration in an MMP-2-dependent manner while leading to a reduction of cell number via their apoptosis, which was also dependent on MMP-2. In addition, hypoxia caused an aberrant tubulike formation on Matrigel that appeared to be unaffected by MMP-2. The hypoxia-induced, MMP-2-dependent migration of ECs is in accordance with the proangiogenic role ascribed to MMP-2, while the involvement of this protease in the hypoxia-related death of ECs supports an additional apoptotic role for this protease. Hence, in the hypoxic microenvironment, MMP-2 appears to have a dual autocrine role in determining the fate of ECs.

gelatinase activity; angiogenesis; apoptosis; tumor necrosis factor-α

Tissue hypoxia, a reduction in Po2, occurs in both physiological and pathological conditions (21). Endothelial cells (ECs) lining the blood vessels respond to hypoxia in two apparently opposing directions: the promotion of angiogenesis, whereby the supply of nutrients and O2 to the tissues is restored or, alternatively, the induction of EC death. Initiation of these pathways may depend on the severity and duration of the primary hypoxic insult and interacting environmental factors (15, 34, 42, 47). Crucial to both the angiogenesis and cell death processes is the focal loosening of cell-cell interactions and their anchorage, as well as the degradation of the basement membrane (BM) and the ECM surrounding the blood vessels (43, 35). Members of the matrix metalloproteinase (MMP) family are pivotal enzymes that participate in the degradation processes, leading to the logical assumption that they are involved in both cell survival and death pathways.

The gelatinase subgroup of MMPs, consisting of MMP-2 and MMP-9, as well as the membrane-type MMPs (MT-MMPs), have been strongly implicated in different stages of the angiogenic process (19). Latent MMP-2 is generally secreted constitutively and undergoes membranal activation mainly through interactions with MT-MMPs, in particular MT1-MMP, and the αβ3 integrin (the vitronectin receptor) (7, 11, 46). Low levels of tissue inhibitor of metalloproteinase (TIMP)-2, the main MMP-2 inhibitor, partake in MT1-MMP-mediated activation of MMP-2 (23), while higher levels of TIMP-2 may block the MT1-MMP-mediated activation of MMP-2 and also directly bind to the active form of MMP-2. The production and activity of MMP-2 appears to be cell and stimulus specific.

The hypoxic environment is a well-known inducer of angiogenesis via upregulation of VEGF; however, the mechanisms involved are still poorly understood (36). TNF-α, the proinflammatory cytokine, produced in the hypoxic microenvironment (30), has independently been reported to promote EC angiogenesis or apoptotic death (35). To the best of our knowledge, despite the reported correlation between the level of MMP-2 activity to accelerated tumor angiogenesis and dissemination as well as to diabetic vascular pathology (12, 43), there is scarce information on the functional involvement of MMP-2 in hypoxia-induced angiogenesis or death of ECs.

Our previous studies using a human EC line (EAhy926) demonstrated that hypoxia upregulates MMP-2 mRNA and protein expression, concomitantly with reduced MT1-MMP and TIMP-2 mRNA expression (3). The hypothesis examined in the present study was that the survival and death pathways of ECs initiated by hypoxia occur in the same cultures and that the MMP-2 induced by hypoxic ECs has an important role in both processes. We particularly examined the effects of hypoxia, or hypoxia within an inflammatory microenvironment (presence of TNF-α), on MMP-2 expression by HUVECs and determined its autocrine participation in crucial stages of angiogenesis and death of ECs. The hypoxia-induced modulation of additional molecules involved in MMP-2 activity, such as MT1-MMP, TIMP-2, αβ3, and paxillin, the cytoskeleton adaptor protein, also was examined.

