ANTICANCER RESEARCH *25*: 2841-2850 (2005)

# Hypoxia Alters Cathepsin B / Inhibitor Profiles in Oral Carcinoma Cell Lines

NALINIE S. WICKRAMASINGHE[1a], KASTURI BANERJEE[1b], NAGATHIHALLI S. NAGARAJ[1], NADARAJAH VIGNESWARAN[3] and WOLFGANG ZACHARIAS[1,2]

*[1]Department of Medicine, James Graham Brown Cancer Center and*
*[2]Department of Pharmacology and Toxicology, University of Louisville, Louisville, Kentucky 40202;*
*[3]Departments of Diagnostic Sciences and Dental Public Health, The University of Texas Health Science Center at Houston, Dental Branch, Houston, Texas 77030, U.S.A.*

**Abstract.** *Background: The tumor microenvironment is believed to contribute to the malignant properties of tumor cells in heterogeneous tumor tissues. We investigated the impact of hypoxia (1% oxygen) on the expression of cathepsin B and its natural inhibitors cystatin B and C. Materials and Methods: Patient-matched oral carcinoma cell lines from primary tumor and lymph node metastasis were used to study the effects of hypoxia on proliferation, protein expression, and proteolytic and inhibitor activities. Results: Hypoxic growth led to elevated cathepsin B expression and activity, and this effect was greater in metastatic than in primary tumor cells. Also, hypoxia led to down-regulation of the inhibitors cystatin C and B, resulting in increased residual activity of cathepsin B. Conclusion: These data suggest that the invasive and/or metastatic potential of cells may be enhanced under hypoxia by increasing cathepsin-mediated proteolysis. The results provide strong evidence for the involvement of cathepsin B and its cystatin inhibitors in hypoxia-enhanced tumor progression.*

The microenvironment within tumor tissues is determined by multiple factors, such as co-existence of tumor cells with non-malignant stromal fibroblast cells, diminished oxygen and nutrient supply due to fast growth of tumor cells or deficient angiogenesis, and altered growth milieu with respect to pH and growth factors. These factors have been found to play an important role in tumor development and organ-specific metastasis (1). Clinical and experimental studies have shown an association of tumor microenvironmental hypoxia with aggressive tumor growth, metastasis and poor response to treatment (2-6). As a response to hypoxia, up-regulation of HIF-1 and -2 transcription factors has been reported in many types of cancer (4, 6-9). HIF-1 binds to a hypoxia-responsive element (HRE) (RCGTG) in the promoter or enhancer of various hypoxia-inducible genes, which include erythropoietin, vascular endothelial growth factor, glucose transporters and glycolytic enzymes, as well as genes involved in iron metabolism and cell survival (5, 9, 10). However, there may be other unidentified genes that are regulated by a hypoxic tumor microenvironment and may contribute to the malignant progression of tumors, presumably by selecting for highly malignant subpopulations of cells (2, 8, 11).

Cathepsins are ubiquitous proteolytic enzymes present in all mammalian cells. Cathepsin B belongs to the lysosomal cysteine protease class and participates in intracellular protein turnover and post-translational processing of biologically important protein precursors (12-14). Such proteolytic enzymes account for a diverse range of physiological processes, including tissue remodeling during embryogenesis and development, wound healing, antigen presentation, bone resorption and programmed cell death. Besides their involvement in normal physiological functions, cathepsins have been implicated in tumor growth, angiogenesis, invasion and metastasis (15, 16). The activity of cathepsin B is regulated by endogenous inhibitors from the cystatin superfamily (17). Decreased expression of cysteine protease inhibitors (CPIs) in tumor tissues and cells

[a]*Present address:* Department of Biochemistry and Molecular Biology, University of Louisville, Louisville, Kentucky 40202, U.S.A.
[b]*Present address:* Laboratory of Cellular and Molecular Biology, Gerontology Research Center, National Institute on Aging/NIH; Baltimore, MD 21224, U.S.A.

*Correspondence to:* Wolfgang Zacharias, Ph.D., Associate Professor, Department of Medicine and Department of Pharmacology and Toxicology, James Graham Brown Cancer Center, University of Louisville, Delia Baxter Bldg. 321F, 580 South Preston Street, Louisville, KY 40202, U.S.A. Tel: 502-852-2579, Fax: 502-852-2356, e-mail: w0zach01@gwise.louisville.edu

*Key Words:* Hypoxia, cathepsin, cystatin, oral carcinoma.

has been associated with some cancers (18). An imbalance between cathepsins and CPIs may be associated with the development of a more aggressive, invasive cell phenotype, secondary tumor formation and metastases (19, 20). Such an imbalance between cathepsins and cystatin inhibitors may be mediated by external as well as intra-tumor microenvironmental factors (19).

Since the effects of hypoxia on oral cancer progression have not been determined, the goal of our current work was to investigate the proteolytic potential of oral cancer cells in response to hypoxia. Here, the effects of micro-environmental hypoxia on the expression of cathepsin B, cystatins B and C, and cell proliferation rates were studied in a panel of uniquely matched primary and metastatic oral carcinoma cell lines.

## Materials and Methods

*Cell lines and reagents.* MDA686Tu (686Tu) and MDA686Ln (686Ln) cell lines were derived from the primary tumor and lymph node metastasis, respectively, of oral squamous cell carcinoma involving the left tonsillar fossa and posterior portion of the tongue in a 49-year-old man (tumor stage T3N3B). MDA1386Tu (1386Tu) and MDA1386Ln (1386Ln) cell lines were obtained from the primary tumor and lymph node metastasis, respectively, of a 71-year-old male patient with primary hypopharynx tumor (tumor stage T4N3B). All cell lines were generous gifts from Dr Peter Sacks, New York University, New York, USA (21). They were routinely maintained in DMEM /F12 1:1 (v/v) mix containing 10% fetal bovine serum and 0.4 μg/ml hydrocortisone at 37˚C with 5% $CO_2$.

