Acidic Extracellular pH Promotes Experimental Metastasis of Human Melanoma Cells in Athymic Nude Mice

Einar K. Rofstad, Berit Mathiesen, Kristil Kindem, and Kanthi Galappathi

Group of Radiation Biology and Tumor Physiology, Department of Radiation Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo, Norway

Abstract

Extracellular pH (pH_e) is lower in many tumors than in the corresponding normal tissue. The significance of acidic pH_e in the development of metastatic disease was investigated in the present work. Human melanoma cells (A-07, D-12, and T-22) were cultured *in vitro* at pH_e 6.8 or 7.4 (control) before being inoculated into the tail vein of BALB/c *nu/nu* mice for formation of experimental pulmonary metastases. Cell invasiveness was studied *in vitro* by using Matrigel invasion chambers and angiogenesis was studied *in vivo* by using an intradermal assay. Protein secretion was measured by ELISA and immunocapture assays. Cells cultured at acidic pH_e showed increased secretion of proteinases and proangiogenic factors, enhanced invasive and angiogenic potential, and enhanced potential to develop experimental metastases. Acidity-induced metastasis was inhibited by treatment with the general matrix metalloproteinase (MMP) inhibitor GM6001, the general cysteine proteinase inhibitor E-64, or blocking antibody against vascular endothelial growth factor-A (VEGF-A) or interleukin-8 (IL-8). Our study indicates that acidic pH_e promotes experimental pulmonary metastasis in A-07, D-12, and T-22 human melanoma cells by a common mechanism involving acidity-induced up-regulation of the proteolytic enzymes MMP-2, MMP-9, cathepsin B, and cathepsin L and acidity-induced up-regulation of the proangiogenic factors VEGF-A and IL-8. One consequence of this observation is that treatment strategies involving deliberate tumor acidification to improve the efficacy of chemotherapy, photodynamic therapy, and hyperthermia should be avoided. Moreover, the possibility that the pH_e of the primary tumor may be an important prognostic parameter for melanoma patients merits clinical investigation. (Cancer Res 2006; 66(13): 6699-707)

Introduction

Tumor tissue gradually accumulates stable and unstable genomic alterations during growth, resulting in the development of an increasing number of aggressive phenotypic traits with time, a process termed malignant progression (1, 2). Concomitantly, most tumors develop a pathophysiologic microenvironment characterized by low oxygen tension (pO_2), elevated interstitial fluid pressure, low glucose concentration, high lactate concentration, low extracellular pH (pH_e), and/or energy deprivation (3, 4). This hostile microenvironment activates several transcription factors, including hypoxia-inducible factor-1, leading to up-regulated expression of a large number of gene products known to promote malignant progression and metastatic dissemination (5, 6).

Clinical investigations have shown that extensive hypoxia, elevated interstitial fluid pressure, and high lactate concentration in the primary tumor are associated with high incidence of regional and distant metastases and, hence, poor disease-free and overall survival rates in several histologic types of cancer (5–12). However, these clinical observations do not necessarily implicate that a hostile tumor microenvironment promotes metastasis and poor survival rates. An alternative interpretation is that the most aggressive and metastatic cell phenotypes develop the most hostile microenvironment.

Experimental studies addressing the mechanisms behind these clinical observations have been initiated in several laboratories and significant evidence that hypoxia (pO_2 <10 mm Hg) may promote metastasis has been provided (13–22). Thus, tumor cells exposed to hypoxia *in vitro* show enhanced invasiveness in Matrigel invasion chambers (13, 14) and enhanced lung colonization potential after i.v. inoculation in mice (15, 16). Moreover, murine and xenografted tumors with high hypoxic fractions metastasize more frequently than genetically equivalent control tumors with low hypoxic fractions, whether high hypoxic fractions are occurring naturally (17, 18) or are imposed by irradiating the tumor bed (19, 20) or by keeping the host mice in a low-oxygen atmosphere (21, 22). The enhanced metastatic potential has been shown to be caused by hypoxia-induced up-regulation of several metastasis-promoting gene products, including proteolytic enzymes, proangiogenic factors, and antiapoptotic proteins (13–23).

Experimental studies designed to investigate whether acidic pH_e (pH_e <7.0) may promote invasive growth and metastatic dissemination have given conflicting results (5, 24). Human melanoma cells cultured at acidic pH_e *in vitro* were found to show enhanced invasiveness in Matrigel invasion chambers (25) whereas enhanced invasiveness following exposure to acidic pH_e was not observed in similar experiments with rodent tumor cells (26). Rodent tumor cells exposed to acidic pH_e *in vitro* were found to show enhanced lung colonization potential when inoculated i.v. in syngeneic host mice (27, 28) whereas acidification of rodent tumors *in vivo*, obtained by administrating meta-iodo-benzylguanidine and/or glucose to the host mice, did not result in enhanced incidence of spontaneous lung metastases (29). Despite these conflicting observations, several studies have indicated that tumor cells exposed to acidic pH_e *in vitro* may show increased expression of several genes known to promote invasive growth and metastasis, including genes encoding matrix degrading enzymes and proangiogenic factors (5, 30). However, it is not clear whether this acidity-induced increase in gene expression is sufficient to enhance the metastatic potential of tumor cells.

Requests for reprints: Einar K. Rofstad, Department of Radiation Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway. Phone: 47-2293-4279; Fax: 47-2293-4270; E-mail: e.k.rofstad@labmed.uio.no.

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In the work reported here, we show unequivocally that growth at acidic pH_e *in vitro* enhances the potential of A-07, D-12, and T-22 human melanoma cells to form experimental pulmonary metastases in athymic nude mice. Because acute exposure to acidic pH_e has been shown to cause up-regulation of the proangiogenic factors vascular endothelial growth factor-A (VEGF-A; refs. 31, 32) and interleukin-8 (IL-8; refs. 33, 34), the matrix metalloproteinases (MMP) MMP-2 (gelatinase A) and MMP-9 (gelatinase B; ref. 35), and the cysteine proteinases cathepsin B and cathepsin L (28, 36), we hypothesized that these metastasis-promoting proteins were involved. To test this hypothesis, we measured the secretion of these proteins in cells cultured in acidic and normal medium and investigated whether treatment with proteinase inhibitors or neutralizing antibodies could inhibit acidity-induced invasiveness, angiogenesis, and experimental metastasis. The experiments gave results consistent with our hypothesis, showing clearly that acidity-induced up-regulation of VEGF-A, IL-8, MMP-2, MMP-9, cathepsin B, and cathepsin L enhanced the metastatic potential of A-07, D-12, and T-22 cells, thus providing significant evidence that acidity-induced up-regulation of these proteins is a possible mechanism for acidity-induced metastasis in malignant melanoma.