MATERIALS AND METHODS

Cell Cultures and Hypoxic Conditions

HUVECs were isolated from umbilical cords according to the method described previously by Jaffe et al. (24) and were cultured in...
medium-199 supplemented with 15% FCS (Biological Industries, Beit Haemek, Israel), 50 μg/ml EC growth supplement (Biomedical Technologies, Stoughton, MA), and 5 U/ml heparin (Kimada, Beit Kama, Israel) on 10 μg/ml fibronectin-coated dishes. Umbilical cords were received from different donors, and cells were harvested, pooled, and used between passages 2 and 5. The Ethical Committee of the Carmel Medical Center approved the study. During experiments, HUVECs were maintained in serum-free medium (Bio-MP1; Biological Industries) with the addition of 0.1% BSA. Trypan blue exclusion demonstrated >95% viability of ECs in culture. The following nontoxic concentrations were added to cultures (evaluated by performing an XTT assay; see below): recombinant VEGF (25 ng/ml), recombinant TIMP-2 (1 μg/ml), caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK, 100 μmol/l; R & D Systems, Minneapolis, MN), MMP-2 inhibitor I (OA-Hy, 25 μmol/l; Ref. 14) and MMP-2/9 inhibitor II (CTT, 100 μmol/l; Ref. 26) (Calbiochem, Darmstadt, Germany), phorbol 12-myristate 13-acetate (PMA, 25 ng/ml; Sigma, St. Louis, MO), and TUNEL (0.1–10 ng/ml; R&D Systems).

ECs were cultured in hypoxic conditions in a sealed chamber (Ruskin Technologies, Guiseley, UK) in which the gas mixture was composed of 95% N2-5% CO2-0.3% O2 with 95% viability of ECs in culture. The following nonradioactive kits (SuperArray Bioscience, Frederick, MD) enabling identification of MMPs and TIMPs and separately for caspase mRNA profiles were used according to the manufacturer’s instructions. Briefly, biotinylated cDNA probes were prepared by reverse transcribing sample RNA (10 μg) in mixtures containing biotinylated dUTP. Membranes were then exposed to the biotinylated cDNA and, after overnight hybridization, to streptavidin-conjugated alkaline phosphatase, followed by the chemiluminescent substrate CDP Star. The OD of specific gene expression was determined on film and compared with β-actin. β-actin was chosen as the reference gene because it is supposedly affected less by hypoxia (49).

Semiquantitative PCR. Semiquantitation of MMP-2 mRNA was performed as previously described (15) using parallel amplification of MMP-2 and β-actin. The integrity of the RNA was assessed by amplifying 1 μl of cDNA using β-actin-specific primers (sense, 5’-CATTGTAGGATCCCGGAGCCG-3’; antisense, 5’-CATCTCTCTGGAGTCTGAAGGAGC-3’). PCR products were electrophoresed and visualized in ethidium bromide-stained agarose gels (Sigma) Three serial dilutions of cDNA (determined empirically in preliminary experiments) were included in each assay to ensure PCR performance in the exponential linear phase of amplification. The OD of PCR products was measured using computerized densitometry (Percy), and the relative expression of MMP-2 to β-actin products was calculated.

TIMP-2 ELISA. A commercial TIMP-2 ELISA kit (Qunatikine: R & D Systems) was used according to the manufacturer’s instructions to evaluate the level of TIMP-2 in supernatants from HUVEC cultures.

Evaluation of mRNA Expression

RNA preparation. Total cellular RNA was extracted from HUVECs (cultured in 10-cm petri dishes) using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. Precipitated RNA samples were treated with DNase I (Pharmacia, Uppsala, Sweden) to remove residual DNA, and enzyme activity was removed using guanidine-thiocyanate extraction. RNA quantity and quality were determined using a spectrophotometer at 260/280 nm emission/excitation.

Mini-gene array. Commercially available, nonradioactive GE array kits (SuperArray Bioscience, Frederick, MD) were used to perform parallel amplification of MMP-2 and β-actin. The integrity of the RNA was assessed by amplifying 1 μl of cDNA using β-actin-specific primers (sense, 5’-CATTGTAGGATCCCGGAGCCG-3’; antisense, 5’-CATCTCTCTGGAGTCTGAAGGAGC-3’). PCR products were electrophoresed and visualized in ethidium bromide-stained agarose gels (Sigma) Three serial dilutions of cDNA (determined empirically in preliminary experiments) were included in each assay to ensure PCR performance in the exponential linear phase of amplification. The OD of PCR products was measured using computerized densitometry (Percy), and the relative expression of MMP-2 to β-actin products was calculated.

Zymography

Superantitins from cells (2 × 10⁴/well) cultured in serum-free medium in microtiter wells were harvested, and the volume proportional to the number of viable cells (determined using XTT assay) were loaded onto gels for the evaluation of MMP-2 and MMP-9 levels as previously described (3). All samples from the same experiment were run on the same gel. Computerized densitometry (BioImaging Gel Documentation System, Dorno and Rhenium, Jerusalem, Israel; and TINA Software, Raytest, Straubenhardt, Germany) was performed to determine the relative OD of digested zones. The results demonstrate the relative activity, which represents the ratio between the activity of a specific sample to the activity of nontreated cells cultured in normoxic conditions.