Antibodies were obtained from the following sources: rabbit polyclonal antibodies for human cathepsin B (Athens Research and Technology, Athens, GA, USA) or human cystatin C (Novus Biologicals, Littleton, CO, USA); mouse monoclonal antibodies for human cystatin B (Axxora, San Diego, CA, USA) or human HIF-1α (Pharmingen, San Diego, CA, USA). Fluorescent-labeled peptide protease substrates were obtained from Sigma (St. Louis, MO, USA), and protease inhibitor L-trans-epoxy-succinyl-Ile-Pro-OH propylamide (CA-074) from Peptides International (Louisville, KY, USA).

*Hypoxia exposure.* Hypoxic conditions were produced by placing logarithmic phase subconfluent cultures in a modular incubator chamber equilibrated with humidified gas containing 1% oxygen, 5% $CO_2$ and 94% nitrogen. The cell lines were grown under hypoxic conditions in serum-free media for periods of 24 or 48 h; control cells were grown in normal oxygen conditions for the same duration.

*Proliferation assay.* MTT-based proliferation assays were performed by seeding 5,000 cells each into 96-well plates. The cells were cultured overnight to attach, and MTT viability assays were performed at 0, 24, 48 or 72 h of hypoxia exposure. MTT (1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; Sigma) was added to each well at 0.5 mg/ml, the reaction was terminated after 4 h by addition of 100 μl of 20% SDS in 50% dimethylformamide and the plates were incubated overnight at 37˚C for total solubilization of reduced MTT. The wells were analyzed on a spectrophotometric ELISA plate reader at 570 nm wavelength, and viable cell numbers determined based on a standard curve.

*Western blotting.* Cellular and secreted proteins were obtained after exposing serum-free cell cultures to 24 or 48 h of hypoxia. To obtain cellular proteins, cells were washed in ice-cold phosphate-buffered saline and protein was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). Media proteins were concentrated using Centriplus centrifugal filter devices (Amicon Bioseparation, Bedford, MA, USA). Protein concentrations were determined using the Bradford assay with the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as standard. After gel separation in 12% polyacrylamide gels under denaturing conditions, proteins (20 to 50 μg/lane) were electroblotted onto PVDF membrane and nonspecific binding was blocked with 5% Carnation non-fat dried milk in Tris-buffered saline (TBS). After washing, the membranes were incubated with antibodies for cathepsin B (1:5,000 dilution), cystatin C (1:4,000 dilution), cystatin B (1:4,000 dilution), or HIF-1α (1:250 dilution). Antibody binding to the respective protein bands was visualized using peroxidase-conjugated goat anti-rabbit or anti-mouse antisera followed by enhanced chemiluminescence detection. Densitometric quantitation of bands was done using a GS-700 Imaging Densitometer and Molecular Analyst imaging program (Bio-Rad). The averages of three independent determinations were calculated for each plot and error ranges calculated with Microsoft Excel.

*Preparation of proteins for activity assays.* Cellular and secreted activities for cathepsin B and cysteine protease inhibitors were measured as described (22). Cells plated in 100-mm dishes were grown to 75% confluence, rinsed three times with phosphate-buffered saline and placed in serum-free medium. After 24 h or 48 h of hypoxic or normoxic growth, the medium was removed, centrifuged at 300 x g to remove cell debris, ammonium sulfate was added to make an 80% solution and the suspension was put on ice for 15 min. After centrifugation at 17,000 x g for 15 min, the pellet was dissolved in cold 0.01 M Tris-HCl, 0.01 M NaCl, pH 8.0 and dialyzed overnight against the same buffer at 4˚C. A 150-μl aliquot of the dialyzed sample was diluted to 450 μl with water, and secreted cathepsin B was activated by adding 50 μl of 1.0 M sodium formate, pH 5.5 for 30 sec at 37˚C. Following activation, cathepsin B activity was measured as described below. For cellular protein fractions, the corresponding cell layers were homogenized with lysis buffer (400 mM $NaH_2PO_4 \cdot H_2O$, 75 mM NaCl, 4 mM EDTA, 0.25% Triton-X 100, pH 6.0), allowed to stand for 1 h on ice, ultrasonicated for 60 sec at 40 W and centrifuged at 25,000 x g (10 min, 4˚C) to remove cell debris (23). For both cell extracts and media concentrates, total protein amounts were determined according to the Bradford assay with bovine serum albumin as standard (24).

*Cathepsin B activity assays.* The activity of cathepsin B was determined fluorimetrically using the cathepsin B-specific methyl-coumarylamide substrate N-CBZ-L-arginyl-L-arginine 7-(4-methyl) coumarylamide (Z-Arg-Arg-NHMec) at pH 6.0 (23, 25). Prior to starting the assays, reaction buffer (250 mM $NaH_2PO_4 \cdot H_2O$, 2.5 mM EDTA, 5 mM dithiothreitol, pH 6.0) was pre-warmed to 37˚C. Then 100 μl of cell extract diluted in lysis buffer (70-100 μg protein) was added and mixed, followed by incubation at 37˚C for 5 min. Finally, 200 μl substrate solution (1 mM) was added to the pre-incubation mixture. The reaction was stopped after 10 min at 37˚C by addition of 2.0 ml of 100 mM monochloroacetic acid in