Materials and Methods

Mice and cell lines. Adult (8-10 weeks of age) female BALB/c *nu/nu* mice, bred and maintained as described elsewhere (18), were used to assess tumor angiogenesis and metastasis. Three human melanoma cell lines (A-07, D-12, and T-22), established as described elsewhere (37, 38), were included in the study. The animal experiments were approved by the Institutional Committee on Research Animal Care and were done according to the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing, and Education (New York Academy of Sciences, New York, NY).

Cell culture. The cells were grown as monolayers in plastic tissue culture flasks added RPMI 1640 (25 mmol/L HEPES and L-glutamine) supplemented with 13% bovine calf serum, 250 mg/L penicillin, and 50 mg/L streptomycin. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Experiments were carried out with cells in exponential growth, cultured in acidic (pH 6.8) or normal (pH 7.4) medium for 24 or 48 hours [i.e., the cells were plated and cultured in normal medium for 24 hours before the medium was removed and replaced by normal (control cultures) or acidic medium (test cultures)]. The pH of the medium was adjusted with 2-(*N*-morpholino)-ethanesulfonic acid and tris-(hydroxymethyl)-aminomethane to be 6.75 to 6.85 or 7.35 to 7.45 during experiments. The plating efficiency of the cells was measured by using a plastic surface colony assay (16).

Protein secretion. Commercial ELISA kits were used as described by the manufacturer to measure medium concentrations of human MMP-2 (R&D Systems, Abingdon, United Kingdom), MMP-9 (R&D Systems), cathepsin B (R&D Systems), cathepsin L (Calbiochem, San Diego, CA), VEGF-A (R&D Systems), and IL-8 (Endogen, Woburn, MA). Furthermore, medium concentrations of the active and latent forms of the MMPs (aMMP and pMMP, respectively) were measured by using the MMP-2 and MMP-9 Biotrak immunocapture enzyme activity assays (GE Healthcare, Piscataway, NJ) according to the instructions of the manufacturer.

Invasion. Cell invasiveness was determined *in vitro* by using 24-well Matrigel invasion chambers with 8-μm pore polycarbonate membranes precoated with a thin layer of Matrigel Basement Membrane Matrix (BD Biosciences, Cowley, United Kingdom). The chambers were rehydrated in serum-free medium as described by the manufacturer. Complete medium (750 μL) was used as chemoattractant. Suspensions of 5×10^4 cells in 500 μL of complete medium (pH 7.4) were added to the wells and incubated for 24 hours at 37°C in 5% CO_2 in air. Cells remaining on the upper membrane surface were removed with a cotton swab whereas the cells on the lower surface were fixed in methanol, stained with hematoxylin, and counted by examining 10 randomly selected fields at ×40 magnification. Effects of proteinase activity inhibition were studied by using a neutralizing anti-human MMP-2 mouse monoclonal antibody (Chemicon, Temecula, CA), a neutralizing anti-human MMP-9 mouse monoclonal antibody (Chemicon), the general MMP inhibitor GM6001 (Chemicon), and the general cysteine proteinase inhibitor E-64 (Sigma, St. Louis, MO). The anti-MMP-2 antibody shows no cross-reaction with aMMP-9 and pMMP-9. The anti-MMP-9 antibody recognizes aMMP-9, pMMP-9, and MMP-9 complexed with tissue inhibitor of metalloproteinase-1 and shows no cross-reaction with aMMP-2 and pMMP-2. The antibodies and proteinase inhibitors were added to the cell suspensions immediately before the cells were transferred to invasion chambers. Nontoxic concentrations of 5 μg/mL of antibody, 15 μmol/L of GM6001, or 10 μmol/L of E-64 were used in all experiments.

Angiogenesis. Angiogenic potential was measured *in vivo* by using an intradermal angiogenesis assay as described elsewhere (39). Aliquots of 1×10^6 cells in 10 μL of serum-free medium were inoculated intradermally in mice. The mice were killed 7 days later when small tumors had developed in the inoculation sites. The tumors with surrounding skin were removed and the capillaries in the dermis oriented towards the tumors were counted by using a stereomicroscope.

Experimental pulmonary metastasis. Experimental metastasis was studied by inoculating 2×10^6 A-07 cells, 3×10^5 D-12 cells, or 1×10^6 T-22 cells into the lateral tail vein of mice. The mice were killed 6 weeks (A-07), 5 weeks (D-12), or 7 weeks (T-22) after the inoculation or when moribund and the lungs were removed and fixed in Bouin's solution. The number of surface colonies was determined by stereomicroscopy.

Treatment with proteinase inhibitors. Effects of proteinase activity inhibition on experimental metastasis were studied by adding 75 μmol/L of GM6001 or 50 μmol/L of E-64 to the cell suspensions 60 minutes before they were inoculated into mice. In addition, the mice were treated with four doses of 150 μg of GM6001 or four doses of 100 μg of E-64, respectively. The treatments were given 4, 24, 48, and 72 hours after the cell inoculation. GM6001 was diluted in 4% carboxymethyl cellulose/PBS and administered i.p in volumes of 0.2 mL. E-64 was dissolved in PBS and administered i.v. in volumes of 0.1 mL. Control mice were treated with four doses of vehicle. Treatment *in vitro* with 75 μmol/L of GM6001 or 50 μmol/L of E-64 for 60 minutes was verified to have no cytotoxic effects on A-07, D-12, and T-22 cells.

Treatment with neutralizing antibodies against proangiogenic factors. Mice inoculated with tumor cells were treated with four doses of 50 μg of anti-human VEGF-A mouse monoclonal antibody (R&D Systems) or four doses of 100 μg of anti-human IL-8 mouse monoclonal antibody (R&D Systems). The first dose was given 1 hour before tumor cell inoculation and the subsequent doses were given in intervals of 24 hours. Control mice were treated with an irrelevant antihuman monoclonal antibody. The antibodies were diluted in PBS and given in volumes of 0.25 mL by i.p. injection.

Statistical analysis. Statistical comparisons of data sets were carried out by using the Student's *t* test (single comparisons) or by one-way ANOVA (multiple comparisons) when the data sets complied with the conditions of normality and equal variance. Under other conditions, comparisons were carried out by nonparametric analysis using the Mann-Whitney rank-sum test (single comparisons) or the Kruskal-Wallis one-way ANOVA on ranks (multiple comparisons). The Bonferroni method (parametric tests) or the Dunett method (nonparametric tests) was used to identify data sets that differed from the control data in multiple comparisons. Probability values of $P < 0.05$, determined from two-sided tests, were considered significant. The statistical analysis was carried out by using SigmaStat statistical software (Jandel Scientific GmbH, Erkrath, Germany).