Viability, Proliferation, and Apoptosis Assays

The number of cells (2 × 10⁴/microtiter well) was determined using an XTT commercial assay [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxyanilide sodium salt; Biological Industries] according to the manufacturer’s instructions. After 2 h, incubation at 37°C optical density (OD) was determined. A cytostatic (2 × 10⁴/ml) was assessed by performing overnight pulsing with [³H]thymidine (Amersham, Piscataway, NJ) (3). The results relative to nontreated cells cultured in normoxic conditions are presented herein.

To distinguish between necrotic and apoptotic death of HUVECs, a commercial ELISA kit (Roche Applied Science, Mannheim, Germany) that detects histone-associated DNA fragments, both cytosolic (apoptosis) and those released into supernatant (necrosis), was used according to the manufacturer’s instructions. Triplicate wells containing 2 × 10⁴ cells/microtiter well were seeded for each sample, and experiments were repeated at least three times.

Apoptosis of the adherent and nonadherent cells (cultured in 6-well plates) was further assessed by performing a flow cytometric terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays (Roche Applied Science) according to the manufacturer’s protocol. Samples (10⁴ cells) were analyzed using flow cytometry (Epics XL/MCL; Beckman Coulter, Miami, FL), and the percentage of TUNEL-positive (i.e., apoptotic) ECs was determined.

Immunofluorescence: Flow Cytometric Analysis and Confocal Microscopy

For flow cytometric evaluation, HUVECs were detached with a cell scraper after addition of cold PBS (pH 7.4) containing EDTA. Membrane-bound MTI-MMP and α,ββ were determined using indirect labeling with mouse anti-MTI-MMP (Calbiochem, San Diego, CA) or α,ββ monoclonal antibodies (Chemicon International, Temecula, CA). Cells washed with PBS/FCS were exposed to phycoerythrin-conjugated goat Fab anti-mouse IgG (Chemicon International). The fluorescence level of 10⁴ cells was evaluated using flow cytometry (Coulter).

For confocal microscopy, HUVECs (2 × 10⁵ cells) cultured on coverslips were fixed with 3.7% paraformaldehyde and membranes were permeabilized with 0.2% Triton X-100. Nonspecific binding sites were blocked with 7% normal goat serum, and cells were labeled with rabbit anti-phosphopaxillin and mouse anti-MTI-MMP, MMP-2, or MTI-αβ monoclonal antibodies. After washes, secondary antibodies anti-rabbit-FITC and anti-mouse rhodamine were added. Isotype controls or secondary antibodies alone served as controls. Coverslips were mounted after the addition of fluoromount G. Serial images were acquired using the MRC 1000 confocal system (Bio-Rad Laboratories, Hercules, CA).

In Vitro Wound Migration Assay

HUVEC monolayers (0.5 × 10⁶ cells) in six-well dishes were wounded with a scratch after overnight incubation, and the line of injury was marked. Detached cells were removed by washing with
PBS and medium without or with additives. At the end of the incubation period, cells were fixed with 4% paraformaldehyde and the number of cells that had migrated past the line of injury were microscopically counted (×100 magnification) in two or three randomly chosen fields for each duplicate treatment and assay was repeated three times.

**In Vitro Tube Formation**

Induction of capillary-like formation was performed using the Matrigel model (17, 39, 48) with reduced growth factor BM extract (50 µl; R & D Systems) in microtiter plates. Because tube formation was observed within <1 h, plates with medium were preincubated in a normoxic or a hypoxic environment for 2 h to attain required O₂ levels. HUVECs (2 × 10⁴ cells/well, with the number of cells having been optimized in preliminary experiments) were then seeded in the different environmental conditions. FCS (2%) was added to HUVEC cultures in this particular assay. At the termination of incubation, cells were fixed and digitized images were obtained. The number of formed lumina per microscopic field (×40 magnification) was assessed in two separate fields. Experiments were performed in triplicate and repeated three times.

**Statistical Evaluation**

Each experiment was repeated three to five times with good agreement found between the results of individual experiments. Data shown are means ± SE. The statistical significance of the results was determined using Student’s t-test when comparing individual treatments against controls and using ANOVA to determine differences within a treated group. Further comparisons within a group were determined using Tukey-Kramer analysis. P < 0.05 was considered significant.

