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Signaling Angiogenesis via p42/p44 MAP Kinase and Hypoxia

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ABSTRACT. Angiogenesis is associated with a number of pathological situations. In this study, we have focused our attention on the role of p42/p44 MAP (mitogen-activated protein) kinases and hypoxia in the control of angiogenesis. We demonstrate that p42/p44 MAP kinases play a pivotal role in angiogenesis by exerting a determinant action at three levels: i) persistent activation of p42/p44 MAP kinases abrogates apoptosis; ii) p42/p44 MAP kinase activity is critical for controlling proliferation and growth arrest of confluent endothelial cells; and iii) p42/p44 MAP kinases promote VEGF (vascular endothelial growth factor) expression by activating its transcription via recruitment of the AP-2/Sp1 (activator protein-2) complex on the proximal region (−88/−66) of the *VEGF* promoter and by direct phosphorylation of hypoxia-inducible factor 1α (HIF-1α). HIF-1α plays a crucial role in the control of HIF-1 activity, which mediates hypoxia-induced VEGF expression. We show that oxygen-regulated HIF-1α protein levels are not affected by intracellular localization (nucleus versus cytoplasm). Finally, we propose a model which suggests an autoregulatory feedback mechanism controlling HIF-1α and therefore HIF-1-dependent gene expression. BIOCHEM PHARMACOL **60**;8:1171–1178, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. VEGF promoter; p42/p44 MAP kinases; hypoxia; HIF-1; angiogenesis; apoptosis

The growth of new blood vessels from the pre-existing vascular tree is known as angiogenesis. In the normal healthy adult, blood vessel growth is practically non-existent, except for certain processes such as the female reproductive cycle. However, deregulated angiogenesis has been associated with a broad number of pathological situations such as wound and fracture healing, arthritis, retinopathies, cardiovascular and cerebral ischemia, and nearly every type of cancer. In this context, several studies have demonstrated that angiogenesis is a rate-limiting step in tumor growth and progression. There is also a direct correlation between intratumoral vessel density and metastasis [1–4].

VEGF† is a critical mediator of angiogenesis. VEGF, initially described as a permeability factor, is a member of a family of secreted growth factors structurally related to platelet-derived growth factor (PDGF) [5, 6]. VEGF is a potent and specific mitogen for vascular endothelial cells, the essential components of blood vessels. Different isoforms of 121, 165, 189, and 206 amino acids result from alternative splicing of the same gene [7]. VEGF is expressed in virtually all tissues and cell types examined, but this expression is highly up-regulated in tumors [8]. The pivotal role played by VEGF in angiogenesis has been elegantly demonstrated. Surprisingly, inactivation of one single *VEGF* allele dramatically stops embryonic development at a very early stage because of a failure in vasculature formation [9, 10]. A similar phenotype was observed after inactivation of both alleles of either VEGFR-1 or VEGFR-2, two receptor tyrosine kinases involved in migration, proliferation, and survival of vascular endothelial cells [11, 12].

The expression of VEGF is tightly regulated at transcriptional and posttranscriptional levels. The precise mechanisms underlying VEGF induction have been extensively investigated in recent years. In this short paper, we summarize recent data obtained in our laboratory concerning the implication of the p42/p44 MAP kinase signaling pathway and hypoxia in the control of angiogenesis and notably in the expression of VEGF.

p42/p44 MAP KINASE CASCADE AND CELL SURVIVAL

MAP kinases are a family of serine/threonine kinases that are activated through a signaling pathway triggered by numerous agonists such as growth factors, hormones, lymphokines, extracellular matrix components, tumor promot-

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† *Abbreviations:* VEGF, vascular endothelial growth factor; MAP, mitogen-activated protein; MKK or MAPKK, MAP kinase kinase; ER, estradiol receptor; MAPKKK, MAP kinase kinase kinase; PARP, poly(ADP-ribose) polymerase; AP-2, activator protein-2; and HIF-1, hypoxia-inducible factor.