Results

Acidic pH_e promotes experimental pulmonary metastasis. Effects of acidic pH_e on cell viability and metastatic potential were studied by culturing A-07, D-12, and T-22 cells at pH_e 6.8 for 0 (control), 24, and 48 hours. Similar results were obtained for the three melanoma lines. Plating efficiency decreased whereas

metastatic potential increased with time at pH_e 6.8 (Fig. 1*A* and *B*). Thus, the number of lung metastases was lower for control cells than for cells exposed to pH_e 6.8 for 24 hours (P = 0.0053-0.043) and cells exposed to pH_e 6.8 for 48 hours (P = 0.0000088-0.00052). The duration of the acidity-induced increase in metastatic potential was studied by culturing cells at pH_e 6.8 for 48 hours and then allowing the cells to recover at pH_e 7.4 for 0 (control), 12, and 24 hours. Similar results were obtained for A-07, D-12, and T-22 cells also in these experiments. Plating efficiency increased whereas metastatic potential decreased with increasing recovery time at pH_e 7.4 (Fig. 1*C* and *D*). Thus, the number of lung metastases was higher for control cells than for cells given a recovery time at pH_e 7.4 of 12 hours (P = 0.0048-0.031) and cells given a recovery time at pH_e 7.4 of 24 hours (P = 0.000040-0.00027). The metastatic potential of cells given a recovery time of 24 hours (Fig. 1*D*) was similar to that of untreated control cells (Fig. 1*B*).

Acidic pH_e increases the secretion of proteolytic enzymes and proangiogenic factors. Effects of acidic pH_e on the secretion of proteolytic enzymes and proangiogenic factors were studied by culturing A-07, D-12, and T-22 cells at pH_e 6.8 or pH_e 7.4 (control) for 24 and 48 hours. The protein concentration in the medium of cells cultured at pH_e 6.8 relative to that in the medium of control cells cultured at pH_e 7.4 was used as a measure of acidity-induced increase in protein secretion. The results were qualitatively similar for A-07 (Fig. 2*A*), D-12 (Fig. 2*B*), and T-22 (Fig. 2*C*) cells. The secretion of MMP-2, MMP-9, cathepsin B, cathepsin L, VEGF-A, and IL-8, as measured by ELISA, was higher at pH_e 6.8 than at pH_e 7.4 by factors of 1.6 to 2.9 for the proteolytic enzymes (P = 0.0016-0.039) and by factors of 2.7 to 4.7 for the proangiogenic factors (P = 0.00035-0.013). The secretion of aMMP-2, pMMP-2, aMMP-9, and pMMP-9, as measured by using enzyme activity assays, was higher at pH_e 6.8 than at pH_e 7.4 by factors of 1.8 to 2.8 (P = 0.0086-0.022). The increase in secretion at pH_e 6.8 was similar for aMMP-2 and pMMP-2 and similar for aMMP-9 and pMMP-9 for all cell lines [i.e., the concentration ratios between aMMP-2 and pMMP-2 and between aMMP-9 and pMMP-9 were not significantly different at pH_e 6.8 and pH_e 7.4 (P > 0.05 for both MMPs for all lines)].

Cell invasiveness is promoted by acidic pH_e and inhibited by proteinase inhibitors and antibodies against proteinases. Effects of acidic pH_e on cell invasiveness were studied by culturing A-07, D-12, and T-22 cells at pH_e 6.8 or pH_e 7.4 (control) for 48 hours before assessing cell invasiveness at pH_e 7.4 in the absence of neutralizing antibody or proteinase inhibitor and in the presence of 5 μg/mL of anti-MMP-2 antibody, 5 μg/mL of anti-MMP-9 antibody, 15 μmol/L of GM6001, or 10 μmol/L of E-64. Number of invading cells relative to that for cells cultured at pH_e 7.4 and assayed without neutralizing antibody or proteinase inhibitor was used as a parameter for cell invasiveness. Qualitatively similar results were obtained for A-07 (Fig. 3*A*), D-12 (Fig. 3*B*), and T-22 (Fig. 3*C*) cells. In the absence of neutralizing antibody, cell invasiveness was higher for cells cultured at pH_e 6.8 than for cells cultured at pH_e 7.4 by factors of ~2.8 (A-07; P = 0.00069), ~2.1 (D-12; P = 0.0015), and ~2.5 (T-22; P = 0.00095). In the presence of anti-MMP-2 antibody or anti-MMP-9 antibody, significant differences between cells cultured at pH_e 6.8 and cells cultured at pH_e 7.4 were not detected. Cell invasiveness was higher in the absence than in the presence of neutralizing antibody by factors of 3.5 to 7.0 (P = 0.000076-0.00080) for cells cultured at pH_e 6.8 and by factors of 1.8 to 2.8 (P = 0.0016-0.0098) for cells cultured at pH_e 7.4. In the absence of proteinase inhibitor, cell invasiveness was higher for cells cultured at pH_e 6.8 than for cells cultured at pH_e 7.4 by factors of ~3.1 (A-07; P = 0.00051), ~1.9 (D-12; P = 0.0018), and ~2.4 (T-22; P = 0.00071). In the presence of GM6001 or E-64, significant differences between cells cultured at pH_e 6.8 and cells cultured at pH_e 7.4 were not detected. Cell invasiveness was higher in the absence than in the presence of GM6001 by factors of 3.3 to 7.0 (P = 0.00023-0.0011) for cells cultured at pH_e 6.8 and by factors of 1.6 to 2.6 (P = 0.0049-0.039) for cells cultured at pH_e 7.4, and higher