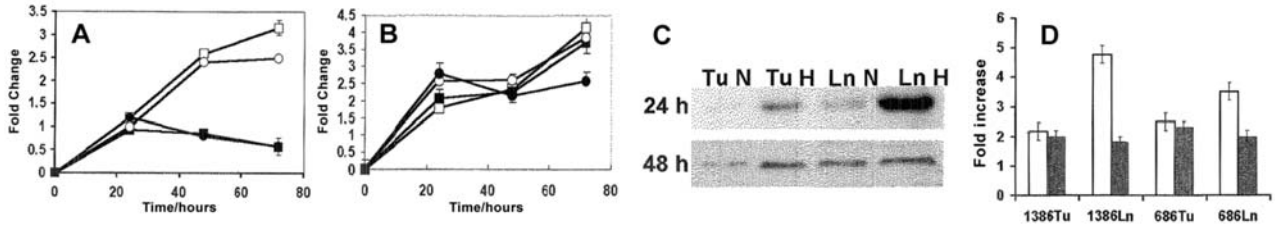


Figure 1. *Cellular response to hypoxia. MTT proliferation assays were performed for A: 686Tu (squares) and 686Ln (circles) cells, and B: 1386Tu (squares) and 1386Ln (circles) cells after growth from 0 to 72 h under either normoxia (open symbols) or hypoxia (closed symbols). The increases in proliferating cells relative to 0 h was plotted for each cell line and condition. Also, HIF-1α induction under hypoxic growth condition was determined by Western blots on cellular protein extracts. C: Western blots for HIF-1α on 686Tu (Tu) and 686Ln (Ln) cell extracts after 24 h or 48 h of normoxic (N) or hypoxic (H) growth; D: HIF-1α induction levels plotted as fold-increases under hypoxia relative to normoxia for 24 h (open bars) or 48 h (closed bars) of exposure.*

100 mM Na-acetate, pH 4.3. Fluorescence F was measured with an excitation wavelength of 360 nm and emission wavelength of 460 nm. The system was calibrated with 80 mM chloroacetic acid (F=0) and 0.1 μM-aminomethylcoumarin (F=100) in Na-acetate buffer. A measured ΔF of 100 represents 25 μ-units of enzyme activity in a 10-min assay (1 unit is the release of 1 μmol of product/min/mg of protein). Since Z-Arg-Arg-NHMec is also a weak substrate for cathepsin L, the cathepsin B-specific inhibitor L-trans-epoxy-succinyl-lle-Pro-OH propylamide (CA-074; 50 μM) was used in all control measurements in order to quantitate the measured activities which were due to cathepsin B.

*Cysteine protease inhibitor activity assays.* Total CPI activity was measured by incubating the cell extract or media proteins with the exogenous cysteine protease papain. Samples were boiled for 5 min to denature the endogenous cysteine protease and the denatured proteins were removed by centrifugation; cystatins are stable under these conditions (26). Sample aliquots were then incubated with 10 μl of 10 mM papain and, assay for papain activity was essentially the same as that used for cathepsin B activity. The units of inhibitory activity were calculated by subtracting the residual papain activity in tubes containing sample aliquots from the total activity of exogenously added papain.

## Results

*Effects of hypoxia on cell proliferation and morphology.* The MTT assays showed that cell proliferation was not significantly affected in the four cell lines by hypoxia treatment up to 24 h. At prolonged exposure, a slight reduction in cell growth rates became apparent for 1386Tu and 1386Ln after 48 h, and for 686Tu and 686Ln after 24 h (Figure 1A, 1B). This indicates that hypoxia treatment for up to 48 h had no severe toxic effects on the cells. However, at 72 h of hypoxia there was some decrease in the number of viable cells in comparison to the normoxic control. No changes in cellular morphologies up to 48 h of hypoxia compared to normoxic controls were observed for all four cell lines (data not shown).

*Effects of hypoxia on HIF-1α expression.* There was measurable expression of HIF-1α in all the cell lines even under normoxic growth conditions; however, under hypoxic conditions, HIF-1α protein was transiently induced to higher levels in comparison to the normoxic control. (Figure 1C, 1D). Maximum increases in HIF-1α expression were seen after 24 h of hypoxia for 1386Tu (2.2-fold), 1386Ln (4.8-fold), 686Tu (2.5-fold) and 686Ln (3.5-fold). After 48 h of hypoxia, the elevated HIF-1α expression for 1386Tu, 1386Ln, 686Tu and 686Ln was found to be 2.0-, 1.8-, 2.3- and 2.0-fold, respectively, relative to normoxic conditions. For both cell line pairs, the Ln cells had a higher HIF-1α induction than the corresponding Tu cells.

*Effects of hypoxia on cathepsin B protein levels.* Under normoxic conditions, the metastatic cell lines consistently secreted more cathepsin B (1386Ln 1.7-fold, 686Ln 2.3-fold) than the corresponding Tu lines. Furthermore, cathepsin B levels were significantly increased at 48 h of hypoxia, and these increases were more pronounced in the Ln lines than in the Tu lines (Figure 2). The metastatic 1386Ln cells showed a higher increase (4.3-fold secreted and 3.8-fold cellular) than 1386Tu (1.5-fold secreted and 1.6-fold cellular). Also, for 686Ln cells, a 2.6-fold increase in secreted and 1.4-fold increase in cellular cathepsin B was observed, whereas for 686Tu cells only minor decreases in the expression of cellular (0.7-fold) and secreted (0.9-fold) cathepsin B compared to the normoxic control were observed (data not shown). These data indicate that both cellular and secreted cathepsin B protein levels were increased due to hypoxia treatment in 1386Tu, 1386Ln and 686Ln cell lines, whereas the 686Tu line did not show any significant response.

*Effects of hypoxia on cystatin B protein levels.* Under normoxic conditions for up to 48 h, the expression of

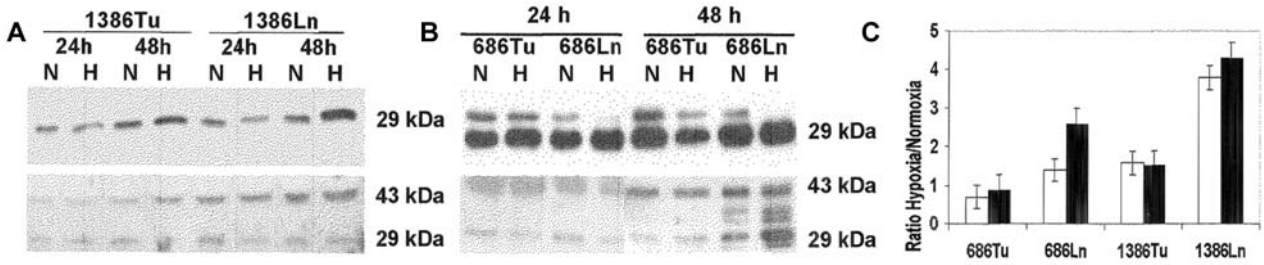


Figure 2. *Expression of cellular and secreted cathepsin B under normoxia and hypoxia. Western blots are shown for the cell lines 1386Tu and 1386Ln (A) or 686Tu and 686Ln (B) at 24 or 48 h of hypoxia (H) or normoxia (N); C: Ratios hypoxia/normoxia of cathepsin B band intensities for the 48 h data. Cell extracts: top gels in A, B, open bars in C; media: bottom gels in A, B, closed bars in C.*

