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Acid-Mediated Tumor Proteolysis: Contribution of Cysteine Cathepsins[1,2]

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Abstract

One of the noncellular microenvironmental factors that contribute to malignancy of solid tumors is acidic peritumoral pH. We have previously demonstrated that extracellular acidosis leads to localization of the cysteine protease cathepsin B on the tumor cell membrane and its secretion. The objective of the present study was to determine if an acidic extracellular pH such as that observed *in vivo* (i.e., pHe 6.8) affects the activity of proteases, e.g., cathepsin B, that contribute to degradation of collagen IV by tumor cells when grown in biologically relevant three-dimensional (3D) cultures. For these studies, we used 1) 3D reconstituted basement membrane overlay cultures of human carcinomas, 2) live cell imaging assays to assess proteolysis, and 3) *in vivo* imaging of active tumor proteases. At pHe 6.8, there were increases in pericellular active cysteine cathepsins and in degradation of dye-quenched collagen IV, which was partially blocked by a cathepsin B inhibitor. Imaging probes for active cysteine cathepsins localized to tumors *in vivo*. The amount of bound probe decreased in tumors in bicarbonate-treated mice, a treatment previously shown to increase peritumoral pHe and reduce local invasion of the tumors. Our results are consistent with the acid-mediated invasion hypothesis and with a role for cathepsin B in promoting degradation of a basement membrane protein substrate, i.e., type IV collagen, in an acidic peritumoral environment.

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Abbreviations: 3D, three-dimensional; ABP, activity-based probe; DQ-collagen IV, dye-quenched collagen IV; pHe, extracellular pH; rBM, reconstituted basement membrane; RFP, red fluorescent protein

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[2]This article refers to supplementary materials, which are designated by Figures W1 to W7 and Movies W1 to W8 and are available online at www.neoplasia.com.
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Introduction

Reprogramming of energy metabolism is one of two emerging cancer hallmarks that have been added to the original six hallmarks of cancer [1]. The concept that metabolism is altered in cancer dates back to the observation made by Otto Warburg that tumor cells exhibit increased glucose fermentation despite the presence of oxygen [2]. Byproducts of elevated glycolytic activity result in an acidic extracellular pH (pHe) that is heterogeneous within a tumor and changes during tumor progression [for review, see [3]]. Acidification has been attributed to a combination of factors including chaotic tumor vasculature, increased glycolysis, and diminished buffering capacity of tumor interstitial fluids. These factors lead to high concentrations of extracellular lactic acid, which may be toxic to normal and cancer cells. Many cancer cells acquire acid-resistant phenotypes that allow them to survive and proliferate when the pHe is acidic [4].

Acidification of the tumor microenvironment has been shown to increase invasiveness and metastasis, leading to the mathematically modeled and tested hypothesis that acidification is critical to an invasive phenotype [5,6]. According to this hypothesis, acidification occurs as a result of glycolysis both in the presence of oxygen (Warburg effect) and during intermittent hypoxia, causing toxicity in the surrounding normal stroma and thereby providing empty space for tumor cell proliferation and invasion. Acidosis or elevated glycolysis has been shown to persist in areas of adequate oxygen supply [7–9]. Treatment of mouse models with orally available buffers neutralized intratumoral pH and reduced spontaneous and experimental metastasis [10,11]. The elevation of tumoral pHe was shown to reduce the activity of cathepsin B, a lysosomal cysteine protease that has an acidic pH optimum [11].

Proteases have been implicated in every stage of cancer progression, from initiation and growth to invasion and metastasis [12]. Cysteine cathepsins participate in proteolytic networks that mediate cancer progression [for reviews, see [13,14]]. We now hypothesize that analyzing cells cultured at neutral pH may have overemphasized the role of proteases that have neutral pH optima. As the acidification of the tumor microenvironment could favor the activity of proteases such as cysteine cathepsins that normally function at an acidic pH, this led us to reexamine proteolysis and the proteases that are secreted and active at the acidic pHe surrounding solid tumors. For these studies, we used a three-dimensional (3D) reconstituted basement membrane (rBM) overlay model that provides a context for mechanisms critical to both mammary gland development and neoplastic processes, as has been described [for reviews, see [15,16]]. We are building on previous studies that have shown that an acidic pHe leads to lysosomal redistribution and secretion of cathepsin B by malignant cells [17]. Our study confirms that cathepsin B is active pericellularly in 3D cultures of breast and colon carcinoma cells and is one of the proteases that participate in the enhanced degradation of collagen IV observed at an acidic pHe.