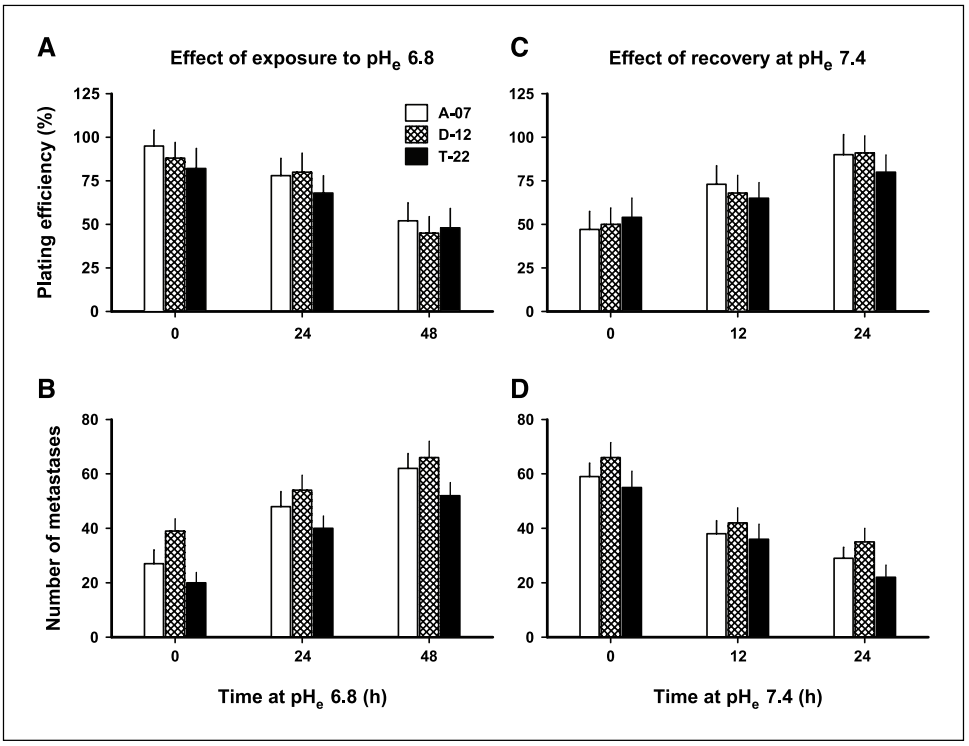

Figure 1. Plating efficiency of A-07, D-12, and T-22 cells assessed *in vitro* (*A* and *C*) and metastatic potential of A-07, D-12, and T-22 cells assessed *in vivo* (*B* and *D*). The cells were cultured *in vitro* at pH_e 6.8 for 0, 24, or 48 hours (*A* and *B*) or at pH_e 6.8 for 48 hours and then allowed to recover at pH_e 7.4 for 0, 12, or 24 hours (*C* and *D*) before being plated *in vitro* or inoculated into the lateral tail vein of BALB/c *nu/nu* mice for formation of pulmonary metastases. Number of experimental metastases after the inoculation of 2×10^6 A-07, 3×10^5 D-12, or 1×10^6 T-22 cells was used as a parameter for metastatic potential. The numbers in (*B* and *D*) have not been corrected for the differences in plating efficiency in (*A* and *C*). *Columns,* mean of four independent experiments involving three culture flasks each (*A* and *C*) and mean of 18 to 20 mice (*B* and *D*). *Bars,* SE.

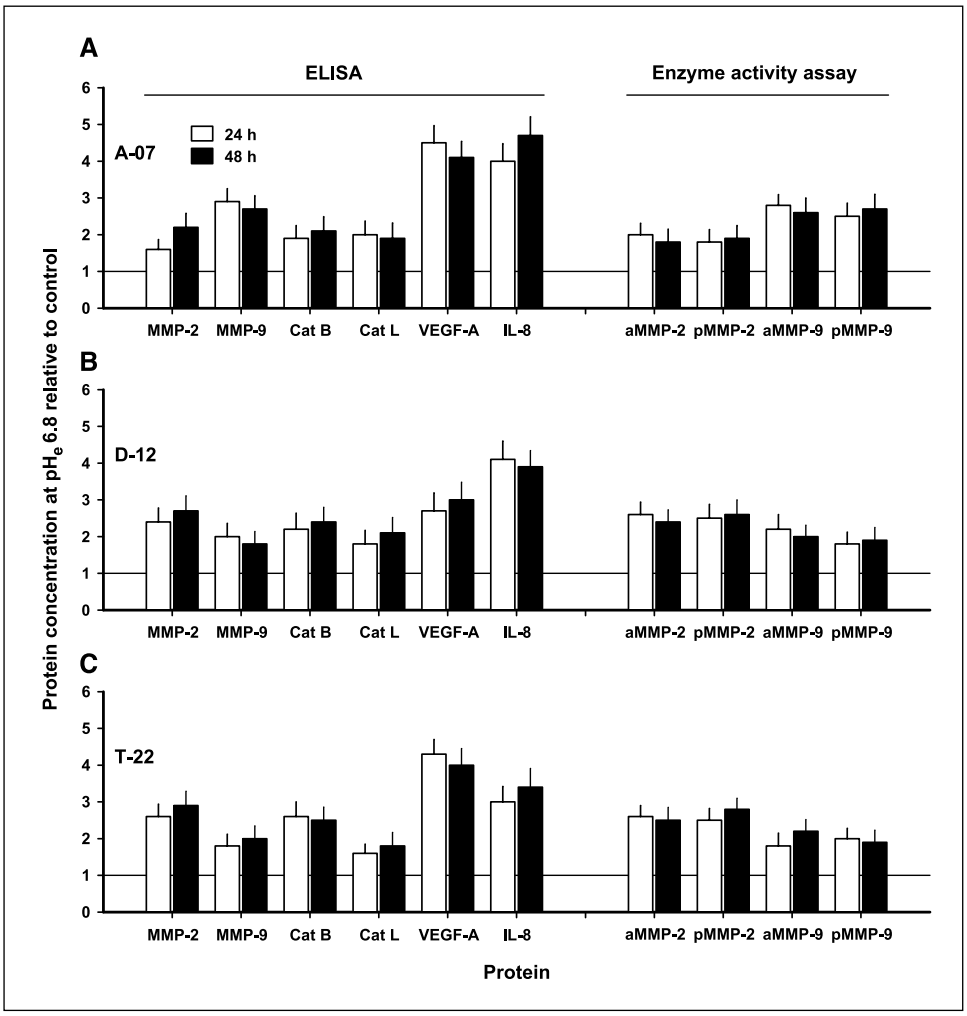

Figure 2. Concentration of MMP-2, MMP-9, cathepsin B, cathepsin L, VEGF-A, and IL-8 (measured by ELISA) and concentration of aMMP-2, pMMP-2, aMMP-9, and pMMP-9 (measured by enzyme activity assay) in the medium of A-07 (*A*), D-12 (*B*), and T-22 (*C*) cells cultured at pH_e 6.8 for 24 or 48 hours relative to that in the medium of control cells cultured at pH_e 7.4 for corresponding times. *Columns,* mean of four independent experiments involving three samples each. *Bars,* SE.

in the absence than in the presence of E-64 by factors of 3.2 to 6.3 (P = 0.00028-0.0011) for cells cultured at pH_e 6.8 and by factors of 1.5 to 2.5 (P = 0.0052-0.048) for cells cultured at pH_e 7.4.