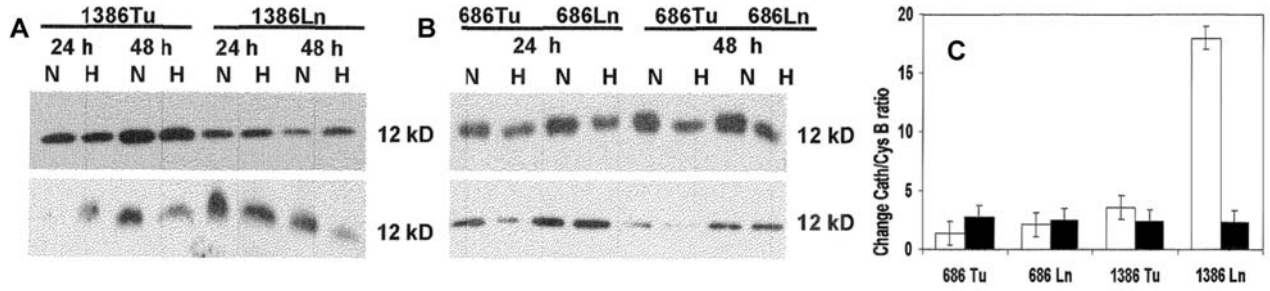


Figure 3. *Expression of cellular and secreted cystatin B under normoxia and hypoxia. Western blots are shown for the cell lines 1386Tu and 1386Ln (A) or 686Tu and 686Ln (B) at 24 or 48 h of hypoxia (H) or normoxia (N). C: Changes of the cathepsin B/ cystatin B ratios for hypoxia relative to normoxia at 48 h of treatment. Cell extracts: top gels in A, B, open bars in C; media: bottom gels in A, B, closed bars in C.*

cellular cystatin B was similar for 686Ln and 686Tu cells, whereas higher cellular expression was seen in 1386Tu (1.8-fold) compared to 1386Ln cells (Figure 3A, 3B). After 48 h of hypoxia, a 2-fold decrease in the expression of cellular cystatin B was found for both 686Tu and 686Ln cells; the secreted cystatin B expression was decreased about 3-fold in 686Tu cells, with no apparent change in expression for 686Ln cells (Figure 3A, 3B). Hypoxia did not have any effect on the expression of this inhibitor in the 1386Tu and 1386Ln cells.

*Changes of cathepsin B / cystatin B ratios.* In order to monitor changes in net residual protease activities, the changes of cathepsin/cystatin ratios among different conditions and cell lines were determined based on intensity values of Western blot bands. Evidently, the band intensity ratios of cathepsin *versus* cystatin bands are only arbitrary since they are based on different antibody-antigen interactions. However, relative fold-changes of such arbitrary ratios can be informative to reveal alterations of cathepsin *versus* cystatin expression, as long as the intensity values to calculate a ratio are taken from the same gel exposure for each target protein. If the cathepsin B band for sample 1 is 4-fold stronger than for sample 2 on one blot, and the cystatin B band for the same sample 1 is 2-fold weaker than for sample 2 on a separate blot, then it is safe to conclude that in sample 1 there is an 8-fold higher cathepsin to cystatin B ratio compared to sample 2. Such changes of cathepsin/cystatin ratios were determined for hypoxic *versus* normoxic conditions. This approach showed that under hypoxic conditions the protease/inhibitor balance is shifted several-fold towards higher protease expression, mainly by lowering the cystatin B levels for both cellular and secreted proteins (Figure 3C). This was consistently observed in repeat experiments for all four cell lines; remarkably, for 1386Ln cell extracts this shift was much higher (~18-fold higher cathepsin/cystatin B ratio than under normoxia) than for all other cells and protein fractions (~2- to 4-fold higher).

*Effects of hypoxia on cystatin C protein levels.* In general, secreted cystatin C levels were higher than cellular levels, and expression levels were significantly higher (2.0 - 2.5-fold) in the primary tumor (686Tu and 1386Tu) than in the metastatic (686Ln and 1386Ln) cell lines (Figure 4A, 4B). Under normoxic conditions, cellular and secreted cystatin C

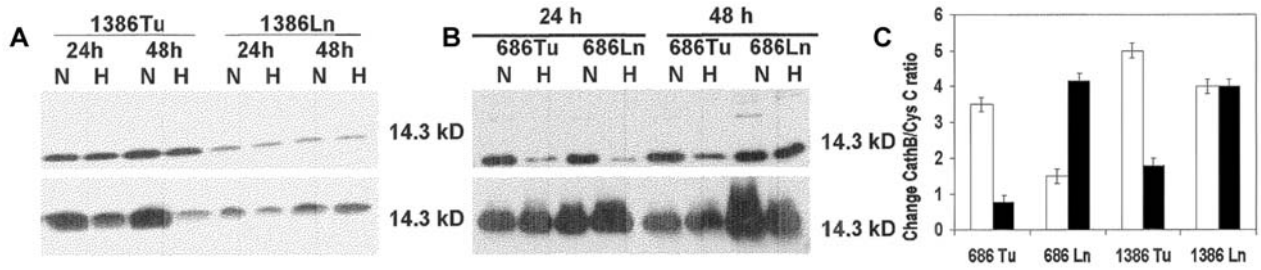


Figure 4. *Expression of cellular and secreted cystatin C under normoxia and hypoxia. Western blots are shown for the cell lines 1386Tu and 1386Ln (A) or 686Tu and 686Ln (B) at 24 or 48 h of hypoxia (H) or normoxia (N); C: Changes of the cathepsin B/cystatin C ratios for hypoxia relative to normoxia at 48 h of treatment. Cell extracts: top gels in A, B, open bars in C; media: bottom gels in A, B, closed bars in C.*

