The Influence of Oxygen Tension and pH on the Expression of Platelet-derived Endothelial Cell Growth Factor/Thymidine Phosphorylase in Human Breast Tumor Cells Grown in Vitro and in Vivo

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Abstract

We report that hypoxia regulates and influences the level of the angiogenic enzyme platelet-derived endothelial cell growth factor (PD-ECGF), also called thymidine phosphorylase, in vitro and in vivo. Levels of PD-ECGF protein increased 6-fold in the breast cancer cell line MDA 231 after 16 h of growth in 0.3% oxygen. A simultaneous increase in enzyme activity was observed. Immunohistochemical staining of MDA 231 tumors grown in nu/nu mice showed increased expression of PD-ECGF in those parts of the tumor that are proximal to the areas of necrosis. In addition, increased and widespread staining for PD-ECGF protein was obtained when the tumor vascular supply was occluded for 2 h by clamping. Lowering the media pH to 6.3—6.7 in vitro also resulted in an increase in PD-ECGF protein levels. This study demonstrates that tumor microenvironmental factors can result in the specific up-regulation of an angiogenic enzyme that can also activate 5-fluorouracil prodrugs and hence is exploitable therapeutically.

Introduction

The development of new blood vessels (angiogenesis) is an essential stage in the growth and metastasis of solid tumors. The vasculature of tumors is relatively poor and disordered when compared to that of normal tissue, leading to insufficient perfusion and diffusion of oxygen and microregional variation in extracellular pH. The existence of hypoxic (low-oxygen) conditions uniquely found within tumors can increase gene expression of a variety of proteins including the angiogenic factor VEGF2 in tumor cells (1).

The expression of VEGF in turn correlates with high vascular density and is a negative prognostic indicator for disease progression and the outcome of therapy for a variety of solid tumors (2, 3). However, the presence of elevated levels of other angiogenic factors in tumors can also be of prognostic importance. In particular, PD-ECGF, which is known to be chemotactic and angiogenic (4), has been shown to be a prognostic indicator in breast (5), ovarian (6), bladder (7), and colorectal tumors (8) and in some instances has been associated with the invasiveness and malignancy of tumors. Indeed, overexpression of PD-ECGF in MCF-7 breast carcinoma cells has markedly enhanced tumor growth and vascular density (9). Very little is otherwise known of the regulation of PD-ECGF within the tumor environment.

PD-ECGF is a 55-kDa polypeptide (10) existing in vivo as a 110-kDa homodimer. It was originally isolated from platelets as an endothelial mitogen. The cDNA sequence predicts a 482-amino acid polypeptide (4). PD-ECGF has been shown to have chemotactic activity in vitro and angiogenic activity in vivo. It is identical to TP, which catalyzes the reversible phospholytic cleavage of thymidine and deoxyuridine to their corresponding bases and 2-deoxyribose-1-phosphate (11). It has been shown that TP activity is critical for the angiogenic activity of PD-ECGF (12) and that 2-deoxy-D-ribose, a dephosphorylated product of the thymidine to thymine breakdown, also has chemotactic and angiogenic properties that may be responsible for the angiogenic activity of PD-ECGF (13).

The purpose of this work was to investigate PD-ECGF/TP protein regulation and expression in tumor microenvironmental conditions and compare the results with what is known of the expression of other angiogenic growth factors such as VEGF.

Materials and Methods

Cell Culture. Because PD-ECGF has been implicated in the pathogenesis of breast cancers, the MDA 231 breast carcinoma cell line, passage 20—30, was used. Cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, penicillin (50 IU/ml), and streptomycin (50 μg/ml) and passaged weekly.

Antibodies. A goat polyclonal antibody was compared to both a monoclonal and a rabbit polyclonal antibody (R. B.) on a Western blot of both wild-type and transfected breast cancer cell lysates (7) and found to produce a single band of 55 kDa, as reported previously (7).

In Vitro Hypoxia. For hypoxic exposure, cells were seeded at 5 × 10^4 on 6-cm permanox plates (Nunc) and gassed with the appropriate oxygen and nitrogen gas concentration containing 5% carbon dioxide at 37°C for 16 h in a humidified incubator.