Angiogenesis is promoted by acidic pH_e and inhibited by antibodies against proangiogenic factors. Effects of acidic pH_e on angiogenic potential were studied by culturing A-07, D-12, and T-22 cells at pH_e 6.8 or pH_e 7.4 (control) for 48 hours before assessing the angiogenic potential in untreated mice and mice treated with anti-VEGF-A antibody or anti-IL-8 antibody. Number of tumor-oriented capillaries relative to that for cells cultured at pH_e 7.4 and assayed in untreated mice was used as a parameter for angiogenic potential. The results were qualitatively similar for A-07 (Fig. 4*A*), D-12 (Fig. 4*B*), and T-22 (Fig. 4*C*) cells. In untreated mice, the angiogenic potential was higher for cells cultured at pH_e 6.8 than for cells cultured at pH_e 7.4 by factors of ~1.5 (A-07; P = 0.0012), ~1.7 (D-12; P = 0.00028), and ~1.8 (T-22; P = 0.00021). In contrast, significant differences between cells cultured at pH_e 6.8 and cells cultured at pH_e 7.4 were not detected when the angiogenic potential was assessed in antibody-treated mice. The angiogenic potential was higher in untreated mice than in anti-VEGF-A-treated mice by factors of 2.1 to 3.8 (P = 0.0000029-0.00012) for cells cultured at pH_e 6.8 and by factors of 1.5 to 1.7 (P = 0.00016-0.0069) for cells cultured at pH_e 7.4, and higher in untreated mice than in anti-IL-8-treated mice by factors of 2.1 to 2.3 (P = 0.00024-0.0020) for cells cultured at pH_e 6.8. For cells cultured at pH_e 7.4, the angiogenic potential was higher in untreated mice than in anti-IL-8-treated mice for A-07 (P = 0.034) and D-12 (P = 0.0025) cells, but not for T-22 (P > 0.05) cells.

Acidity-induced metastasis is inhibited by proteinase inhibitors and antibodies against proangiogenic factors. Effects of treatment with proteinase inhibitors and anti-proangiogenic factor antibodies on acidity-induced experimental pulmonary metastasis were studied by culturing A-07, D-12, and T-22 cells at pH_e 6.8 or pH_e 7.4 (control) for 48 hours before assessing the metastatic potential in untreated mice and mice treated with GM6001, E-64, or antibodies against VEGF-A or IL-8. Qualitatively similar results were obtained for A-07, D-12, and T-22 cells, whether the host mice were treated with proteinase inhibitors (Fig. 5) or antibodies against proangiogenic factors (Fig. 6). In untreated mice, the number of metastases was higher for cells cultured at pH_e 6.8 than for cells cultured at pH_e 7.4 by factors of 1.9 to 2.0 (A-07; P = 0.000011-0.000071; Figs. 5*A* and 6*A*), 1.5 to 1.6 (D-12; P = 0.00029-0.0071; Figs. 5*B* and 6*B*), and 2.3 to 2.7 (T-22; P = 0.0000016-0.000010; Figs. 5*C* and 6*C*). In treated mice, significant differences between cells cultured at pH_e 6.8 and cells cultured at pH_e 7.4 were not detected although the number of metastases tended to be higher for cells cultured at pH_e 6.8 than for cells cultured at pH_e 7.4 for all treatments. The number of metastases was higher in

untreated mice than in mice treated with proteinase inhibitors by factors of 1.7 to 3.5 (P = 0.00000064-0.00012) for cells cultured at pH_e 6.8 and by factors of 1.4 to 1.8 (P = 0.0017-0.047) for cells cultured at pH_e 7.4 (Fig. 5*A-C*), and higher in untreated mice than in mice treated with antibodies against proangiogenic factors by factors of 2.0 to 3.7 (P = 0.00000010-0.000032) for cells cultured at pH_e 6.8 and by factors of 1.5 to 2.4 (P = 0.0013-0.044) for cells cultured at pH_e 7.4 (Fig. 6*A-C*).

Discussion

Metastatic spread of malignant cells from the primary tumor to distant organ sites is the major cause of death of cancer patients. Patients with advanced melanoma may develop metastases in several distant organs, including lung (40). The pH_e of human melanoma, measured with electrodes, has been reported to be within the range of 6.4 to 7.3 (3) or 6.6 to 7.0 (41). These values are substantially lower than those of 7.3 to 7.6 measured for human skin (3, 41). The study reported here shows that acidic pH_e promotes experimental pulmonary metastasis of A-07, D-12, and T-22 human melanoma cells in BALB/c *nu/nu* mice. It is thus conceivable that acidic pH_e in the primary tumor may promote metastasis of melanomas in humans and, therefore, the pH_e may seem to be an important prognostic parameter for melanoma patients.

This suggestion is based on the assumption that the melanoma lines used in our preclinical study are adequate models of human melanoma. This assumption is probably valid because the A-07, D-12, and T-22 melanomas have been shown to retain several important biological features of the donor patients' tumors after transplantation to BALB/c *nu/nu* mice, including several pathophysiologic parameters, sensitivity to treatment, angiogenic potential, and the ability to form lung metastases (37, 38). However, in the study reported here, the melanoma cells were inoculated directly into the blood circulation of the mice after having been exposed to acidic or normal pH_e *in vitro*. Thus, our experiments consider only the last part of the metastatic process (i.e., the arrest and growth of melanoma cells at the secondary site). Therefore, studies of possible relationships between the pH_e of the primary tumor and the development of spontaneous metastases in human melanoma xenografts are highly warranted.

Our study is the first study showing unequivocally that human tumor cells exposed to acidic pH_e *in vitro* may acquire an enhanced potential to form experimental metastases *in vivo*. However, it has been shown previously that exposure to acidic pH_e *in vitro* may enhance the lung colonization potential of rodent tumor cells