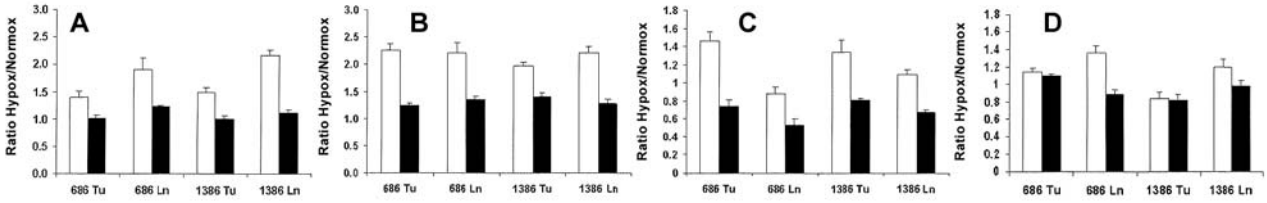


Figure 5. *Activity assays for cathepsin B and CPIs in cell lines. The ratios of specific activities obtained for hypoxia versus normoxia are plotted for the four cell lines, either for cellular extracts (open bars) or conditioned media (closed bars). A: cathepsin B activity, 24 h; B: cathepsin B activity, 48 h; C: CPI activity, 24 h; D: CPI activity, 48 h. Means±S.D. of three independent experiments in duplicate assays are shown.*

expression levels were comparable in 686Tu and 686Ln cells, whereas 1386Tu cells produced higher cellular (3.6-fold) and secreted (2.5-fold) cystatin C than 1386Ln cells. Densitometric quantitations of the respectiveWestern blots revealed that, under hypoxic conditions for up to 48 h, total cystatin C expression (cellular and secreted) was significantly decreased in all four cells (686Tu: 30%; 686Ln: 34%; 1386Tu: 55%; 1386Ln: 18%). No significant change in cellular cystatin C levels due to hypoxia treatment was observed in 1386Ln and 1386Tu cell lines, but their secreted cystatin C levels were lowered several-fold under hypoxia. About a 4-fold decrease in cellular cystatin C expression, without significant change for the secreted form (1.1-fold increase), was found in 686Tu at 48 h hypoxia, and 686Ln showed decreases in both the cellular (1.3-fold) and secreted (1.5-fold) forms.

*Changes of cathepsin B / cystatin C ratios.* The ratios of cathepsin B to cystatin C under 48 h hypoxia, determined as described above, were significantly increased in cellular extracts (1.5 to 5 times) and in secreted proteins (1 to 4 times) of all cell lines (Figure 4C). Interestingly, these increased ratios were higher for the secreted forms in the metastatic lines, but were higher for the cellular forms in the primary tumor lines, bearing in mind that, in general, most of the cystatin C produced was secreted. These findings showed that the extracellular cathepsin B activity of metastatic cell lines under hypoxia appears to be higher than those of the primary tumor cell lines.

*Cathepsin and CPI activities.* Functional cathepsin B proteolytic activity was assayed at pH 6.0 by using Z-Arg-Arg-NHMec as a substrate after a pre-activation incubation step; under this condition, total (active and pro-from) cathepsin B activity was measured. Since the peptide substrate also has some weak activity for other cysteine proteases, overlapping activity was controlled for by assaying in the presence of the cathepsin B-specific inhibitor L-trans-epoxy-succinyl-lle-Pro-OH propylamide (CA 074). In this way, cathepsin B activities, and in parallel CPI activities, in cells grown under hypoxia or normoxia for 24 h or 48 h, were determined (Figure 5).

Cathepsin B activities consistently increased for all cell lines under hypoxic conditions relative to normoxia, and these increases were more pronounced after prolonged exposures (Figure 5A, 5B; 24 *versus* 48 h). The highest increases were seen in cellular cathepsin activities (up to 2.4-fold) for cell extracts, and also as secreted activities at 48 h of hypoxia (up to 1.5-fold). The CPI activities were not affected as much as the protease values for hypoxia *versus* normoxia, yet showed minor decreases for some of the cell lines (Figure 5C, 5D). For the inhibitors, the decreases were

most pronounced in the secreted proteins (down to 0.6-fold). Overall, these combined activity assays demonstrated that the net cathepsin B proteolytic activities increased in response to hypoxia, in agreement with the above described protein analyses by Western blots.

Interestingly, the initial increases relative to normoxia were higher in 686Ln and 1386Ln than in 686Tu and 1386Tu in the cell extracts, and to a smaller degree in the media (Figure 5A, 5B). These changes were accompanied by generally larger decreases for the Ln relative to the Tu cells in their CPI activities (Figure 5C, 5D). Thus, the net increases in cathepsin B protease activities after 24 or 48 h hypoxia, and also the differential changes increase in Ln *versus* Tu phenotypes, correlated well with the respective decreases in inhibitor activities. Altogether, the combined protease and CPI activity assays support the conclusion from Western blot data and confirm that the cathepsin/CPI balance and net protease activity are altered in hypoxia-exposed cells towards more proteolytic extracellular activity compared to normoxic growth.

## Discussion

*Cell proliferation or morphology.* The proliferation rates and cellular morphologies of these cells were not significantly affected by hypoxia, indicating that short-term exposure to hypoxia exerts no toxic effects on these cells. Their ability to survive and proliferate under hypoxia is in agreement with other studies (27). An *in vivo* study on murine anaplastic sarcoma showed that malignant cells have the ability to proliferate in a hypoxic environment (28). A similar study showed that, in contrast to normal cells, a subset of leukemic cells proliferated during hypoxia, and this subpopulation subsequently renewed and expanded the leukemic cell load (28). Here, no increase in cell proliferation of 686Tu and 686Ln cells was observed after 24 h hypoxia in comparison to normoxia, which might be due to the slowing down of the cells in S- and G2/M-phase. Hypoxic cells can progress through the cell cycle, although their rate of progression is slower than that of normally oxygenated cells (27). Although extended hypoxia is lethal as a direct stress trigger, hypoxic zones in solid tumors harbor viable cells which are resistant to treatment and contribute to disease relapse (29). It is well established that hypoxia can drive the tumors into a more aggressive mode by promoting apoptosis, thereby selecting against p53-competent cells which are unable to follow this pathway and also promote the formation of new blood vessels (29).