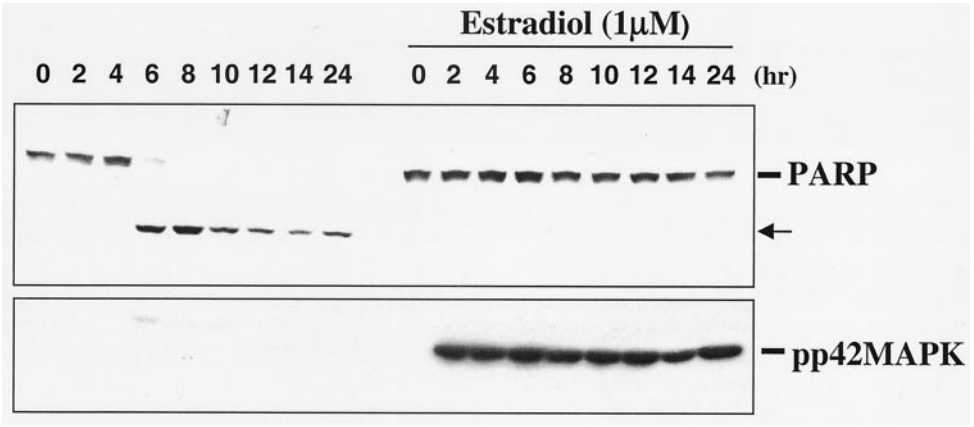

FIG. 1. Inhibition of apoptosis following anchorage and serum withdrawal by p42/p44 MAP kinase activation. CCL39-Raf-1:ER cells were deprived of anchorage and serum in the presence or absence of 1 μM estradiol for the indicated times. PARP cleavage (top) and p42 MAP kinase activation (bottom) following the detachment of cells in the presence or absence of estradiol are shown. (Reproduced from [19]).

ers, and stress factors. This activation is attained after dual phosphorylation on threonine and tyrosine residues. Their specific upstream activators represent a set of dual specificity protein kinases, called MKK or MAPKK, which are in turn activated by upstream MAPKKK. These kinase cascades, extremely well conserved from yeast to man, have been extensively investigated. Our laboratory has devoted much effort to the characterization of the spatio-temporal control of the p42/p44 MAP kinase pathway. These two closely related MAP kinase isoforms (also called ERK [extracellular signal-regulated kinase] 2 and ERK 1, respectively) have been implicated in a wide range of cellular functions including the control of protein synthesis, nucleotide metabolism, cell growth, cell fate, and differentiation [13–18].

Recently, we have shown that besides controlling cell proliferation, sustained activation of the p42/p44 MAP kinase pathway efficiently protects CCL39 fibroblasts and Madin–Darby canine kidney (MDCK) epithelial cells from apoptosis [19]. In order to specifically evaluate the contribution of the p42/p44 MAP kinase pathway in cell survival, we used a derivative of the CCL39 and MDCK cell lines stably expressing the Raf-1:ER chimera. This protein is a fusion between the catalytic domain of Raf-1, the MAPKKK upstream activator of the p42/p44 MAP kinase pathway, and the ligand-binding domain of the estradiol receptor. Addition of estradiol to these cells induces a rapid and strong stimulation of p42/p44 MAP kinases that persists as long as estradiol is present in the medium [20–22]. Our results show that the addition of estradiol to Raf-1:ER cells markedly inhibits the apoptotic process triggered by anchorage and serum removal that is determined by the cleavage of PARP (Fig. 1). In our model, suppression of apoptosis by Raf-1 activation occurs without detectable activation of Akt, suggesting that the phosphoinositide 3-kinase/Akt pathway is not necessary for promoting survival of anchorage-deprived cells [19]. The finding that the antiapoptotic activity of estradiol correlates well with activation of the p42/p44 MAP kinases is supported by the fact that constitutively active MKK1 SS/DD (p42/p44 MAPKK)-expressing cells are clearly protected from apoptosis [19]. Furthermore, pharmacological inhibition of activated MKK1 with PD 98059 reverses Raf-1:ER-mediated inhibition of PARP cleavage and cell survival [19]. These results are particularly interesting since matrix breakdown occurs during the first stages of angiogenesis and loss of anchorage dependence correlates with tumor growth *in vivo*. Although these results have not yet been directly demonstrated to take place in vascular endothelial cells, we have reason to believe that the contribution of the p42/p44 MAP kinase signaling cascade is a general process in cell survival.

p42/p44 MAP KINASE CASCADE AND CONFLUENCY IN ENDOTHELIAL CELLS

As predicted, activation of the p42/p44 MAP kinase cascade also plays a central role in the control of vascular endothelial cell proliferation. Thus, p42/p44 MAP kinases are stimulated by classical endothelial cell mitogens such as VEGF or fibroblast growth factor-2 (FGF-2) and the specific inhibition of this pathway blocks cell proliferation [23–27]. However, we have found that after growth factor stimulation, p42/p44 MAP kinase activation is restrained to confluent endothelial cells as compared to non-confluent cells [28]. This apparent resistance to activate p42/p44 MAP kinases in confluent cells correlates well with their poor proliferation capacity. On the contrary, the forced activation of p42/p44 MAP kinases by transfection of the Raf-1:ER chimera is sufficient to restore the entry of confluent cells into the cell cycle measured by BrdU (bromodeoxyuridine) or thymidine incorporation (Fig. 2 and [28]). The effect of cell confluency on p42/p44 MAP kinase activity seems to be absent in other model systems such as NIH3T3 or Swiss 3T3 fibroblasts. Our results predict that the overexpression of an orthovanadate-sensitive phosphatase should be implicated in the "repression" of