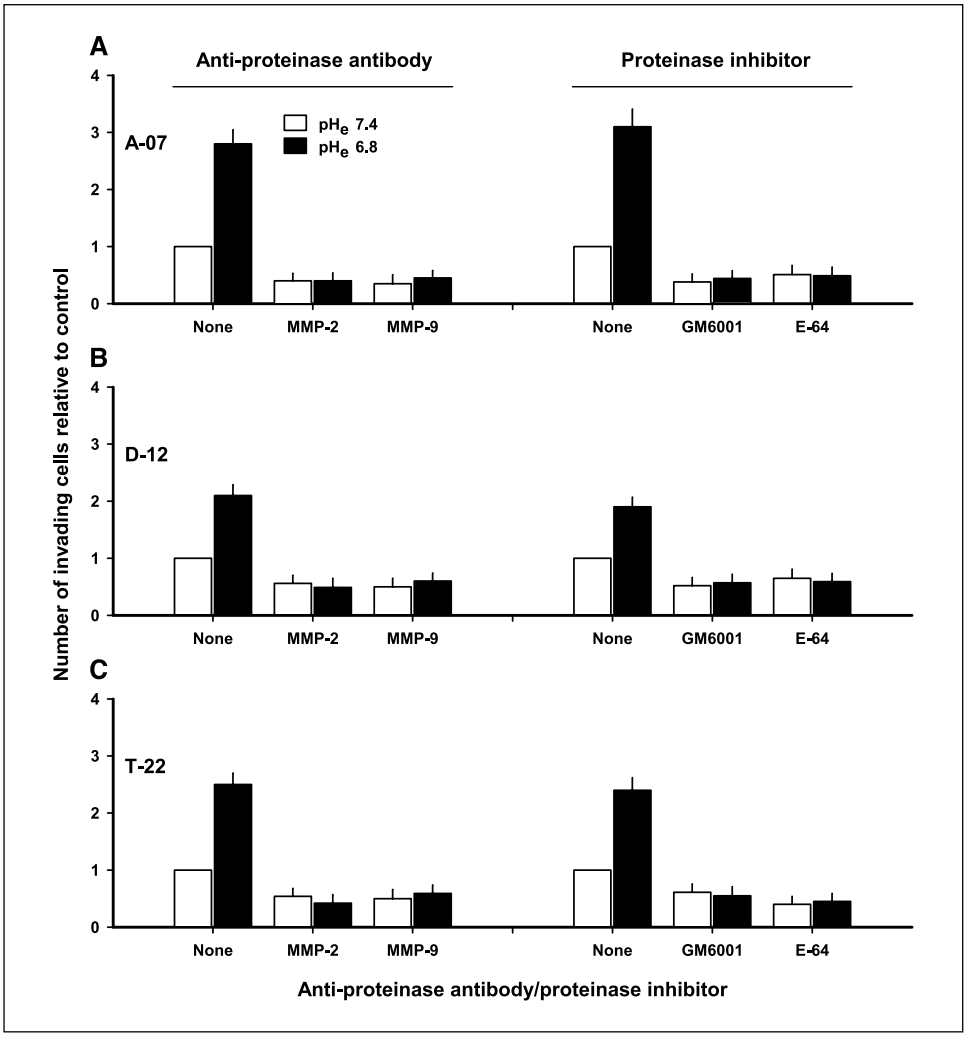

Figure 3. Invasiveness of A-07 (*A*), D-12 (*B*), and T-22 (*C*) cells assessed *in vitro* by using Matrigel invasion chambers. The cells were cultured at pH_e 6.8 or pH_e 7.4 for 48 hours before analysis. Cell invasiveness was assessed at pH_e 7.4 in the absence of neutralizing antibody or proteinase inhibitor and in the presence of 5 μg/mL of anti-MMP-2 antibody, 5 μg/mL of anti-MMP-9 antibody, 15 μmol/L of GM6001, or 10 μmol/L of E-64. Number of invading cells relative to that for cells cultured at pH_e 7.4 and assayed without neutralizing antibody or proteinase inhibitor was used as a parameter for invasiveness. *Columns,* mean of four independent experiments done in triplicate. *Bars,* SE.

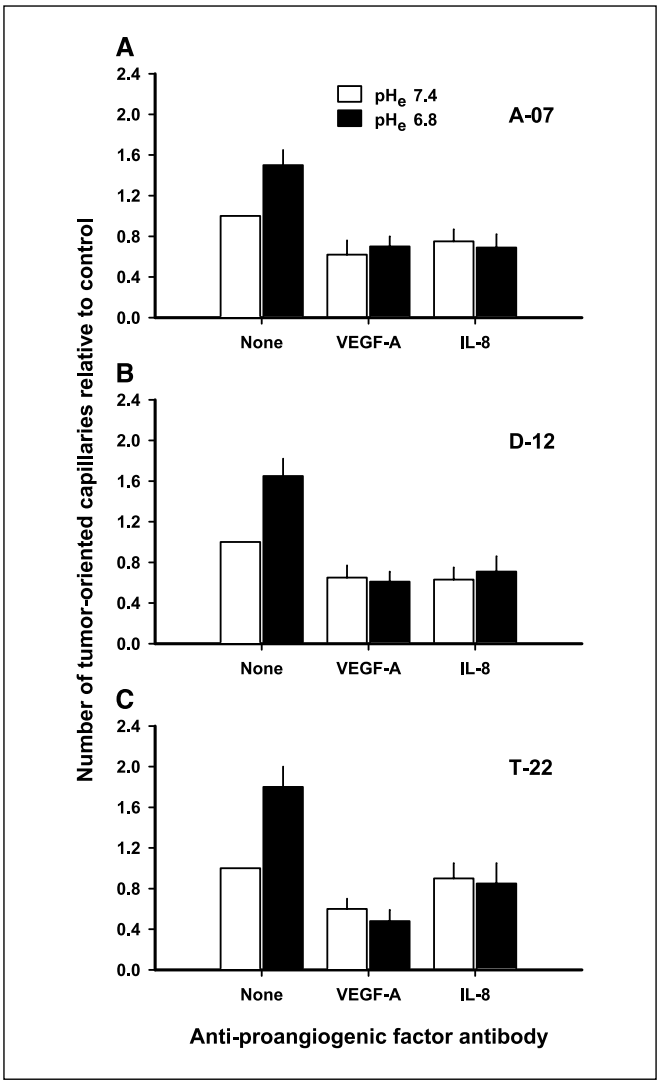

Figure 4. Angiogenic potential of A-07 (*A*), D-12 (*B*), and T-22 (*C*) cells assessed *in vivo* by using an intradermal angiogenesis assay. The cells were cultured *in vitro* at pH_e 6.8 or pH_e 7.4 for 48 hours before being inoculated into the dermis of BALB/c *nu/nu* mice for evocation of angiogenesis and tumor formation. The angiogenic potential was assessed in untreated mice and mice treated with anti-VEGF-A antibody or anti-IL-8 antibody by counting the capillaries in the dermis oriented towards the tumors that developed in the inoculation sites. Number of tumor-oriented capillaries at day 7 after the inoculation of 1.0×10^6 cells relative to that for cells cultured at pH_e 7.4 and assayed in untreated mice was used as a parameter for angiogenic potential. *Columns,* mean of 16 to 20 cell inoculations. *Bars,* SE.

(27, 28). In these experiments, KHT and B16 cells were exposed to pH_e 6.5 for 48 hours and then allowed to recover at pH_e 7.4 for 0, 24, or 48 hours before being inoculated i.v. in syngeneic mice. The lung colonization potential of the cells was not enhanced immediately after the exposure to acidic pH_e but increased significantly with increasing recovery time at normal pH_e. Our melanoma cells, on the other hand, showed a significantly enhanced metastatic potential immediately after having been exposed to pH_e 6.8 for 48 hours, and then the metastatic potential decreased with increasing recovery time at pH_e 7.4. The kinetics of the acidity-induced increase in metastatic potential was thus completely different for the human melanoma cells and the rodent tumor cells, suggesting that fundamentally different mechanisms were involved.