*HIF-1α expression.* HIF-1α has been identified as a principal transcription factor that regulates cellular responses to physiological and pathological hypoxia (30, 31). All oral carcinoma cell lines had measurable expression of HIF-1α even under normoxic growth conditions; however, under hypoxic conditions, HIF-1α protein is transiently induced to higher levels. This is consistent with the up-regulation of HIF-1α expression observed in other human cancers such as breast, lung, head and neck, or brain relative to the benign counterparts, and marked differences were also observed between the malignant and metastatic forms of these cancers (8, 9, 32). Significant association between HIF-1α overexpression and patient mortality was shown in cancers of the brain, breast, cervix, oropharynx, ovary and uterus (32, 33). Under normoxic conditions, HIF-1α protein is rapidly degraded, presumably *via* the ubiquitin-proteasome pathway such that very low levels of protein are detected in the cytoplasm. However, HIF-1α protein significantly accumulates in response to hypoxia (34). In the oral cell lines used here, exposure to hypoxia resulted in higher levels of HIF-1α protein, and this effect was more prominent in the metastatic cells than in the primary tumor cells. This is a novel finding since no such comparison of primary and metastatic oral cancer cell lines has been reported so far. This finding may indicate that the effects of hypoxia on HIF-1α regulation or processing may be different between primary and metastatic oral carcinomas.

*Cellular expression and secretion of cathepsin B.* Cellular cathepsin B expression and activities were increased when exposed to 48 h hypoxia in the cell lines 1386Tu, 1386Ln and 686Ln. However, in 686Tu cells, both the expression and activity of cathepsin B were slightly decreased at 48 h hypoxia. Under normoxic conditions, the metastatic cell lines consistently secreted more cathepsin B than their corresponding primary tumor cells. Under hypoxia, secreted cathepsin B expression was found to increase in the metastatic (1386Ln, 686Ln) and the primary tumor 1386Tu cell lines, whereas 686Tu did not show a similar effect. These observations indicate that hypoxia has varying effects depending on the cell line and phenotype, consistent with other studies showing that increases in the abundance of cathepsin B transcript and protein correlated with an increase in tumor grade and alterations in subcellular location and activity of cathepsin B (35). The increases in the secreted cathepsin B levels under hypoxic conditions also agree with observations that hypoxic treatment led to an increase in cathepsin B expression and enhanced metastatic potential of murine tumor cells (36). It has been proposed that extracellular cathepsin B, together with other classes of proteases, *i.e.* cathepsin D, plasminogen activators and matrix metalloproteinases, are all subject to an activation cascade. Because of altered or compromised regulation of such cascades in tumor tissue, enzyme activation may

result in uncontrolled proteolysis of the extracellular matrix components believed to be necessary for local and metastatic spread (14, 37, 38). This also agrees with the previously described association of cathepsin B with tumor progression (14, 39).

*Cystatin B and C changes.* Since the activities of cathepsin proteases are regulated by their endogenous cystatin inhibitors, the balance between protease and cystatin may be important in tumor progression (23). We observed that the total expression (cellular and secreted forms) of cystatins C and B were down-regulated under hypoxia in 686Tu, 686Ln and 1386Tu cells, which resulted in increased net activities of cathepsin B. However, no such reduction in cystatin expression was seen in 1386Ln cells at 48 h. In the latter cells, the cathepsin B expression was so high that down-regulation of inhibitors may not be necessary for increasing the activity of cathepsin B. In 686Tu and 686Ln cells, the cellular expression of both cystatins B and C was diminished by exposure to hypoxia; however, their secreted cystatin levels were not affected. In contrast, hypoxia treatment of 1386Tu and 1386Ln cells decreased the secreted levels of the two inhibitors without showing any apparent effect on cellular expression. These results indicate that, although there are cell-specific effects of hypoxia, the net result is a decrease in the total cystatin levels which, in turn, increases the activity of cathepsin B.

There is evidence that cathepsin B and its endogenous cystatin inhibitors are implicated in the invasive behavior of squamous cell carcinoma of the head and neck, showing that higher levels of cystatins correlated significantly with longer survival probability (37). Overexpression of cystatin C has been shown to diminish the invasiveness of murine B16 melanoma cells (19, 40).

Our data showed that in all four cell lines, secreted cystatin C levels were higher than the cellular levels, and its expression levels were significantly higher in the primary tumor cell lines (686Tu and 1386Tu) than in the metastatic cell lines (686Ln and 1386Ln). More importantly, the ratio of secreted cathepsin B to secreted cystatin C under 48 h hypoxia was higher in both metastatic Ln cell lines than their respective primary tumor cell lines Tu. These findings document that the net cathepsin B activity of the metastatic cell lines was higher than that of the primary tumor cell lines.

*Changes of Cathepsin B versus CPI levels and residual proteolytic activities.* Under hypoxic conditions, we observed increases in cathepsin B proteolytic activity levels in both cell types, and these correlated well with the respective protein expression levels determined by Western blots. Overall, CPI activity was essentially decreased if cathepsin B activity was increased, and reduced CPI activity was observed in the metastatic Ln cells compared to primary Tu cells. Our results demonstrated that total cellular and secreted CPI activity decreased for hypoxia-treated 686 and 1386 cultures, resulting in a net increase in cathepsin B proteolytic activity. Depending on the cell type and treatment period, the cells released a variable portion of pro-cathepsins into the medium. The portion of secreted relative to total cathepsin activity was apparently reduced as a consequence of increased accumulation of cathepsin activity within the cells. Our cathepsin B and CPI activity results correlated well for both cell lysates and secreted proteins, and confirmed that the overall net activity of cathepsin B was altered in these cell types. There is prior evidence that an increase of CPI reduces the levels of cysteine-type cathepsins, and that the enzymatic activity of tumor-associated proteases is counter-balanced by specific inhibitors (41, 42).