Materials and Methods

Materials

DMSO, Dulbecco's modified Eagle's medium (DMEM), Hepes, $NaHCO_3$, Pipes, and all other chemicals not otherwise noted were obtained from Sigma (St Louis, MO). FBS was purchased from Hyclone (Logan, UT), CA074 and E64 from Peptides International (Louisville, KY), Z-Arg-Arg-NHMec from Bachem (Torrance, CA), pimonidazole HCl from Hypoxyprobe (Burlington, MA), and Ventana OmniMap anti-Rb HRP from Roche (Indianapolis, IN). rBM: *in vitro* studies used reduced growth factor Cultrex from Trevigen (Gaithersburg, MD); *in vivo* studies used Matrigel (BD Biosciences, San Jose, CA). Click-iT EdU (5-ethynyl-2′-deoxyuridine) microplate assays, dye-quenched collagen IV (DQ-collagen IV), CellTracker Orange, Hoechst 33342, live/dead assay kit, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and trypsin EDTA were purchased from Life Technologies (Grand Island, NY). Pierce Protein A/G Plus Agarose beads (Thermo Scientific, Rockford, IL) and anti-rabbit cathepsin B, anti-rabbit pre-immune IgG [18], anti-rabbit cathepsin S (a kind gift of Dr Boris Turk, Jozef Stefan Institute, Ljubljana, Slovenia), and anti-rabbit cathepsin L (a kind gift of Dr Ekkehard Weber, Martin Luther University, Halle, Germany) antibodies were used for immunoprecipitation. GB123, an activity-based probe (ABP) for cysteine cathepsins [19], was provided by Dr Matthew Bogyo (Stanford University, Palo Alto, CA).

Tissue Culture

Low passages of MDA-MB-231 [American Type Culture Collection (ATCC), Manassas, VA] and MDA-MB-231 stably transfected with red fluorescent protein (RFP) and luciferase [11], and HCT116 (ATCC) and Hs578T (ATCC) cells were maintained in phenol red–free DMEM supplemented with 10% FBS and penicillin/streptomycin at pH 7.4 for no more than 4 weeks. Cells were monitored by microscopy to confirm that they maintained their original morphology and were regularly screened for mycoplasma by microscopy (MycoFluor; Life Technologies) and reverse transcription–polymerase chain reaction (LookOut; Sigma). Media for pH experiments were additionally buffered to maintain pH 7.4 or 6.8 with 2 g/l sodium bicarbonate, 25 mM Pipes, and Hepes and then incubated overnight at 37°C in 5% CO_2 [11] and adjusted to either pH 7.4 or 6.8. Cultures were maintained in humidified conditions under atmospheric oxygen levels and 5% CO_2. For subculturing, cells were detached from uncoated tissue culture flasks with 0.05% trypsin EDTA.

Live Cell Assays for Proteolysis and Active Cysteine Cathepsins

A detailed protocol for the live cell proteolysis assay has been published [20]. Briefly, glass coverslips in 35-mm dishes were coated with 45 μl of Cultrex containing 25 mg/ml DQ-collagen IV and placed in a 37°C incubator for 10 minutes to allow solidification. Approximately 50,000 cells were seeded on top of the Cultrex and incubated at 37°C and pH 7.4 for 30 to 60 minutes until adherent, at which point either pH 7.4 or 6.8 culture media containing 2% rBM were applied. Media were changed the following day. For inhibitor studies, 20 μM CA074/E64 or an equal volume of diluent (DMSO) was added to the Cultrex before solidification as well as to the medium overlay. To visualize active cysteine cathepsins in the 3D cultures, 1 μM GB123, an ABP [19], was added to the media 16 to 18 hours before imaging. Cells were washed in phosphate-buffered saline (PBS) to remove any unbound probe and returned to probe-free complete growth medium at either pH 7.4 or 6.8 for at least 1 hour before imaging. After nuclei were labeled with Hoechst 33342, imaging was performed with a Zeiss LSM 510 META NLO confocal microscope using a 40× water-dipping objective. Under all conditions, the parameters for acquisition of images were identical (laser power, detector gain and offset, and so on). Quantification by the software is based on true pixel intensities, which are raw data from