In Vitro Acidic Exposure. Acidic exposure was achieved by adjusting the pH of NaHCO_3-deficient medium using 100 mM HCl and 7.5% NaHCO_3 in 75-cm² cell culture vessels seeded with 2 × 10^6 cells (with or without 10% FCS) and incubated at 37°C in a humidified 5% carbon dioxide and air mix for 16 h.

In Vitro Cobalt Chloride Exposure. Cells were seeded as for acidic exposure using 10% FCS-supplemented RPMI 1640 (pH 6.8) or serum-free RPMI 1640. The cells were exposed to 50—250 μM cobalt chloride for 16 h.

Cell Lysate Preparation and TP Activity Measurement. Cell lysates were prepared by harvesting and washing unfixed cells in PBS buffer, followed by sonication in lysis buffer [0.1 mM DTT, 0.15 mM NaCl, and 50 mM Tris-HCl (pH 7.4)] and centrifugation at 12,000 rpm for 1 min. The supernatant was snap-frozen in liquid nitrogen. TP activity measurements were made on cell lysates by incubating with 10 mM thymidine and 50 mM K_2HPO_4 for 16 h, quenching with 500 mM NaOH, and measuring the formation of thymine spectrophotometrically at 300 nm.

In Vivo Localization of PD-ECGF. Male nu/nu mice were injected s.c. with 5 × 10^6 MDA 231 cells in the dorsal area in a volume of 0.1 ml. Ten animals...
were used per group. When the tumors reached 600 mm³ (6–7 weeks), they were clamped for 2 h to occlude the blood supply. Controls were not clamped. Tumors were excised immediately after clamp removal and fixed and paraffin-embedded for histology. Sections were stained with hematoxylin and anti-PD-ECGF antibody (R & D Systems) conjugated with a bridging biotinylated horseradish peroxidase antibody (DAKO, United Kingdom) and developed with diaminobenzidine (Sigma) to produce a visible brown precipitate.

Procedures were performed with approved protocols and in accordance with the Scientific Procedures Act (1986), United Kingdom Home Office License No. PPL30/00386.

Results

PD-ECGF levels were raised in MDA 231 cells 1.5-fold by 16 h of catalyst-induced anoxia, levels increased linearly with increasing oxygen concentration up to a maximum of 6-fold at 0.3% oxygen (Fig. 1). Little or no induction was seen from a concentration of 0.3–1% oxygen. Similar effects were seen with the human non-small cell lung line A549 and the human colon carcinoma cell line HT29 (results not shown).

In one repeated experiment in which both fold induction and enzyme activity were measured in the same sample, hypoxic cells showing a 2.85-fold increase in staining for PD-ECGF showed increased TP activity from 514–1359 ng thymidine cleaved/mg protein/h, indicating that the elevated protein was enzymatically active. The RNase protection assay indicated a small but significant increase (17%) in TP mRNA after a 16-h exposure. Thus there is a hypoxically induced increase of PD-ECGF protein with an accompanying increase in TP activity in vitro. It is not known whether PD-ECGF is regulated by the same hypoxia-enhancer elements that have been found in the genes encoding erythropoetin (14) and VEGF (15); these elements are known to respond to cobalt stimulation and to hypoxia in vitro in a similar fashion. Cells treated with cobalt chloride for 16 h (50–250 μM) in the absence of serum show an increase in PD-ECGF levels (2.0 ± 0.25-fold; n = 3), indicating that the response may be similar to that seen in the above. The addition of serum blocks this response.

In addition, lowering of extracellular pH has long been considered a physiological condition of many solid tumors. An increase in staining (FACS analysis) for PD-ECGF protein in MDA 231 cells is observed when extracellular pH is reduced from 6.8–7.0 to 6.3–6.7 in the absence of serum (Fig. 2); cells cultured in the absence of serum for 16 h seemed adherent and viable. This increase in PD-ECGF levels at low pH is lost in the presence of serum. These levels of pH may be found in microregions of solid tumors (16).

A combination of 0.3% oxygen exposure with serum-free medium resulted in a much-reduced viability of the cells, so the effect of serum depletion in hypoxia could not be investigated.

MDA 231 xenograft tumors in nude mice showed high levels of staining for PD-ECGF protein in areas proximal to necrotic tissue where both low pH and oxygen tension are likely to exist. (Fig. 3a). A 2-h clamping to occlude the tumor blood supply produced a marked, uniform increase in PD-ECGF staining throughout the tumor (Fig. 3b). FACS analysis on disaggregated tumor cells indicated an overall increase in the levels of staining for PD-ECGF in the clamped tumors (1.85 ± 0.60-fold compared to that of unclamped controls; n = 3).