*Possible regulatory mechanisms.* HIF-1α has been shown to function as a master regulator of numerous hypoxia-inducible genes under hypoxic conditions (4, 5). More than 60 putative target genes of HIF-1 have been identified, including cathepsin D (43). Most probably, other genes that have not been reported yet are regulated by the hypoxic environment, and it is possible that hypoxia may exert its effects on cathepsin B and cystatin B/C in these oral cancer cells *via* alteration of HIF-1α. However, direct evidence for HIF-1α-induced transcriptional up-regulation of cathepsin B is still lacking. Microarray-based gene expression profiling experiments on hypoxia-dependent gene expression changes in oral carcinoma cell lines may provide such evidence and are in progress.

*Pathological implications.* The redistribution of cathepsin B within tumor cells, as well as the increased expression in tumor cells adjacent to the extracellular matrix, suggest that proteases can be mobilized to regions of tumor invasion. On the other hand, *in vitro* and *in vivo* studies have shown that protease inhibitors can reduce the invasive and metastatic capabilities of tumor cells (44). Our results revealed that hypoxic growth leads to elevated cathepsin B expression and activity in oral cancer cell lines, and that this effect is greater in metastatic than in primary tumor cell lines, indicating that high levels of cathepsin B may play a role in tumor metastatic spread. Additionally, hypoxia leads to down-regulation of the cysteine protease inhibitors cystatin C and B, which contributes to the increased net activity of cathepsin B. Our results provide strong support for the involvement of the cathepsin B / cystatin balance in hypoxia-enhanced tumor cell malignancy. Our data convey, for the first time, that cathepsins and their inhibitors may be responsive to HIF-1α protein by increasing the net proteolytic activity, and that this response may be dependent on the malignant phenotype of the differently-

staged oral carcinoma cell lines. These findings will be most useful for the development of new therapies for oral cancer targeted at cathepsin B or, more apically, at HIF-1α.

## Acknowledgements

Supported by NIH grant DE#13150, by Philip Morris USA Inc. and Philip Morris International (W.Z.), and by a postdoctoral fellowship award from the University of Louisville Brown Cancer Center U.S.A. (N.N.). N.W., K.B. and N.N. contributed equally to this work.

## References

1 Witz IP: The tumor microenvironment – introduction. Sem Cancer Biol *12*: 87-88, 2002.

2 Subarsky P and Hill RP: The hypoxic tumor microenvironmental and metastatic progression. Clin Exp Metast *20*: 237-250, 2003.

3 Wykoff CC, Beasley NJP, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talk KL, Maxwell PH, Pugh CW, Ratcliffe PJ and Harris A: Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res *60*: 7075-7083, 2000.

4 Le Q-T, Denko NC and Giaccia AJ: Hypoxic gene expression and metastasis. Cancer Metast Rev *23*: 293-310, 2004.

5 Michiels C: Physiological and pathological responses to hypoxia. Am J Pathol *164*: 1875-1882, 2004.

6 Krishnamachary B, Berg-Dixon S, Kelly B, Agani F, Feldser D, Ferreira G, Iyer N, LaRusch J, Pak B, Taghavi P and Semenza GL: Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. Cancer Res *63*: 1138-1143, 2003.

7 Burke B, Giannnousdis A, Corke KP, Gill D, Wells M, Ziegler-Heitbrock L and Lewis CE: Hypoxia-induced gene expression in human macrophages. Implication for ischemic tissue hypoxia regulated gene therapy. Am J Pathol *163*: 1233-1243, 2003.

8 Koukourakis MI, Giatromanolaki A, Sivridis E, Pastorek J, Karapantzos I, Gatter KC and Harris AL: Hypoxia-activated tumor pathways of angiogenesis and pH regulation independent of anemia in head-and-neck cancer. Int J Radiat Oncol Biol Physics *59*: 67-71, 2004.

9 Mohamed KM, Le A, Duong H, Wu Y, Zhang Q and Messadi DV: Correlation between VEGF and HIF-1α expression in human oral squamous cell carcinoma. Exp Mol Pathol *76*: 143-152, 2004.

10 Huang E and Bunn HF: Hypoxia-inducible factor and biomedical revelance. J Biol Chem *278*: 19575-19578, 2003.

11 Nagaraj NS, Vigneswaran N and Zacharias W: Hypoxia-mediated apoptosis in oral carcinoma cells occurs *via* two independent pathways. BMC Mol Cancer *3*: 38, 2004.

12 Agarwal SK: Cathepsins - a view. Biochem Edu *18*: 67-72, 1990.

13 Turk B, Turk D and Turk V: Lysosomal cysteine proteases: more than scavengers. Biochim Biophys Acta *1477*: 98-111, 2000.

14 Roshy S, Sloane BF and Moin K: Pericellular cathepsin B and malignant progression. Cancer Metast Rev *22*: 271-286, 2003.

15 Coulibaly S, Schwihla H, Abramhamson M, Albini A, Cerni C, Clark JS, Ng KN, Katunuma N, Schlappack O, Glossl J and Mach L: Modulation of invasion properties of murine squamous carcinoma cells by heterologous expression of cathepsin B and cystatin C. Int J Cancer *83*: 526-531, 1999.

16 Vigneswaran N, Zhao W, Dasanayake A, Muller S, Miller DM and Zacharias W: Variable expression of cathepsin B and D correlates with highly invasive and metastatic phenotype of oral cancer. Human Pathol *31*: 931-937, 2000.

17 Turk V and Bode W: Human cysteine proteinases and their inhibitors, stefins and cystatins. *In:* Innovations in Proteases and their Inhibitors (Avile FX, ed), Berlin: Walter de Gruyter, pp. 47-59, 1993.