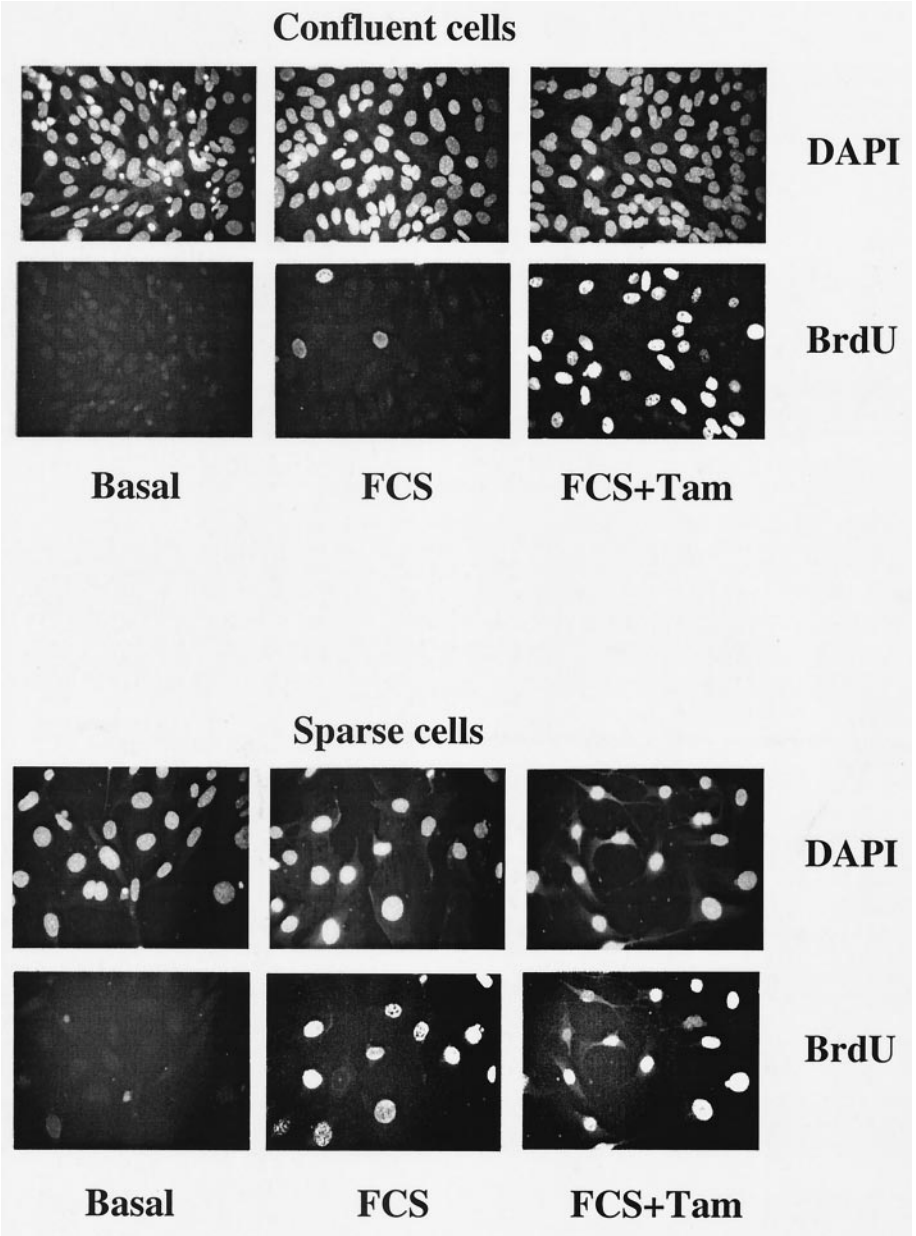

FIG. 2. Forced activation of p42/p44 MAP kinases induces confluent endothelial cell entry into the cell cycle. 1G11-Raf-1:ER cells were grown under conditions promoting sparseness or confluency and serum-deprived for 24 hr. Cells were stimulated or not (basal) with 20% FCS (fetal calf serum) or 1 mM tamoxifen plus 20% FCS (FCS + Tam) for 24 hr. During the last 4 hr, cells were labeled with BrdU (bromodeoxyuridine). DNA synthesis was assessed by immunodetection of cells that had incorporated BrdU. Nuclei were stained with DAPI (4′,6-diamidino-2′-phenylindole dihydrochloride). (Reproduced from [28]).