**RESULTS**

**Hypoxia Enhances MMP-2 but Reduces MT1-MMP and TIMP-2 mRNA Expression in HUVECs**

In the present study, we have examined the mRNA expression of MMP-2, MT1-MMP, and TIMP-2 in ECs exposed to hypoxia. Our preliminary results revealed hypoxia-mediated modulation of MMPs and TIMPs already at 24 h of hypoxia (data not shown), which became more pronounced at 48 h. The results of the GE assay demonstrated that exposure of HUVECs to 48-h hypoxia compared with cells cultured under normoxic conditions led to a 50% increase in MMP-2 mRNA, accompanied by mRNA decreases of 45% in MT1-MMP and 17% in TIMP-2 (Fig. 1). The mRNA expression of the additional gelatinase MMP-9 was barely detected in both normoxic and hypoxic conditions. The results also demonstrated the abundance of MMP-2 mRNA expression in ECs compared with the mRNA levels of MT1-MMP and TIMP-2.

**TNF-α Enhances the Hypoxia-Induced Uptregulation of MMP-2 Protein but Not Its mRNA Expression**

The zymography assay demonstrated hypoxia-mediated enhanced expression of pro-MMP-2 protein in accordance with the elevation of its mRNA expression. Hypoxic to normoxic cultured cells showed a 40% increase at 24 h (P = 0.005) and a 100% increase at 48 h (P < 0.001) (Fig. 2). Although the active form of MMP-2 was observed in zymography gels after 24-h culture in hypoxic conditions, its level was too low to allow for quantification using densitometry at this time point. After 48 h, the level of the MMP-2 active form was higher in hypoxic than in normoxic cultures (P = 0.002).

The addition of TNF-α (0.1–1 ng/ml) to HUVEC cultures for 24 h enhanced the level of latent MMP-2, and at 48 h, it induced a dose-dependent elevation of MMP-2 latent protein in normoxic (P < 0.003; ANOVA) as well as in hypoxic EC cultures (P ≤ 0.002; ANOVA) (Fig. 2, A and C). The highest concentration of TNF-α (10 ng/ml) led to a 4.2-fold elevation of latent MMP-2 protein in hypoxic (48 h) compared with nontreated normoxic culture (P < 0.001). In addition, TNF-α led to a time- and dose-dependent increase in the MMP-2 active form that was more prominent in hypoxic than in normoxic culture at 48 h (P < 0.0001; ANOVA) (Fig. 2D). Low MMP-9 gelatinase activity was observed in HUVEC supernatants from a few batches of cells after the addition of TNF-α (1 ng/ml).

Similar to the gene-array results, semiquantitative RT-PCR analysis demonstrated that hypoxia (at 24 and 48 h) significantly upregulated MMP-2 mRNA (Fig. 2E). However, the addition of TNF-α to both normoxic and hypoxic HUVEC cultures (6–48 h) did not further alter MMP-2 mRNA levels.

**Hypoxia Reduces MT1-MMP and TIMP-2 While Enhancing αvβ3 Protein Expression**

ELISA demonstrated consecutive secretion of TIMP-2 in supernatants (Fig. 3A). Hypoxia of ECs resulted in the gradual decrease (data not shown) of TIMP-2 levels, which at 48 h had decreased by 35% compared with the levels in normoxic cultures (P < 0.001).

