Advances in Brief

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 nude 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 VEGF 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 chemoattractant 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 chemoattractant 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 clonal and a rabbit polyclonal antibody (R. B.) on a Western blot of both.

In Vitro Hypoxia. For hypoxic exposure, cells were seeded at 5 × 10⁵ 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 NaHCO₃-deficient medium using 100 μM HC1 and 7.5% NaHCO₃ in 75-cm² cell culture vessels seeded with 2 × 10⁶ 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) and serum-free RPMI 1640. The cells were exposed to 50–250 μM cobalt chloride for 16 h.

FACS Analysis. After exposure, the cells were fixed in 70% ethanol for 30 min, washed with PBS containing 0.01% FCS, and stained with anti-PD-ECGF polyclonal antibody raised in goat (R & D Systems, United Kingdom) at a dilution of 1:1000 for 30 min at room temperature. The cells were washed, and anti-goat FITC conjugate was raised in rabbit (Poole, Sigma, United Kingdom) added at a dilution 1:100 for 30 min at 20°C before a final wash. FACS analysis on samples was performed using excitation at 488 nm using a Becton Dickinson FACS sort machine and expressed as fold increase in fluorescence over control.

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 K₂PO₄ 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 nude mice were injected s.c. with 5 × 10⁶ MDA 231 cells in the dorsal area in a volume of 0.1 ml. Ten animals

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: VEGF, vascular endothelial growth factor; PD-ECGF, platelet-derived endothelial cell growth factor; TP, thymidine phosphorylase; FACS, fluorescence-activated cell sorting.
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 nu/nu 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 of carbon dioxide 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

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3 J. Crew, personal communication.
Extracellular pH may be low. Clamping the xenografts produces hypoxic or anoxic conditions that can affect the angiogenic status and extracellular pH within the tumor (22, 23). The presence of hypoxia and the potential for exploitation in cancer therapy. Br. J. Cancer, 74: 5126—5132, 1996.


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