p42/p44 MAP kinase activity at confluency and also suggest that p42/p44 MAP kinase activity tightly controls the state of proliferation of endothelial cells [28].

p42/p44 MAP KINASE CASCADE AND *VEGF* PROMOTER ACTIVITY

A variety of cytokines and growth factors, including EGF (epidermal growth factor), TGF (transforming growth factor)-α, TGF-β as well as oncogenic agents like *v-H-Ras* and *v-Raf* have been shown to induce VEGF expression in several cell lines [29–36]. However, the precise mechanisms leading to this increased expression have not been fully elucidated. We compared the expression of VEGF in quiescent, serum-stimulated, or oncogenically transformed CCL39 fibroblasts. Starved and exponentially growing non-transformed cells express barely detectable levels of VEGF mRNA. As expected, serum stimulation of growth-arrested cells triggers the induction of VEGF mRNA [37]. More importantly, this expression is strongly elevated in cells transformed with polyomavirus, *v-H-Ras*, or MKK1 SS/DD. These results suggest that the regulation of VEGF expression occurs through a p42/p44 MAP kinase-dependent mechanism. In order to confirm the role of this kinase pathway, we have again used our CCL39 cell line stably

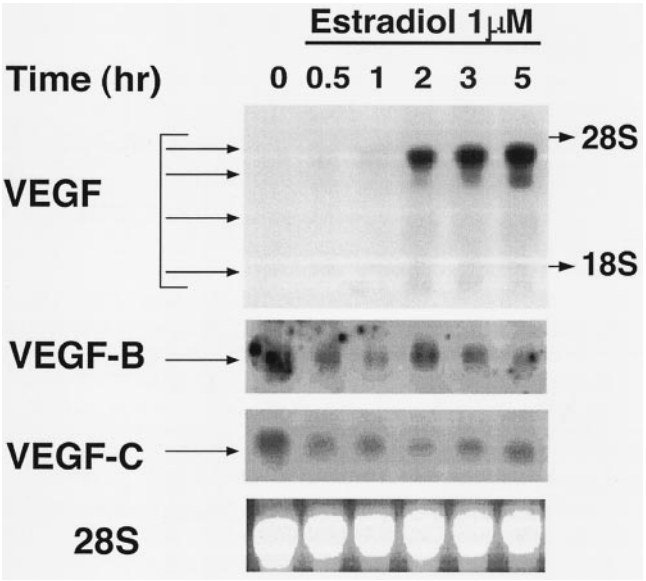

FIG. 3. Regulation of VEGF expression in CCL39-Raf-1:ER stably expressing cells. Twenty micrograms of RNA isolated from quiescent or estradiol-stimulated cells (1 μM) for the indicated periods of time were analyzed by Northern blot. The blot was hybridized with probes corresponding to VEGF, VEGF-B, and VEGF-C. 28S ribosomal RNA is shown as loading control. (Reproduced from [37]).