The development of experimental metastases is a complex process composed of a cascade of linked, sequential, and highly selective steps including survival of tumor cells in the blood circulation, arrest in the capillary bed of the secondary organ, invasion into the secondary organ interstitium and parenchyma, proliferation, and induction of angiogenesis. The acidity-induced metastasis of our melanoma cells was most likely a consequence of enhanced invasion as well as enhanced angiogenesis in the lungs, as cells grown at acidic pH_e were found to show enhanced invasiveness in Matrigel invasion chambers and to evoke enhanced angiogenesis in the dermis of BALB/c *nu/nu* mice.

Acidic pH_e induced up-regulation of aMMP-2, pMMP-2, aMMP-9, pMMP-9, cathepsin B, and cathepsin L in all melanoma lines. Treatment with blocking antibody against MMP-2 or MMP-9 inhibited cell invasion *in vitro*, and cell invasion as well as experimental metastasis *in vivo* were inhibited by treatment with the general MMP inhibitor GM6001 or the general cysteine proteinase inhibitor E-64. Moreover, cells cultured at pH_e 6.8 and cells cultured at pH_e 7.4 did not differ significantly in invasiveness and metastatic potential when assayed in the presence of GM6001 or E-64. Consequently, the acidity-induced metastasis of our melanoma cells was in all likelihood partly a result of enhanced invasion caused by acidity-induced up-regulation of MMP-2, MMP-9, cathepsin B, and cathepsin L. This conclusion is consistent with a large number of experimental and clinical studies suggesting that these proteolytic enzymes may play important roles in the metastatic process in a wide variety of tumor types (42).

The proangiogenic factors VEGF-A and IL-8 were also up-regulated by acidic pH_e, and the angiogenic as well as the metastatic potential of all melanoma lines were reduced when assessed in mice treated with blocking antibody against VEGF-A or IL-8, resulting in no significant difference between cells cultured at pH_e 6.8 and cells cultured at pH_e 7.4. Consequently, acidity-induced up-regulation of VEGF-A and IL-8 may have contributed to the acidity-induced metastasis of our melanoma cells by promoting angiogenesis. This conclusion is consistent with experimental and clinical data showing positive correlations between metastasis, angiogenesis, and the expression of VEGF-A or IL-8 in several histologic types of cancer including malignant melanoma (43).

The acidity-induced up-regulation of VEGF-A and IL-8 may have promoted experimental metastasis of our melanoma cells not only by promoting angiogenesis but also by promoting extravasation and invasion into the lungs. Thus, it has been shown that increased expression of IL-8 may lead to significant up-regulation of MMP-2 in human melanomas (44) and that up-regulation of VEGF-A may lead to increased expression of endothelial cell proteinases and, hence, enhanced basement membrane degradation (45, 46). Moreover, VEGF-A, also known as vascular permeability factor, has the ability to induce vascular leakage in several organs by inducing interendothelial cell gaps and endothelial fenestration (47).

The acidity-induced metastasis observed here was a transient phenomenon. When the melanoma cells were allowed to recover at pH_e 7.4 after having been exposed to pH_e 6.8 for 48 hours, the metastatic potential returned to that of untreated control cells within 24 hours, implying that the number of metastases scored 5 to 7 weeks after the cell inoculation was determined by the state of the cells during the first 24 hours in the mouse. Therefore, it was necessary to inoculate the cells directly into the blood circulation for formation of experimental metastases to be able to detect the acidity-induced increase in metastatic potential. The enhanced metastatic potential could probably not have been detected by

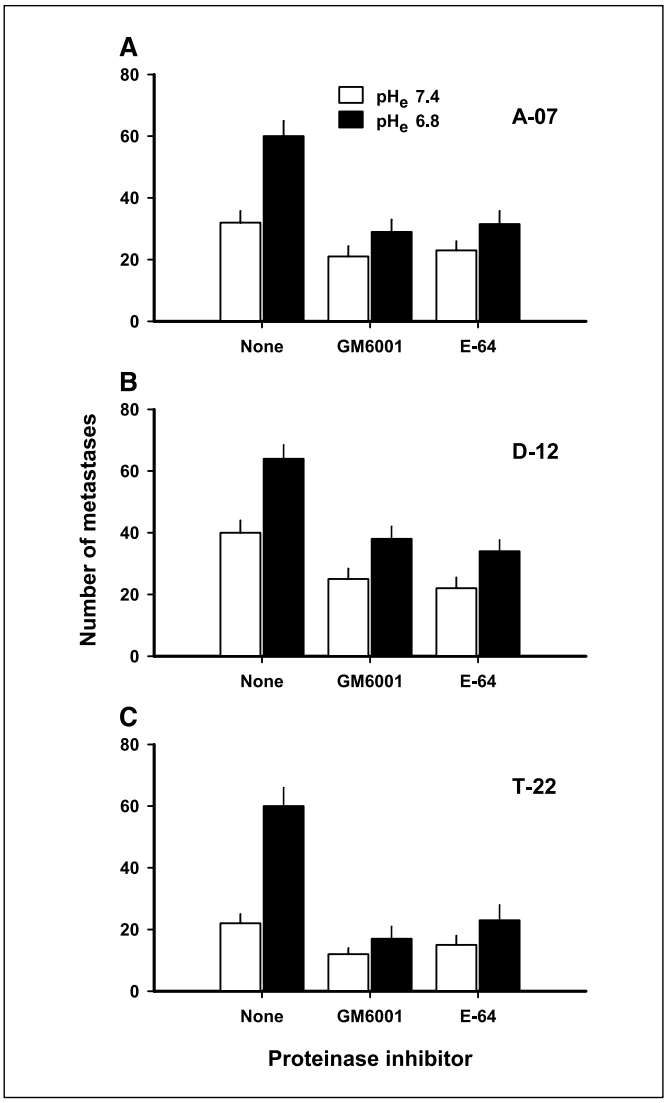

Figure 5. Metastatic potential of A-07 (*A*), D-12 (*B*), and T-22 (*C*) cells assessed in BALB/c *nu/nu* mice. The cells were cultured *in vitro* at pH_e 6.8 or pH_e 7.4 for 48 hours before being inoculated into the lateral tail vein for formation of pulmonary metastases. The metastatic potential was assessed in the absence of proteinase inhibitor and in the presence of GM6001 or E-64. Number of experimental metastases after the inoculation of 2×10^6 A-07, 3×10^5 D-12, or 1×10^6 T-22 cells was used as a parameter for metastatic potential. *Columns,* mean of 18 to 20 mice. *Bars,* SE.

inoculating the cells intradermally for development of primary tumors and spontaneous metastases, owing to the short half-life of the phenomenon.

