Hypoxia-Induced Vascular Endothelial Growth Factor Transcription and Protection from Apoptosis Are Dependent on α6β1 Integrin in Breast Cancer Cells

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Abstract

The α6β1 integrin has been implicated in breast carcinoma progression, but the mechanisms involved remain elusive. MDA-MB-435 cells engineered to be deficient in α6β1 expression form primary tumors that are highly apoptotic and unable to metastasize, although they exhibit no increased apoptosis in vitro under standard culture conditions. Based on the hypothesis that α6β1 is necessary for the survival of these cells in the tumor microenvironment, we report here that hypoxia protects these cells from apoptosis induced by serum deprivation and that hypoxia-mediated protection requires α6β1 expression. We investigated the influence of α6β1 on vascular endothelial growth factor (VEGF) expression because autocrine VEGF is necessary for the survival of serum-deprived cells in hypoxia. The results obtained indicate that α6β1 is necessary for VEGF expression because the ability of hypoxia to activate HIF-1 and to stimulate VEGF transcription in MDA-MB-435 cells is dependent on α6β1 expression by a mechanism that involves protein kinase C (PKC)-α.

Introduction

An important role for the α6β1 integrin in breast cancer progression has been indicated by several studies (1–3). The involvement of this integrin in progression was suggested first by the finding that high expression of the α6 subunit in women with breast cancer correlated significantly with reduced survival times (1). In an analysis of 119 patients with invasive breast carcinoma, all of the patients with low or absent α6 expression survived, whereas the mortality rate of the patients with a high level of α6 expression was 19%. Of note, 30 of 34 of the patients that presented with distant metastases were highly positive for α6 expression. This study is consistent with the report that the metastatic potential of MDA-MB-435 cells correlates with their level of α6β1 expression (3). Moreover, when α6β1-deficient MDA-MB-435 cells were inoculated into the mammary fat pads of nude mice, primary tumor size was significantly diminished compared with the parental cells because of increased apoptosis (2). The α6β1-deficient cells did not form metastases in the lung, as did the parental cells, because of their inability to survive in this organ (2). Interestingly, these α6β1-deficient cells did not differ in their ability to survive in vitro under standard culture conditions, suggesting that α6β1 is needed for survival within the tumor microenvironment. Given that the microenvironment of solid tumors is often hypoxic and lacks the rich growth factor milieu present in culture medium, we examined the hypothesis that this integrin contributes to the survival of MDA-MB-435 cells in such conditions. Interestingly, the data obtained indicate that hypoxia protects these cells from apoptosis induced by serum deprivation and that this protection depends on α6β1 expression. Protection from apoptosis under these conditions requires autocrine vascular endothelial growth factor (VEGF), and additional analysis revealed that α6β1 is necessary for VEGF expression because it functions in concert with hypoxia to activate hypoxia inducible factor (HIF)-1 and to stimulate VEGF transcription by a mechanism that involves protein kinase C (PKC)-α.

Materials and Methods

Cells and Reagents. MDA-MB-435 breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University (Washington, DC). MDA-MB-435 cells that had been sorted by fluorescence-activated cell sorting to obtain populations that express relatively high or low α6β1 expression were provided by Beth Israel Deaconess Medical Center (Boston, MA). Myrisylated PKC-α and PKC-ζ constructs were obtained from Dr. Alex Toker (Beth Israel Deaconess Medical Center). The HIF-1 dominant-negative mutant constructs A26E and K29E (4, 5) were provided by Dr. Dev Mukhopadhyay (Beth Israel Deaconess Medical Center).

The following antibodies were used: an HIF-1α monoclonal antibody (Novus Biological, Newington, NH); a rabbit p300 polyclonal antibody (Upstate Biotechnology, Lake Placid, NY); an α6 integrin monoclonal antibody, clone 2B7 (prepared in our laboratory); and a rabbit actin antibody (Sigma, St. Louis, MO). Rabbit polyclonal anti-VEGF serum (clone 618) was obtained from Dr. Don Senger (Beth Israel Deaconess Medical Center).

Small Interfering RNA (siRNA) Experiments. A siRNA specific for the α6 integrin subunit (GGUGCUGACAUGUGCUCAU) and a scrambled-sequence control (UUGCAGAGGCCGUCCUCUU) were synthesized by Dharmaco, Inc. (Lafayette, CO). A siRNA specific for α5 integrin subunit (UGGCCUCAGACAUUGCAUC) and control siRNA were synthesized by Qiagen, Inc. (Valencia, CA). Cells (1 × 10^6) were plated onto 35-mm tissue culture dishes a day before the transfection of 200 nM siRNA duplex with 25 mg of TransIT-TKO transfection reagent (Mirus, Madison, WI) in the presence of serum. A day after transfection, the transfection medium was aspirated from cells, and fresh complete medium was added and incubated for an additional 48–72 h. For each transfection, flow cytometry was used to assess α6 integrin expression.