18 Zajc I, Sever N, Bervar A and Lah TT: Expression of cysteine peptidase cathepsin L and its inhibitors stefin A and B in relation to tumorigenicity of breast cancer cell lines. Cancer Lett *18*: 185-190, 2002.

19 Kos J and Lah TT: Cysteine proteinases and their endogenous inhibitor: target protein for prognosis, diagnosis and therapy in cancer. Oncol Rep *5*: 1349-1361, 1998.

20 Vigneswaran N, Wu J and Zacharias W: Upregulation of cystatin M during the progression of oral squamous cell carcinoma from primary tumor to metastasis. Oral Oncol *39*: 559-568, 2003.

21 Sacks PG: Cell, tissue and organ culture as *in vitro* models to study the biology of squamous cell carcinomas of the head and neck. Cancer Metast Rev *15*: 27-51, 1996.

22 Troen BR, Ascherman D, Atlas D and Gottesman MM: Cloning and expression of the gene for the major excreted protein of transformed mouse fibroblasts. J Biol Chem *263*: 254-261, 1988.

23 Ulbricht B, Spiess E, Albiez RS and Ebert W: Quantification of intracellular cathepsin activities in human lung tumor cell lines by flow cytometry. Biol Chem Hoppe-Seyler *376*: 407-414, 1995.

24 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*: 248-254, 1976.

25 Barrett AJ and Krischke H: Cathepsin B, cathepsin H and cathepsin L. *In:* Methods in Enzymology Vol. 80. (Lorand L, ed.) New York, Academic Press, pp. 535-561, 1981.

26 Sen CL and Whitaker JR: Some properties of ficin-papain inhibitor from avian egg white. Arch Biochem Biophys *158*: 623-632, 1973.

27 Webster L, Hodgkiss RJ and Wilson GD: Cell cycle distribution of hypoxia and progression of hypoxic tumour cells *in vivo*. Br J Cancer *77*: 227-234, 1998.

28 Jensen PO, Mortensen BT, Hodgkiss RJ, Iversen PO, Christensen IJ, Helledie N and Larsen JK: Increased cellular hypoxia and reduced proliferation of both normal and leukaemic cells during progression of acute myeloid leukaemia in rats. Cell Prolif *33*: 381-395, 2000.

29 Schmaltz C, Hardenbergh PH, Wells A and Fisher DE: Regulation of proliferation-survival decisions during tumor cell hypoxia. Mol Cell Biol *18*: 2845-2854, 1998.

30 Wang GL, Jiang BH, Rue EA and Semenza GL: Hypoxia-inducible factor is a basic-helix-loop-helix-PAS heterodimer regulated by cellular $O_2$ tension. Proc Natl Acad Sci USA *92*: 5510-5514, 1995.

31 Greijer AE and van der Wall E: The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. J Clin Pathol *57*: 1009-1014, 2004.

32 Zhong H, De Marzo MD, Laugher E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL and Simons JW: Overexpression of hypoxia-inducible factor 1 α in common human cancers and their metastasis. Cancer Res *59*: 5830-5835, 1999.

33 Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ and Harris AL: The expression and distribution of hypoxia-inducible factor HIF-1α and HIF-2α in normal human tissues, cancers, and tumor-associated macrophages. Am J Pathol *157*: 411-421, 2000.

34 Jiang BH, Semenza GL, Bauer C and Martin HH: Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of oxygen tension. Am J Physiol *271*: 1172-1180, 1996.

35 Mohanam S, Jasti SL, Kondraganti SR, Chandrasekar N, Lakka SS, Kin Y, Fuller GN, Yung AWK, Kyritsis AP, Dinh DH, Oliver WC, Gujrati M, Ali-Osman F and Rao J: Down regulation of cathepsin B expression impairs the invasive and tumorigenic potential of human glioblastoma cells. Oncogene *20*: 3665-3673, 2001.

36 Cuvier C, Jang A and Hill RP: Exposure to hypoxia, glucoses starvation and acidosis: effect on invasive capacity of murine tumor cells and correlation with cathepsin (L+B) secretion. Clin Exp Metast *15*: 19-25, 1997.

37 Strojan P, Budihna M, Smid L, Svetic B, Vrhovec I, Kos J and Skrk J: Prognostic significance of cysteine proteinases cathepsin B and L and their endogenous inhibitors stefins A and B in patients with squamous cell carcinoma of the head and neck. Clin Cancer Res *6*: 1052-1062, 2000.

38 Kawasaki G, Kato Y and Mizuno A: Cathepsin expression in oral squamous cell carcinoma: relationship with clinicopathologic factors. Oral Surg Oral Med Oral Pathol Oral Radiol Endod *93*: 446-454, 2002.

39 Yan S, Sameni M and Sloane BF: Cathepsin B and human tumor progression. Biol Chem *379*: 113-23, 1988.

40 Sexton PS and Cox JL: Inhibition of motility and invasion of B16 melanomas by over-expression of cystatin C. Melanoma Res *7*: 97-101, 1997.

41 Saleh Y, Ziolkowski P, Siewinski M, Milach J, Marszalik P and Rybka J: The combined treatment of transplantable solid mammary carcinoma in Wistar rats by use of photodynamic therapy and cysteine proteinase inhibitor. In Vivo *15*: 351-357, 2001.

42 Siewinski M, Saleh Y, Popiela A, Ziolkowski P, Jelen M and Grybos M: Expression of high molecular weight cysteine proteinase inhibitor in ovarian cancer tissues: regulation of cathepsin B expression by placental CPI. Biol Chem *384*: 1103-1107, 2003.

43 Lee J-W, Bae S-H, Jeong J-W, Kim S-H and Kim K-W: Hypoxia-inducible factor (HIF-1)α: its protein stability and biological functions. Exp Molec Med *36*: 1-12, 2004.

44 Koblinski JE, Ahram M and Sloane BF: Unraveling the role of proteases in cancer. Clin Chim Acta *291*: 113-135, 2000.

*Received May 9, 2005*
*Accepted May 18, 2005*