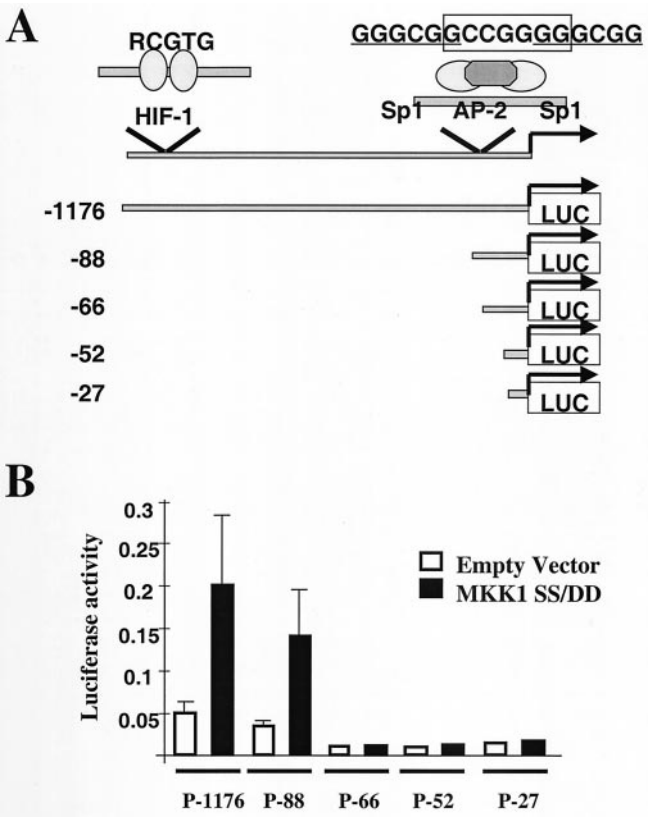

FIG. 4. (A) Description of the *VEGF* promoter. Shown are the different domains of the promoter and the constructs used in transient transfection assays. (B) Identification of a p42/p44 MAP kinase pathway-responsive region on the *VEGF* promoter. CCL39 cells were transfected with 250 ng of the different reporter gene constructions and 100 ng of a control plasmid coding for β-galactosidase in the presence or absence of 200 ng of MKK1 SS/DD. After 16 hr of transfection, cells were rinsed with PBS and grown in medium supplemented with 7.5% FCS (fetal calf serum) for 48 hr. Cells were lysed and luciferase activity measured. These results are representative of three independent experiments performed in triplicate. (Reproduced from [37]).

expressing the Raf-1:ER chimera. VEGF mRNA is induced in response to estradiol in a dose-dependent manner [37]. As shown in Fig. 3, VEGF is expressed at a detectable level after 2 hr with maximal expression attained after 3 hr stimulation. Furthermore, inhibition of MKK1 by the specific inhibitor PD 98059 blocks p42/p44 MAP kinase-induced VEGF expression. In contrast to VEGF, VEGF-B and VEGF-C isoforms are poorly regulated by growth and oncogenic factors. These results have recently been confirmed by Rak and co-workers. They have demonstrated that in Ras-transformed fibroblasts, VEGF expression was inhibited by treatment with PD 98059, whereas the PI3-K inhibitor LY 294002 had no effect [36]. Taken together, these results clearly demonstrate the specific role of the p42/p44 MAP kinase pathway in *VEGF* gene expression.

To further evaluate the mechanism by which the p42/p44 MAP kinase cascade stimulates *VEGF* expression, we directly analyzed the activation of the VEGF promoter coupled to a luciferase reporter gene. Constitutively active Ras (Ras-Val^{12}) or MKK1 (MKK1 SS/DD) can strongly stimulate the *VEGF* promoter even in the absence of serum. Thus, two upstream members of the p42/p44 MAP kinase cascade can activate *VEGF* gene transcription. Transfection of p38 and c-Jun N-terminal kinase (JNK) in the presence of activating agents or their constitutively active activating kinases (MKK3 or MKK4) had no effect on *VEGF* promoter activity, confirming the specific role played by the p42/p44 MAP kinase cascade in the control of *VEGF* gene transcription. We next characterized the specific p42/p44 MAP kinase-responsive region on the *VEGF* promoter. Figure 4A shows the detailed structure of this promoter and the different constructs used in transfection assays. The *VEGF* promoter contains binding sites for AP-2, Sp1, or Sp1-related factors as well as for HIF-1, which regulates the transcription of the gene under hypoxia. We compared the activities of the different truncations in the presence or absence of MKK1 SS/DD. Our results demonstrate that sequences between −88 and −66 are absolutely required for basal and p42/p44 MAP kinase-stimulated promoter activity (Fig. 4B). Based on these data, we evaluated the potential contribution of putative AP-2 or Sp1-binding sites present in this region (GC-rich region) in p42/p44 MAP kinase-induced *VEGF* gene transcription. Wild-type or mutant constructs for the AP-2, both Sp1, or the three binding sites were transfected and activation of the *VEGF* promoter was analyzed by luciferase assays. Interestingly, only the combined mutation of the AP-2 and both Sp1-binding sites blocked basal and MKK1 SS/DD-dependent transcriptional activation [37]. This finding suggests a cooperative effect of both transcription factors for

maximal VEGF induction. We have further analyzed the −88/−66 region by electrophoretic mobility shift assay (EMSA) and supershift experiments. With the use of specific antibodies, we confirmed that AP-2 and Sp1 are components of the more intense complex that is increased upon estradiol stimulation [37].