VEGF Antisense Strategy. Either a VEGF antisense 2'-O-methyl phosphorothioate oligodeoxynucleotide (5'-CACCCAAAGACAGCAAGA-3') or a VEGF sense 2'-O-methyl phosphorothioate oligodeoxynucleotide (5'-CTTCGGCTGCTTTGCTG-3') at a concentration of 0.3 μM was transfected into MDA-MB-435 cells as described previously (6).

Protein Analysis. For the analysis of protein expression, cells were extracted in radioimmunoprecipitation assay buffer [20 mM Tris buffer (pH 7.4) containing 0.14 M NaCl; 1% NP40; 10% glycerol; 1 mM sodium orthovanadate; 2 mM phenylmethylsulfonyl fluoride; and 5 μg/ml aprotonin, pepstatin, and leupeptin], and immunoblotting was performed as described previously (7). For experiments that assessed HIF-1 expression and HIF-1 association with p300, nuclei were isolated and extracted as described previously (8, 9) and used for immunoblotting and immunoprecipitation experiments.

Apoptosis Assay. Adherent and nonadherent cells were harvested and assayed for apoptosis using annexin V-FITC (BioSource, Sunnyvale, CA) and propidium iodide (BioSource) as described previously (7). In some experiments, cells were incubated in low serum [0.5% fetal bovine serum (FBS)] in hypoxia with or without recombinant VEGF (final concentration, 100 ng/ml; R&D Systems) along with 1 μg/ml heparin (Sigma) before the apoptosis assay.

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Quantitative Real-Time PCR. Quantitative analysis of VEGF mRNA expression was performed by real-time PCR as described previously (7).

Transcription Assays. Human VEGF promoters (either the full-length 2.6-kb promoter or 0.35-kb deletion mutant lacking the hypoxia response element [HRE]) were cloned into pGL3-basic vector (Promega, Madison, WI) and used to assess VEGF promoter activity using firefly luciferase as the reporter gene (10). Cells at a confluency of 85–95% were used for all experiments and incubated with 20 μM ZVAD-FMK (Promega) to prevent apoptosis during the experiment. Plasmids were transiently transfected with the Effectene transfection kit (Qiagen, Inc.) according to the manufacturer’s protocol. Thirty h after transfection, cells were washed with PBS and lysed with reporter buffer (Promega) at room temperature for 15 min followed by the luciferase assay. Luciferase activity was measured with a luminometer (MicroLumat LB96P; Berthold Technologies, Bad Wildbad, Germany) using the Dual-Glo luciferase assay kit (Promega). Renilla luciferase construct was used as an internal control to normalize the result. Triplicate readings were taken for each experiment, and SDs were calculated.

Results and Discussion

Hypoxia Protects Serum-Deprived MDA-MB-435 Cells from Apoptosis: Role of α6β1 Integrin. To assess the importance of the α6β1 integrin for the survival of tumor cells in stress conditions, we used MDA-MB-435 cells, which express α6β1 but not α6β4 (2, 11). Two approaches were used to modulate α6β1 expression in these cells. In the first approach, cells were sorted by fluorescence-activated cell sorting to obtain two distinct clonal populations that exhibited either relatively high or low α6 integrin expression. The second approach involved expression of a siRNA oligonucleotide specific for the α6 integrin subunit. Expression of this siRNA in MDA-MB-435 cells that expressed high levels of α6β1 resulted in an approximate 70% reduction in the surface expression of this integrin (Fig. 1B). This siRNA did not alter expression of the α5 integrin subunit (Fig. 1B).