Acute exposure of tumor cells to acidic pH_e has been shown to cause transient up-regulation of a large number of genes (5, 24, 30, 33), consistent with the transient nature of the acidity-induced increase in metastatic potential in our melanoma cells. The acidity-induced metastasis observed here was most likely a result of effects induced by proteolytic enzymes and proangiogenic factors within the first 24 hours after the cell inoculation. The vascular permeabilization effect of VEGF-A becomes evident within several minutes after microvessels are exposed to VEGF-A and leads to the formation of an extravascular fibrin gel that serves as a substrate for endothelial and tumor cell growth (47). This effect of VEGF-A may have contributed to the acidity-induced metastasis seen here, not only by facilitating melanoma cell extravasation but also by stimulating melanoma cell proliferation and promoting angiogenesis. Moreover, intravital microscopy studies have shown that single metastatic tumor cells and endothelial cells may attract each other, probably by secreting chemotactic signal molecules involving proangiogenic factors and proteolytic enzymes, and there is significant evidence that inhibiting this communication at an early stage (i.e., before the onset of visible angiogenesis) may lead to extensive tumor cell killing (48). It is thus possible that acidity-induced up-regulation of proangiogenic factors and proteolytic enzymes may have contributed to the acidity-induced metastasis seen here by facilitating the communication between melanoma and endothelial cells.

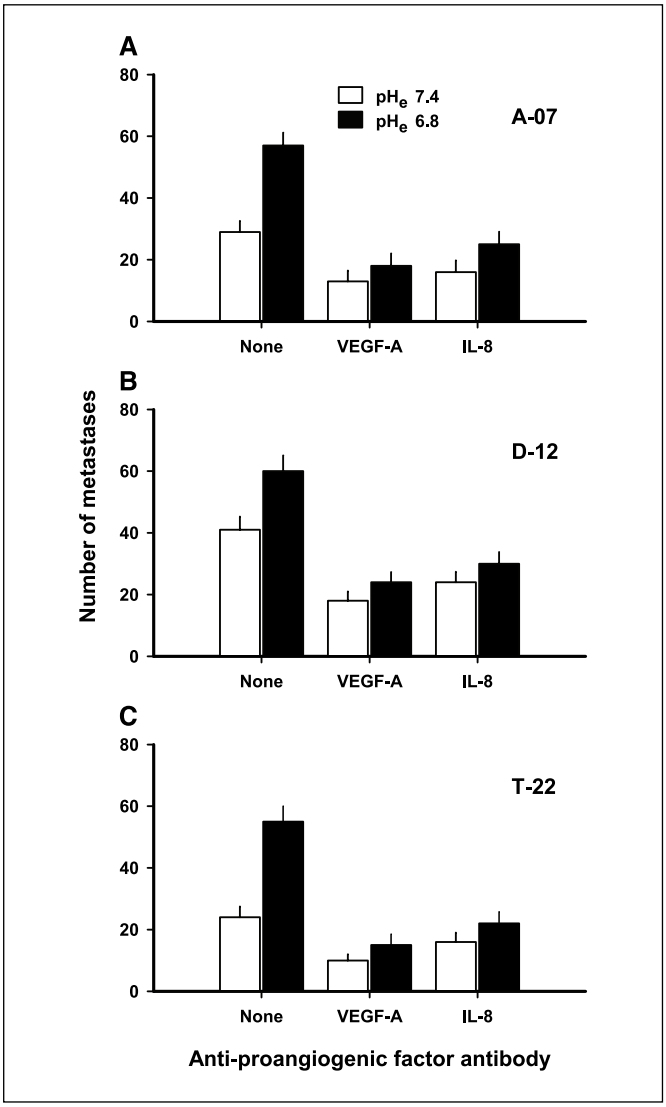

Figure 6. Metastatic potential of A-07 (*A*), D-12 (*B*), and T-22 (*C*) cells assessed in BALB/c *nu/nu* mice. The cells were cultured *in vitro* at pH_e 6.8 or pH_e 7.4 for 48 hours before being inoculated into the lateral tail vein for formation of pulmonary metastases. The metastatic potential was assessed in untreated mice and mice treated with anti-VEGF-A antibody or anti-IL-8 antibody. Number of experimental metastases after the inoculation of 2×10^6 A-07, 3×10^5 D-12, or 1×10^6 T-22 cells was used as a parameter for metastatic potential. *Columns,* mean of 18 to 20 mice. *Bars,* SE.

Growth at acidic pH_e induced similar changes in A-07, D-12, and T-22 cells in terms of secretion of proteinases and proangiogenic factors, cell invasiveness *in vitro*, angiogenic potential *in vivo*, and potential to develop experimental metastases. This observation suggests that pulmonary metastasis of human melanomas may be promoted by acidic pH_e by a common mechanism involving up-regulation of the proteolytic enzymes MMP-2, MMP-9, cathepsin B, and cathepsin L and the proangiogenic factors VEGF-A and IL-8. It is reasonable to assume that proteolytic enzymes and proangiogenic factors other than those studied here also may be up-regulated at acidic pH_e and, hence, may contribute to enhancing the metastatic potential of melanoma cells under acidic conditions. Moreover, our study, of course, does not exclude the possibility that fundamentally different mechanisms also may be involved.

Experimental studies have shown that acidic pH_e may enhance the therapeutic effects of some cancer treatments, including hyperthermia, certain forms of chemotherapy, and certain forms of photodynamic therapy (49). Several strategies for reducing the pH_e of tumors are currently being evaluated to improve the efficacy of these treatments and it has been shown that tumor acidification with glucose and/or mitochondrial inhibitors may enhance the antitumor effects of heat, weakly acidic chemotherapeutic agents, and aminolevulinic acid–mediated photodynamic therapy (29, 49, 50). Our study suggests that treatment strategies involving deliberate tumor acidification should be avoided as acidic pH_e may enhance the metastatic potential of tumor cells.

Studies of cells in culture and experimental tumors have shown that specific inhibitors of the Na^+/H^+ antiporter and other membrane-bound proton pumps may increase the antitumor effects of a large number of chemotherapeutic agents, either by enhancing their cytotoxic or apoptosis-inducing effects or by inhibiting processes causing multidrug resistance (4, 30, 41). In light of the work reported here, it is possible that proton pump inhibitors also may inhibit metastasis by enhancing the pH_e of tumors. Experimental studies investigating this possibility should be carried out.

In summary, the present study shows that acidic pH_e promotes experimental pulmonary metastasis of A-07, D-12, and T-22 human melanoma cells in athymic nude mice by up-regulating the expression of the proteolytic enzymes MMP-2, MMP-9, cathepsin B, and cathepsin L and the proangiogenic factors VEGF-A and IL-8. One significant implication of this observation is that treatment strategies involving deliberate tumor acidification may have undesirable metastasis-promoting side effects and thus should be avoided. Furthermore, clinical studies investigating the possibility that the pH_e of the primary tumor may be an important prognostic parameter for melanoma patients are highly warranted.

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