HIF-1α AND THE HYPOXIA-DEPENDENT EXPRESSION OF VEGF

As previously mentioned, hypoxia is also a major inducer of VEGF expression. Transcriptional up-regulation has been shown to play a pivotal role in the hypoxic induction of *VEGF* gene [38, 39]. In this case, another distal region (hypoxia-responsive element [HRE]) of the *VEGF* promoter is regulated by fixation of HIF-1 (see Fig. 4A). HIF-1 is a heterodimer composed of the HIF-1α (120 kDa) and HIF-1β subunits (94 kDa) (Fig. 5A). Both HIF-1 subunits belong to the subfamily of bHLH (basic helix–loop–helix) transcription factors containing a PAS (PER, ARNT, SIM) motif [40]. HIF-1β is the already-characterized ARNT (aryl hydrocarbon receptor nuclear translocator) previously shown to heterodimerize with the AHR (aryl hydrocarbon receptor). In contrast, HIF-1α specifically mediates hypoxic responses. HIF-1 plays a central role in oxygen homeostasis by inducing the expression of a broad range of genes in a hypoxic-dependent manner (*VEGF*, *VEGFR*-1, inducible nitric oxide synthase, transferrin, tyrosine hydroxylase, glucose transporter-1, and almost every gene in the glycolytic pathway) [41]. The molecular mechanisms by which cells sense low oxygen concentration and activate HIF-1 are still largely unknown. Several studies have demonstrated that HIF-1 activation is a multistage and complex process. However, hypoxia-mediated induction of HIF-1α appears to be the primary mode of regulation, since the expression of the rate-limiting α subunit determines the activity of the HIF-1 complex [42]. HIF-1α is a short-lived protein that is maintained at low and often undetectable levels in normoxia, whereas it is strongly induced in hypoxic cells [43–46]. As shown in Fig. 5B and C, after 3 hr of incubation in a hypoxic environment (1% O_2), the HIF-1α protein is strongly induced and found in the nucleus. However, as quickly as HIF-1α is induced, it is even more rapidly degraded upon return to normoxic conditions (Fig. 5D). Previous results have shed light on the mechanism by which HIF-1α protein levels are so tightly regulated by oxygen concentration [47–49]. They have shown that, in normoxic cells, HIF-1α is quickly ubiquitinated and degraded by the proteasome system, whereas hypoxia induces HIF-1α by relaxing its ubiquitin–proteasome degradation (Fig. 5E). In order to evaluate the role of subcellular localization in the HIF-1α stabilization/degradation pattern, we prevented HIF-1α nuclear translocation by expressing a membrane-bound HIF-1α chimeric protein. Interestingly, the kinetics of induction and/or degradation of the chimeric protein are similar to those of the endogenous HIF-1α protein. Our results indicate both