The cell populations described above were maintained in either a normoxic or hypoxic environment in the presence of either 10 or 0.5% FBS for 24 h, and apoptosis was assessed by annexin-V-FITC staining (Fig. 1C). In normoxia, serum deprivation (0.5% FBS) increased the level of apoptosis by approximately 3–4-fold compared with cells maintained in 10% serum (Fig. 1C). Hypoxia, however, protected cells from apoptosis induced by serum deprivation as a function of α6β1 expression. More specifically, both the low-expressing α6 cells and the α6 siRNA-treated cells were significantly more apoptotic under these conditions than were the corresponding high-α6β1-expressing control cells (Fig. 1C). To assess the integrin specificity of these results, we used a siRNA to reduce expression of the α5β1 integrin, which is expressed in MDA-MB-435 cells at levels comparable with α6β1. As shown in Fig. 2A, expression of a siRNA specific for the α5 integrin subunit reduced surface expression of α5β1 by approximately 30%. This reduction in α5β1 expression, however, had no impact on apoptosis induced by serum deprivation under either normoxic or hypoxic conditions as assessed by annexin-V-FITC staining (Fig. 2B).

Hypoxic Stimulation of VEGF Transcription Is Dependent on α6β1. Given the recent reports that autocrine VEGF is necessary for the survival of breast and other carcinoma cells (6, 7, 12–15), we assessed whether the ability of hypoxia to prevent apoptosis is VEGF dependent. As shown in Fig. 3A, expression of an antisense VEGF oligonucleotide in high-α6-expressing cells reduced hypoxia-induced VEGF expression by approximately 60% in comparison with the sense oligonucleotide, and it increased the apoptosis of serum-deprived cells in hypoxia 2-fold. Furthermore, incubation of low-α6-expressing cells with exogenous recombinant VEGF165 inhibited the apoptosis induced by serum deprivation significantly (Fig. 3A). These findings led us to examine the hypothesis that α6β1 influences hypoxia-induced VEGF expression. In the presence of 10% serum, VEGF expression is relatively high and independent of α6β1 expression in MDA-MB-435 cells (data not shown). Serum deprivation for
Fig. 2. Expression of α6β1 but not α5β1 integrin is needed for protection from apoptosis by hypoxia. A, cells that express high levels of α6β1 were transfected with siRNAs specific for α6 or α5 or with scrambled sequences (scr). After 72 h, integrin expression was assessed by flow cytometry as in Fig. 1A. B, the populations of cells described above were assessed for apoptosis under the conditions described in Fig. 1C. Apoptosis is reported as the percentage of annexin V-FITC−propidium iodide (PI−) cells. The data shown are mean values (±SD) of a representative experiment performed in triplicate. Ab, antibody.

24 h, however, reduced VEGF expression to nearly undetectable levels in normoxia (Fig. 3B). In contrast, hypoxia sustained both total VEGF and secreted VEGF as a function of α6β1 expression (Fig. 3B). These data indicate that serum induces VEGF expression independently of α6β1, but in the absence of serum, VEGF expression decreases and apoptosis increases. Hypoxia prevents this increase in apoptosis in serum-deprived cells by inducing VEGF expression in an α6β1-dependent manner.

To address the mechanism by which α6β1 influences hypoxia-induced VEGF expression, we quantified VEGF mRNA expression by real-time PCR. Hypoxia increased VEGF mRNA expression approximately 2-fold in serum-deprived cells with high α6β1 expression but not in cells with reduced expression of this integrin (Fig. 3C). This finding prompted us to evaluate the mechanism by which this integrin contributes to VEGF transcription. For this purpose, cells were transfected with a reporter construct that consisted of the intact VEGF promoter conjugated to luciferase. The relative luminescence of the reporter construct increased approximately 3-fold in hypoxia but only in those populations of cells that expressed high levels of α6β1 (Fig. 3D). The ability of hypoxia to increase VEGF promoter activity in cells selected for low α6β1 expression or treated with the α6 siRNA was negligible (Fig. 3D). To exclude the possibility that the apoptosis induced by these conditions affects VEGF promoter activity, cells were incubated with the general caspase inhibitor ZVAD-FMK. The use of this inhibitor prevented the apoptosis triggered by serum deprivation and low α6 expression (data not shown). The effect of α6β1 on VEGF transcription appears to be independent of its ligation because we observed comparable VEGF expression when cells were plated on either laminin, an α6β1 ligand, or other matrix proteins that are not ligands for this integrin such as collagen (data not shown).

MDA-MB-435 cells lack expression of VEGFR-1 (flt-1) and VEGFR-2 (KDR, flk-1; Refs. 16 and 17). However, they express neuropilin-1 (17), and the expression of this VEGF receptor did not differ between high- and low-α6-expressing cells, as evidenced by immunoblotting (data not shown) suggesting that the α6β1 integrin does not influence the expression of VEGF receptors.