Flow cytometric analysis was used to assess the effects of hypoxia and TNF-α on the expression of membrane MT1-MMP and αvβ3 expression (Fig. 3, B–E). Because MT1-MMP expression by HUVECs was found to be low, PMA was added to cultures as a positive control to demonstrate possible changes of EC MT1-MMP expression (Fig. 3C). Hypoxia (24 h) led to a significant (25%; P < 0.05) reduction in the level of MT1-MMP compared with its level in ECs cultured under normoxic conditions. In contrast, exposure of HUVECs to hypoxia led to 40% (P < 0.01) enhancement in the level of αvβ3 expression compared with its level in normoxic cultures. TNF-α (1 ng/ml) had no additional effect on the levels of either MT1-MMP or αvβ3 expressed by ECs.
MMP-2 is involved in hypoxia-induced migration of ECs. We examined the secretion of VEGF by cultured HUVECs and found that hypoxia significantly upregulated its level (mean ± SE: 32 ± 4.1 pg/ml in supernatants from hypoxic cultures and 4.5 ± 1.09 pg/ml in normoxic cultures). In addition, a threefold increase in VEGF mRNA level was observed in hypoxic ECs compared with normoxic ECs as evaluated in a GE assay. The in vitro addition of exogenous VEGF to normoxic cultures (positive control) demonstrated the capacity of HUVECs to migrate (Fig. 4). Hypoxia (24 h) compared with normoxia significantly enhanced the number of migrating HUVECs 2.9-fold (*P < 0.001). The addition of VEGF or TNF-α to hypoxic cultures did not further enhance the number of migrating cells (data not shown). The addition of the MMP-2 inhibitors OA-Hy and CTT as well as the physiological inhibitor TIMP-2 significantly decreased the number of migrating cells in hypoxic cultures compared with normoxic cultures. Higher concentrations of the inhibitor could not be used because of its cytotoxicity. The pan-caspase inhibitor Z-VAD-FMK did not alter the hypoxia-mediated decreased tubelike formation.

Hypoxia Leads to Aberrant Tubelike EC Formation In Vitro

In the in vitro Matrigel model, hypoxia led to a decrease in the number of tubelike structures formed after 24-h hypoxia compared with normoxic cultures. In hypoxic cultures, lumen formation was distorted and blunt-ended and nondifferentiated cell cumuli were observed (Fig. 5). The addition of TNF-α to both normoxic and hypoxic cultures did not significantly alter the number of lumina in either of the cultures. Similarly, the MMP-2 inhibitor OA-Hy did not affect the number of lumina formed. Higher concentrations of the inhibitor could not be used because of its cytotoxicity. The pan-caspase inhibitor Z-VAD-FMK did not alter the hypoxia-mediated decreased tubelike formation.

Hypoxia and TNF-α Modulate the Viability and Proliferation of ECs

The XTT assay that measures mitochondrial enzymatic activity and is directly proportional to cell number demonstrated that hypoxia led to a time-dependent reduction in the overall number of HUVECs. This reduction was significant (*P < 0.01) from 18 h compared with cells cultured in normoxic conditions.
The addition of TNF-α (1 ng/ml) to normoxic cultures reduced the number of ECs, while its presence in hypoxic cultures led to a further decrease in the number of cells (TNF-α plus hypoxia vs. normoxia cultured cells; \( P = 0.0001 \), ANOVA). The addition of a higher concentration of TNF-α (10 ng/ml) resulted in a more pronounced reduction in the number of cells at each of the time points (data not shown).

To evaluate whether the effect of hypoxia on the number of ECs was the result of reduced proliferation, thymidine uptake was measured. Shorter exposure to hypoxia (6 and 18 h) led to a transient increase in the proliferation of HUVECs compared with normoxic conditions (Fig. 6B). Extended (24–72 h) exposure of HUVECs to hypoxia resulted in a time-dependent decrease of thymidine uptake and thus in the proliferation of cells (hypoxia vs. normoxia of ECs; \( P < 0.0001 \), ANOVA). The addition of 1 ng/ml TNF-α suppressed proliferation of ECs in a similar manner in both hypoxic and normoxic cultures (\( P < 0.0001 \); ANOVA).

**Hypoxia and TNF-α Induced MMP-2-Dependent Apoptosis but Not Necrosis of ECs**

In light of the reduced number of cells observed during hypoxia, we evaluated whether the induced death of ECs resulted from either necrosis or apoptosis. Compared with normoxia, hypoxia induced prominent levels of apoptosis in a time-dependent manner (6–48 h) (data shown only for 24 h; at 24 h, a 1.6-fold increase was noted; \( P = 0.006 \)) and very low levels of necrosis (Fig. 7A). The addition of TNF-α significantly (vs. nontreated normoxic cells; \( P < 0.001 \)) enhanced apoptosis of ECs cultured in hypoxic cultures (Fig. 7A). The addition of the pan-caspase inhibitor Z-VAD-FMK to cultures inhibited spontaneous and hypoxia-induced apoptosis. The results of a TUNEL assay supported the ELISA findings of hypoxia-enhanced apoptosis that was further increased by TNF-α at 48 h (with vs. without TNF-α; \( P < 0.0001 \)). In addition, the TUNEL assay demonstrated that hypoxia alone influenced the whole population of ECs in addition to the small population of cells that had undergone complete apoptosis; that is, the mean fluorescence values for the majority of cells were higher compared with background and compared with normoxia, suggesting partial DNA fragmentation among this cell population (Fig. 7D).