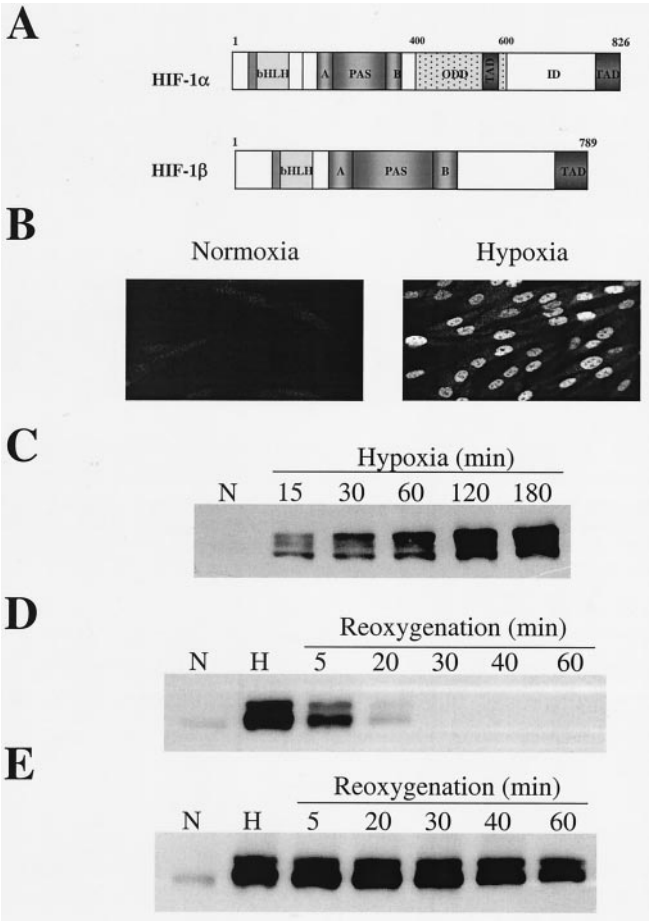

FIG. 5. (A) Schematic representation of the HIF-1 transcription factor subunits HIF-1α and HIF-1β bHLH, basic helix–loop–helix; PAS, PER, ARNT, SIM; ODD, oxygen-mediated degradation domain; TAD, transactivation domain. (B) HIF-1α induction. CCL39 cells were incubated for 3 hr in a normoxic (20% O_2) or hypoxic environment (1% O_2) and HIF-1α was revealed by immunofluorescence with a specific antibody. (C) Time–course of HIF-1α induction. HeLa cells were incubated in normoxia or for different periods of time in hypoxia and cellular proteins were resolved by SDS-PAGE and immunoblotted with a specific anti-HIF-1α antibody generated in our laboratory. (D) Time–course of HIF-1α degradation. After a 3 hr incubation in a hypoxic environment, HeLa cells were returned to normal oxygen concentrations (20%) for different periods of time. (E) Proteasome inhibition blocks HIF-1α degradation. HeLa cells were incubated as in D in the presence of 20 μM lactacystin. Similar results have been shown in Refs. 43–45. (Reproduced from [46]).

that nuclear translocation is not necessary for HIF-1α stabilization upon hypoxia and that both "nuclear" and "cytoplasmic" proteasomal activities are competent to degrade HIF-1α in an oxygenated environment.

Regulation of the proteasome-dependent degradation of HIF-1α appears to be a pivotal step in the hypoxic activation of HIF-1. Giaccia's group has recently shown that PTEN and Akt participate in this process [50]. Indeed, acute regulation of protein levels by the proteasome machinery often requires a targeting mechanism [51–53]. In this context, two proteins, the von Hippel–Lindau tumor

suppressor protein (pVHL) and MDM2, have recently been suggested to target the proteasomal degradation of HIF-1α [54, 55]. In addition, according to our unpublished results, we propose a model in which an HIF-1α proteasome targeting factor, referred to here as HPTF, is involved in this process. We have evidence that HIF-1α degradation depends on HIF-1 transcriptional activity. Therefore, we speculate that HPTF expression, not yet molecularly identified, is controlled by HIF-1-dependent transcriptional activity. This model suggests the presence of an autoregulatory feedback mechanism which controls the activity of HIF-1.

HIF-1α IS ALSO A TARGET FOR p42/p44 MAP KINASES

Phosphorylation has often been suggested to be implicated in HIF-1 activation. In this context, we have demonstrated that HIF-1α is a highly phosphorylated protein and that HIF-1α phosphorylation induces strong changes in its electrophoretic migration pattern [56]. Previous results suggested that serine/threonine protein kinase(s) could be responsible for HIF-1α phosphorylation [47, 57, 58]. Since p42/p44 MAP kinases are two serine/threonine kinases that modulate the activity of a number of transcription factors, we wanted to evaluate the capacity of p42/p44 MAP kinases to phosphorylate HIF-1α. Interestingly, incubation of HIF-1α with active p42 MAP kinase induced a rapid shift in the electrophoretic mobility of the *in vitro* translated protein with a $t_{1/2}$ of 5 min and a complete shift at 20 min (Fig. 6A). We therefore analyzed the specificity of this phosphorylation by assessing whether the stress-activated kinases (p38 and JNK) could phosphorylate HIF-1α. Our results indicate that the phosphorylation of HIF-1α by MAP kinases is specific to the p42 et p44 MAP kinase isoforms [56]. More interestingly, we demonstrate that HIF-1α is phosphorylated by p42/p44 MAP kinases *in vivo*. To this purpose, we used the previously described CCL39-Raf-1:ER cells. As seen in Fig. 6B, HIF-1α is induced upon hypoxia and migrates as a single band at approximately 104 kDa. In contrast, after 30 min of stimulation with estradiol, a form of HIF-1α appeared at 116 kDa. The induction of the HIF-1α shift closely followed the activation of p42/p44 MAP kinases. Furthermore, this shift was inhibited by treatment of cells with the specific MKK1 inhibitor, PD 98059 [56]. We next evaluated the possible effect of HIF-1α phosphorylation by p42/p44 MAP kinases on HIF-1 transcriptional activity. To perform this experiment, we used a luciferase reporter plasmid driven by the *VEGF* promoter in which the combined mutation of the AP-2 and both Sp1-binding sites had eliminated the previously shown p42/p44 MAP kinase response element. This construction was co-transfected with HIF-1α and HIF-1β plasmids in the Raf-1:ER cells. Addition of estradiol strikingly activated the mutated *VEGF* promoter by almost 6-fold over untreated cells without affecting basal levels in cells transfected with the reporter only [56]. These results demonstrate that strong activation of p42/p44 MAP kinases is sufficient to effectively promote the transcriptional activity of HIF-1.