The α6β1 Integrin Regulates the Transcriptional Activity of HIF-1. We examined the hypoxic induction of HIF-1α expression as a function of α6β1 expression because HIF-1 plays a pivotal role in stimulating the transcription of VEGF and many other genes in hypoxia (18). Interestingly, as shown in Fig. 4A, the level of HIF-1α induction in hypoxia was comparable between the high and low α6 clones, suggesting that α6β1 integrin is not involved in either the expression or stabilization of HIF-1α. To assess HIF-1 activation directly, we monitored the association of HIF-1 with its coactivator, p300 (Fig. 4A). HIF-1 binding to HRE itself is not sufficient to activate target gene transcription, and HIF-1 association with p300 is required to recruit the RNA polymerase II complex to initiate transcription (19). Therefore, association of HIF-1 with p300 is an indicator of HIF-1 activation. Association of HIF-1 with p300 was maximal at 6 h in cells that expressed high levels of α6β1 and diminished by 24 h (Fig. 4A). In cells that expressed low levels of α6β1, however, association of HIF-1 with p300 was barely detectable (Fig. 4A). The total level of p300 expression is similar in both populations of cells (Fig. 4A). These data indicate that the α6β1 integrin influences HIF-1 activation but not its expression.

To confirm the involvement of HIF-1 in VEGF transcription under hypoxia, we used a luciferase construct conjugated with a VEGF promoter (0.35 kb) that lacks the HRE (Fig. 4B). We also expressed HIF-1 dominant-negative mutants that block HIF-1 binding to HRE. Expression of this mutant promoter, as well as the two different dominant-negative HIF-1 mutants, in high-α6-expressing cells level did not result in hypoxia-induced increase in VEGF promoter activity (Fig. 4B), indicating that HIF-1 is playing a major role in α6β1-mediated VEGF transcription in hypoxia.

Our consideration of possible mechanisms involved in α6β1 regulation of HIF-1 activation led us to PKC because of the report that this integrin associates with specific PKC isoforms (20) and other studies that have linked PKCs to HIF-1 activation, VEGF expression, apoptosis, and survival signaling (21–24). Pharmacological inhibition of PKC-α activity using Go6976 prevented α6β1-mediated HIF-1 activation as measured by p300 association (Fig. 4C). The finding that PKC-α activity is necessary for HIF-1 activation raised the possibility that HIF-1 activation could be induced in cells that expressed low levels of α6β1 by expression of a constitutively active form of PKC-α. Indeed, as shown in Fig. 4C, expression of a constitutively active form of PKC-α increased HIF-1 activity dramatically in cells that normally could not activate HIF-1 under hypoxia (Fig. 4A). Expression of a constitutively active form of PKC-α, in contrast, did
not rescue the ability of these cells to activate HIF-1 under hypoxia (Fig. 4D). Collectively, these data highlight a key role for PKC-α in HIF-1 activation and VEGF transcription.

To date, the mechanism of HIF-1 activation has focused on two different posttranslational modifications at the COOH-terminal activation domain of HIF-1α that are known to regulate the association of p300 with HIF-1α. One modification is the hydroxylation of a specific asparagine residue (amino acid 803 of HIF-1α) at
the COOH-terminal activation domain that prevents the association of p300 with HIF-1 in normoxia (25, 26). The other modification is the phosphorylation of a threonine residue (amino acid 844 of HIF-1/α) that is required for their association (27). An important issue that arises, therefore, is how PKC-α facilitates HIF-1 activation and how this signaling pathway is linked to these other signaling pathways that have been implicated in HIF-1 activation. Our data indicate that PKC-α regulates the association of HIF-1 and p300 in hypoxia, which excludes a role for PKC-α in regulating asparagine hydroxylase because it is inactive in hypoxia. More likely, PKC-α is directly or indirectly involved in COOH-terminal activation domain phosphorylation to activate HIF-1.

Overall, our studies highlight a potential function for the α6β1 integrin in stimulating VEGF transcription and providing a selective survival advantage for carcinoma cells in the tumor microenvironment in which both nutrients and oxygen supply are limited. Moreover, these findings reveal a mechanism that could account for the involvement of the α6β1 integrin in tumor survival that has been observed in vivo, and they are consistent with the finding reported here and elsewhere (2, 3) that breast carcinoma cells depend upon autocrine VEGF for survival. These findings may also bear on other aspects of VEGF function in cancer, as well as other targets of VEGF transcription that could contribute to tumor survival. The best-characterized functions of VEGF, produced by both tumor and stromal cells, are to increase vascular permeability (28) and to stimulate angiogenesis (18, 29). Thus, it can be inferred that the α6 integrin influence on VEGF transcription will also impact these core VEGF functions.

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