The evaluation of the expression of mRNA molecules associated with apoptosis demonstrated hypoxia-induced upregulation of caspase-3 and caspase-6 (45% and 60%, respectively, vs. normoxic cultures; Fig. 7C).
prominently enhanced MT1-MMP in normoxic and hypoxic cultured cells. Hypoxia conditions. No differences were observed in the localization of the periphery of the cells (Fig. 8). Examined. Hypoxia led to partial dispersion of MMP-2 toward conjunction with phosphorylated (i.e., activated) paxillin was more pronounced in ECs cultured in hypoxic environmental conditions (see arrows in Fig. 8). Induction of phosphopaxillin leading to its aggregated distribution in patches was also induced by hypoxia (Fig. 8C1).

Distinct colocalization of MMP-2 and αvβ3 with phosphopaxillin was more pronounced in ECs cultured in hypoxic conditions (see arrows in Fig. 8), C3 and G3, respectively), but not in normoxic conditions. In contrast, partial colocalization of MT1-MMP with phosphopaxillin was prominent in normoxic but not hypoxic cultured cells (arrows Fig. 8D3).

**DISCUSSION**

The major novel finding of the present study is that MMP-2 induced by hypoxia of ECs participates in autocrine processes that determine the migration as well as the apoptotic death of ECs. The present results also demonstrate enhanced production and/or activation of EC MMP-2 in a hypoxic inflammatory environment (with the addition of TNF-α) that is associated with a skewing toward apoptosis of ECs while not affecting their migration. The hypoxia-induced production of MMP-2 appears to involve both transcriptional and posttranscriptional events, while the additional elevation of MMP-2 in the hypoxic inflammatory milieu comprises mainly posttranscriptional activity. Furthermore, this study suggests that the hypoxia-induced autocrine influences on EC fate involve αvβ3 and paxillin, two major components of focal adhesion sites, rather than MT1-MMP activity.

First, we determined that EC viability and proliferation, which are integral reflections of EC survival. We observed that by excluding an early, transient period of enhanced proliferation, in agreement with a previous study (38), hypoxia led to a time-dependent reduction of proliferation. Because proliferation and death of ECs could occur concomitantly in the same cell population, the influence of hypoxia on the number of viable cells was determined. Despite the transient enhanced proliferation of cells before 18 h, no increment in the

**Hypoxia Enhances Phosphopaxillin Expression and Induces Its Colocalization with MMP-2 and αvβ3**

The spatial distribution of MMP-2, MT1-MMP, and αvβ3 in conjunction with phosphorylated (i.e., activated) paxillin was examined. Hypoxia led to partial dispersion of MMP-2 toward the periphery of the cells (Fig. 8B2, short arrows) compared with its clustered localization in cells cultured in normoxic conditions. No differences were observed in the localization of MT1-MMP in normoxic and hypoxic cultured cells. Hypoxia prominently enhanced αvβ3 staining, leading to its localization in patches of high fluorescence intensity. The enhanced expression of phosphopaxillin leading to its aggregated distribution in patches was also induced by hypoxia (Fig. 8C1).

Distinct colocalization of MMP-2 and αvβ3 with phosphopaxillin was more pronounced in ECs cultured in hypoxic conditions (see arrows in Fig. 8, C3 and G3, respectively), but not in normoxic conditions. In contrast, partial colocalization of MT1-MMP with phosphopaxillin was prominent in normoxic but not hypoxic cultured cells (arrows Fig. 8D3).
hypoxia-induced factor-1 demonstrated enhanced activating protein-1-dependent (4) and importance of endogenous MMP-2 in ECs during hypoxia. The lack of influence, the small changes in their level of expression observed in hypoxia or combined hypoxia and TNF-α, and the upregulation of MMP-2 were further significantly enhanced by the stimulation of hypoxic cultures with TNF-α. The death of ECs, which occurred primarily via an apoptotic pathway, was prevented by MMP-2 inhibitors, supporting the involvement of hypoxia-induced MMP-2 in EC death. The death of ECs and the upregulation of MMP-2 were further significantly enhanced by the stimulation of hypoxic cultures with TNF-α. However, the death-associated role of MMP-2 in the inflammatory milieu appears to be less prominent, because the death of MMP-2, which at a low level induces mainly the migration of ECs and over a certain threshold level induces apoptosis, explaining the observed migration and apoptosis in the same culture conditions.