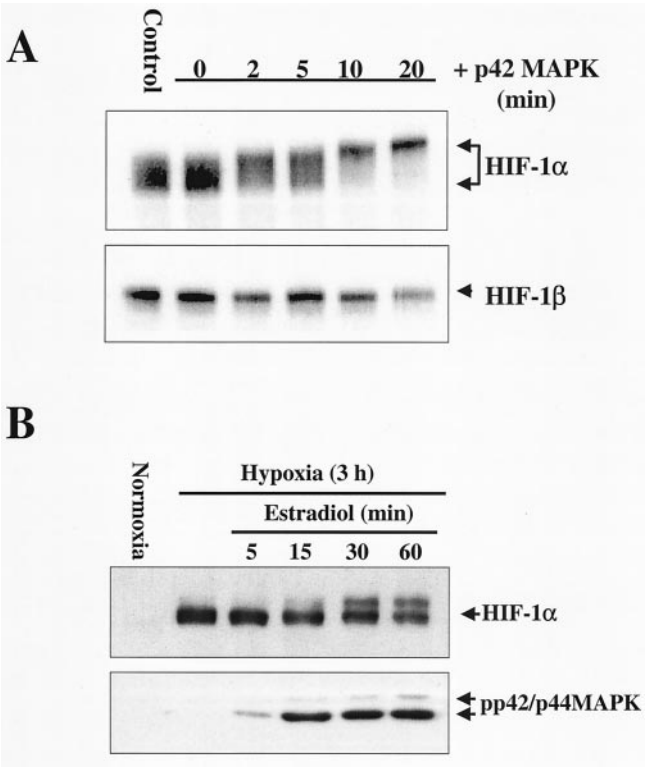

FIG. 6. (A) *In vitro* phosphorylation of HIF-1α by p42 MAP kinase. *In vitro*-translated hemagglutin (HA) epitope-tagged forms of either HIF-1α or HIF-1β (labeled) were immunoprecipitated using an anti-HA antibody and incubated in the presence of active purified recombinant p42 MAP kinase for the times indicated at 30°. The samples were then analyzed by SDS–PAGE (7.5% gel) and autoradiography. (B) p42/p44 MAP kinase activation induces HIF-1α phosphorylation *in vivo*. CCL39-Raf-1:ER cells were FCS (fetal calf serum)-starved for 24 hr before 3 hr of hypoxia. Before the end of the hypoxic period, Raf-1:ER cells were stimulated with 100 nM estradiol for the times indicated. Whole cell extracts (50 μg) were analyzed by SDS–PAGE (7.5% gel) and immunoblotted. (Reproduced from [56]).

CONCLUSIONS

Angiogenesis occurs in a number of pathological situations and in particular is a promoting event for tumor growth and metastasis. Hence, tumor angiogenesis has been one of the most heavily studied subjects in oncology in recent years. In this study, we have summarized our more recent findings focusing on the control of VEGF expression and action. We have demonstrated that the activation of p42/p44 MAP kinases, a key signaling pathway in the control of cell growth and differentiation, can also play a central role at different levels during angiogenesis. In fact, forced activation of this kinase pathway offers protection from apoptosis, promotes endothelial cell entry into the cell cycle, and induces VEGF expression by increasing transcription. We

have demonstrated that this transcriptional activation is mediated by recruitment of AP-2 and Sp1 to a proximal region of the *VEGF* promoter and also by phosphorylation of HIF-1α. HIF-1α is the rate-limiting subunit of HIF-1 responsible for the hypoxia-induced expression of VEGF. HIF-1α expression is tightly regulated by oxygen concentrations. We have shown that HIF-1α can be stabilized even if nuclear translocation is impaired. Finally, we propose a model in which HIF-1α degradation is mediated by a HIF-1α proteasome targeting factor, HPTF.

The finding that HIF-1α is phosphorylated by p42/p44 MAP kinases suggest a cooperation between hypoxic and growth factor signals, which ultimately leads to an increase in HIF-1-dependent gene expression. Altogether, these data highlight the importance of the p42/p44 MAP kinase pathway and hypoxia in controlling different steps of the angiogenic program. This cooperation is very important since angiogenesis normally occurs under conditions of low oxygen concentrations. The further comprehension of the molecular mechanisms regulating the proliferation of new blood vessels would undoubtedly help us in the development of new therapeutic targets.

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