Discussion

Hypoxia and aberrant angiogenesis are conditions characteristic of disease states including rheumatoid arthritis (17), psoriasis (18), and solid tumors. The presence of PD-ECGF is a common feature in all of these diseases.

Increased expression of VEGF has been reported with oxygen concentrations ranging from catalyst-induced anoxia to 1% O₂ (see Ref. 19 for review). PD-ECGF has a more restricted response range to oxygen, at least in the MDA 231 cell line. That the mechanism of hypoxic induction of PD-ECGF may be similar to that of VEGF and erythropoetin in vitro is indicated by its response to cobalt stimulation. It is unclear, however, why the addition of serum ablates the response of PD-ECGF to cobalt, because both VEGF and erythropoetin are reported to respond in the presence of serum (20).

Acidic pH is not necessarily a condition associated with the incidence of hypoxia in solid tumors because tumor cells are known to preferentially convert glucose and other substrates to lactic acid even under aerobic conditions. Recently it has been clarified that it is extracellular rather than intracellular pH that is acidic in solid tumors and that tumor cells maintain neutrality by enhanced extrusion and lactate retention (16). Hence, increases in PD-ECGF caused by acidic pH may be another, separate contributor to tumor angiogenesis.

A study of PD-ECGF/TP activity in breast cancer reported the activities in 138 patients to range over 2 orders of magnitude and concluded that activity in cancer tissue is correlated to malignancy (21). Increases in PD-ECGF protein and TP activity may be one of the reasons that areas of hypoxia are a negative influence on the outcome of some therapies and overall prognosis.

The localization of staining for PD-ECGF around necrotic regions in MDA 231 xenografts suggests that it is also up-regulated by hypoxia in vivo. Without precise measurement by oxygen electrodes, it is difficult to accurately assess the level of hypoxia in this environment, but it is probable that oxygen tensions approaching anoxia are reached. This

Fig. 3. a, tissue section of MDA 231 tumor xenograft immunostained with anti-PD-ECGF antibody. Magnification, ×16. b, tissue section of MDA 231 tumor xenograft immunostained with anti-PD-ECGF antibody after a 2-h occlusion of the blood supply. Magnification, ×16.

would to some extent concur with the in vitro data, especially because the extracellular pH may be low. Clamping the xenografts would produce radiobiological hypoxia (less than 0.1% O2) and may also affect ATP status and extracellular pH within the tumor (22, 23). The presence of PD-ECGF staining within necrosis may be due to the release of PD-ECGF from dead tumor cells, from the viable tumor cells around the necrosis, or from the migration of PD-ECGF-producing immune cells into the necrotic tissue (macrophages and so forth). The PD-ECGF gene does not encode a classical secretion signal, and the question of how it exerts its mitogenic, chemotactic, and angiogenic properties remains. Suggestions that the breakdown product 2-deoxystreptozotocin ribose may be responsible have been made, but it is possible that PD-ECGF is released by a nonclassical pathway.

The importance of PD-ECGF as an angiogenic substance with increased expression in many human tumors, together with its enzymatic activity, makes PD-ECGF a potential target both for antiangiogenic and enzyme-directed drug development. Drugs such 5-fluorouracil or Furtun does not encode a classical secretion signal, and the question of how it exerts its mitogenic, chemotactic, and angiogenic properties remains. The importance of PD-ECGF as an angiogenic substance with increased expression in many human tumors, together with its enzymatic activity, makes PD-ECGF a potential target both for antiangiogenic and enzyme-directed drug development. Drugs such as 5-fluorouracil or Furtun may be potentiated by TP enzyme activity (24, 25), are already under clinical investigation. The environmentally regulated tumor-specific increases in the levels of PD-ECGF demonstrated in this paper lend further consideration to its therapeutic potential.

Acknowledgments

We thank S. Townsend for assistance with FACS analysis, T. M. Hacker for histology, D. Pocock and J. Clarke for technical assistance with the in vivo work, A. Patterson for analysis of antibody specificity, and J. Crew for RNA protection analysis.

References


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*Cancer Res* 1997;57:570-572.

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