Despite the proposed housekeeping function attributed to MMP-2 and its regulators because of their constitutive expression, the small changes in their level of expression observed in this study were shown to be of critical importance for physiological and pathological processes (41, 46). The lack of influence of hypoxia or combined hypoxia and TNF-α on the expression of the additional gelatinase MMP-9 (both RNA and protein) in the majority of EC batches further emphasizes the importance of endogenous MMP-2 in ECs during hypoxia. Recent studies of hypoxic cardiac and colon cancer cells have demonstrated enhanced activating protein-1-dependent (4) and hypoxia-inducible factor-1α-dependent (27) transcription of MMP-2, encouraging further investigation of the signaling pathways leading to MMP-2 expression and activity in ECs.

In the present study, TNF-α alone was found to elevate both latent and active forms of secreted MMP-2 while not influencing its mRNA accumulation. Therefore, in contrast to hypoxia, only translational or posttranslational modulation of MMP-2 by TNF-α can be suggested in this context. In addition, the influence of TNF-α on MMP-2 seems to be independent of MT1-MMP, because TNF-α did not affect the membrane expression of this molecule. Although TNF-α enhanced the MMP-2 activity, it did not significantly alter the number of migrating cells induced by hypoxia. This may have resulted from TNF-α-initiated independent death signals that counterbalanced the MMP-2-mediated migration of ECs. Alternatively, the TNF-α-induced elevation of MMP-2 may have led to excessive BM and ECM degradation and EC detachment (anoikis). These results may point to a dose-related effect of MMP-2, which at a low level induces mainly the migration of ECs and over a certain threshold level induces apoptosis, explaining the observed migration and apoptosis in the same culture conditions.

Plasma, the physiological microenvironment surrounding ECs, contains MMPs, a variety of cytokines, and growth factors, which in various combinations affect both cell migration and apoptosis. This in vivo surrounding is unpredictable and does not allow the evaluation of a single defined parameter. Because the goal of this in vitro research was to evaluate the specific effects of hypoxia and TNF-α via MMP-2 activity on the determination of EC destiny, a serum-free culture medium had to be used.

The evaluation of all experiments in the present study was performed after 6, 12, 24, or 48 h of hypoxia. The highest levels of MMP-2 as well as the lowest levels of TIMP-2 were observed after 48 h of hypoxia, when enhanced cell death occurred, thus technically interfering with the performance of certain assays. Because both MMP-2 and TIMP-2 have a long half-life and accumulate in the supernatants, the assessment of their levels at the end of the experiment does not actually reflect the time of their production. We thus chose to present data at a time point (24 h) when apoptosis existed, and functional assays, such as for cell migration and tubelike formation, could still be evaluated clearly. These kinetic results also support dose-related autocrine effects of hypoxia-induced MMP-2, because at a low ratio of MMP-2 relative to TIMP-2 (24 h of hypoxia) both migration and apoptosis were detected, whereas at higher ratios, after extended hypoxia (48 h), upregulated cell death was prominent. The preliminary results of the TUNEL assay performed at 48 h (Fig. 7D) suggest that hypoxia influenced the whole EC population rather than the existence of a subset of cells predestined toward death or survival within the hypoxic culture.

A variety of different in vitro methodologies have been used by other researchers to study tubelike formations (10, 17, 25, 28, 29, 39, 48). In the present study, hypoxia led to a significant reduction in the number of tubelike structures spontaneously formed in the culture and to atypical, open-ended formations (13). However, specific MMP-2 inhibitors as well as the pan-caspase inhibitor Z-VAD-FMK, which prevented MMP-2-dependent apoptosis, did not restore the normal tubelike formation. Thus hypoxia-induced antiangiogenic effects, not mediated by MMP-2 or caspase-dependent apoptosis, seem to be responsible for the observed reduction in tubelike forma-
Indeed, in an in vitro fibrin model, tubelike formation in different cell types was shown to depend directly on MT1-MMP and not on MMP-2 (1, 25, 29). Therefore, the hypoxia-induced reduction of MT1-MMP may be relevant to the impaired tubelike formation in the present study. The observed lack of effect of TNF-α on tubelike formation is supported by additional research using ECs in normoxic (29, 39) or hypoxic conditions (2). The present results may suggest the involvement of hypoxia-triggered MMP-2 autocrine activity at specific points of the angiogenic pathway, not including tubelike formation. Nonetheless, the Matrigel itself enhanced MMP-2 (data not shown), which, when added to that induced by hypoxia, could result in excessive degradation and a lack of tubelike structures. Of note, higher concentrations of MMP-2 inhibitors could not be added because of their cytotoxicity (data not shown). Thus the participation of MMP-2 in the hypoxia-induced tubelike damage still needs clarification.

The hypoxia-induced activation and aggregate formation of paxillin, as well as its colocalization with both enhanced αvβ3 and enhanced MMP-2, suggest hypoxia-related accrual of these three molecules at points of focal adhesion in ECs. The integrin αvβ3 was previously shown to localize (7) and activate MMP-2 (23) at points of focal adhesion on malignant cells and ECs, facilitating cellular motility and angiogenesis (37, 40). On the other hand, the loss of EC adhesion via unligated αvβ3 results in apoptosis that is dependent on anoikis (9) or caspase-8 recruitment and activation at the cell membrane (31, 44). The multidomain adaptor protein paxillin, which is found at the interface between the plasma membrane and actin cytoskeleton (45) after activation by phosphorylation via integrin and growth factor triggering, transduces extracellular signals to the cell interior and the cytoskeleton. It is not clear whether previous activation of MMP-2 by hypoxia leading to matrix degradation may have altered the distribution of focal adhesion points, resulting in the observed colocalization of MMP-2 with αvβ3 and paxillin, or whether the hypoxia-induced redistribution of focal adhesion points resulted in the activation of MMP-2. Thus activation of MMP-2 by hypoxia may be upstream or downstream of focal adhesion reorganization. To the best of our knowledge, this report is the first to demonstrate hypoxia-mediated colocalization of MMP-2 with phosphopaxillin and suggests functional association of MMP-2 with...
Fig. 8. Hypoxia induces colocalization of phosphopaxillin with MMP-2 and with α,β3. HUVECs were exposed to normoxia (B, D, and F) or hypoxia (C, E, and G) for 24 h and were indirectly immunostained with rabbit anti-phosphopaxillin (B1–G1), followed by secondary goat anti-rabbit labeled with FITC or mouse-anti-MMP-2 (B2 and C2), anti-MT1-MMP (D2 and E2), and anti-α,β3 (F2 and G2), followed by secondary goat anti-mouse labeled with rhodamine. Cells exposed only to secondary antibodies served as controls for background staining (FITC, A1, and rhodamine, A2). The merged serial images obtained using confocal laser microscopic analysis of phosphopaxillin and MMP-2 (B3 and C3), MT1-MMP (D3 and E3), or α,β3 (F3 and G3) images are shown. Areas of colocalization are stained yellow. Short arrows indicate the distribution of MMP-2 at the periphery of the cells (B2), and long arrows point to areas containing colocalization of phosphopaxillin and MMP-2, MT1-MMP, or α,β3 (B3 and G3). Magnification, ×400.
with cytoskeletal alterations and the provocation of intracellular signaling pathways.

Generally, activation of pro-MMP-2, which occurs on the cell membrane, is mostly dependent on MT1-MMP and associated with a high MMP-2-to-TIMP-2 ratio (41, 46). The hypoxia-induced reduction of TIMP-2 is in line with known activation pathways and upregulated MMP-2 activity after prolonged hypoxia. However, the hypoxia-mediated decrease of MT1-MMP mRNA, as well as its dissociation from phosphopaxillin, negates the participation of MT1-MMP and/or TIMP-2 in the activation of pro-MMP-2 in the hypoxic microenvironment.

In summary, in the present study, we have demonstrated an interesting role for autocrine MMP-2 in the balance between the survival and death of ECs during hypoxia. Further evaluation of the molecular mechanisms that lead to MMP-2 expression and activation, as well as the autocrine influence of this protease on the destiny of ECs, is of critical therapeutic relevance for improving physiological neovascularization of ischemic or injured tissues as well as for interventions in the pathological angiogenesis of hypoxic tumors and other vascular pathologies.

GRANTS

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REFERENCES


