Hypoxia Promotes Lymph Node Metastasis in Human Melanoma Xenografts by Up-Regulating the Urokinase-Type Plasminogen Activator Receptor

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ABSTRACT

Clinical studies have shown that metastatic spread is associated with hypoxia in the primary tumor. The mechanism behind this association has not been identified and, in fact, it has not been established whether hypoxia induces metastasis or whether the most metastatic cell phenotypes develop the most hypoxic tumors. The present study demonstrates that hypoxia promotes spontaneous lymph node metastasis in R-18 human melanoma xenografts by up-regulating the urokinase-type plasminogen activator receptor (uPAR). Pimonidazole was used as a hypoxia marker, and hypoxia and uPAR expression were detected by immunohistochemistry. R-18 cells were capable of up-regulating uPAR under hypoxic conditions in vitro, as revealed by Western and Northern blot analyses, and uPAR-positive regions showed a high degree of colocalization with hypoxic regions in R-18 tumors. There was a strong correlation between uPAR-positive fraction and hypoxic fraction in individual tumors (P < 0.00001). Incidence of metastases, hypoxic fraction, and uPAR-positive fraction increased with the size of the primary tumor with similar kinetics. Metastatic tumors showed a 1.5-fold higher hypoxic fraction (P = 0.00004) and a 1.4-fold higher uPAR-positive fraction (P = 0.0005) than nonmetastatic tumors of the same size. Moreover, treatment with neutralizing antibody against uPAR prevented metastasis almost completely. Only 1 of 30 treated mice developed metastases, whereas 14 of 30 control mice were metastasis positive, suggesting that functional uPAR is a prerequisite for lymph node metastasis in R-18 tumors. The study reported here suggests that metastatic spread may be promoted by hypoxia in the primary tumor and identifies the plasminogen activation system as an important target for the treatment of malignant melanoma.

INTRODUCTION

Most human tumors develop regions of chronically or transiently hypoxic cells during growth (1). Hypoxic tumor regions may show increased expression of many genes because of hypoxia-induced activation of DNA transcription factors (2–4). Hypoxia may also lead to increased gene expression in tumor tissue by inducing amplifications, rearrangements, translocations, and genomic instability (4–6). Several of the gene products that are induced or up-regulated under hypoxic conditions may play an important role in the metastatic process (4, 7, 8). Therefore, it has been suggested that hypoxia may promote the development of metastatic disease in human cancer (4, 8).

This suggestion is supported by recent clinical studies that have shown that invasive growth and metastatic spread are associated with tumor hypoxia (9–11). Thus, human cervical carcinomas with a median pO2 < 10 mm Hg were found to have larger tumor extensions, more frequent parametrical infiltration, and more extensive lymphovascular space involvement than those with a median pO2 > 10 mm Hg (9). Pretreatment median pO2 was shown to be lower in soft tissue sarcomas that gave rise to pulmonary metastases after treatment than in those that did not metastasize (10). The primary tumors of cervical carcinoma patients with regional lymph node metastases at presentation were found to have higher hypoxic fractions, i.e., higher fractional volumes with pO2 < 5 mm Hg, than those of the patients without metastases (11). However, these clinical studies do not necessarily implicate that hypoxia promotes metastasis. An alternative interpretation is that the most aggressive tumors develop the most extensive hypoxic regions.

Some recent studies of experimental tumors are also consistent with the suggestion that hypoxia may promote cancer metastasis (12–15). First, cells from murine tumors exposed to hypoxic conditions in vitro were found to show unspecific gene amplification and increased lung colonization efficiency after i.v. inoculation in syngeneic hosts (12). However, the gene products responsible for the increased lung colonization efficiency have not been identified (13). Second, human melanoma cells exposed to hypoxia in vitro were found to show enhanced secretion of VEGF and increased lung colonization efficiency after i.v. inoculation in nude mice. The hypoxia-induced increase in lung colonization efficiency was prevented by treatment with neutralizing antibody against VEGF (14). Third, studies of the KHT-C fibrosarcoma have indicated that mice with tumors having high hypoxic fractions may show a slightly higher number of lung micrometastases than those with tumors having low hypoxic fractions (15). However, studies of experimental tumors giving conclusive evidence that hypoxia may promote the development of macroscopic spontaneous metastases have not been reported thus far. In fact, experiments attempting to establish correlations between hypoxia and spontaneous metastasis in the KHT-C and SCC-VII tumors have given negative results (15, 16).

The plasminogen activation system, which leads to the formation of the serine protease plasmin, has been shown to play an important role in metastasis (17–19). tPA, uPA, PAI-1, PAI-2, and uPAR are members of the plasminogen activation system (20). Plasminogen is synthesized in the liver and deposited in tumors in response to hyperpermeability. Plasmin is formed by a conversion of zymogen plasminogen, which is regulated by plasminogen activators. Two distinct plasminogen activators have been recognized, tPA and uPA. The activity of the activators is regulated by interactions with specific inhibitors, of which two have been described, PAI-1 and PAI-2. uPA can be localized at the tumor cell surface by binding to a specific receptor, uPAR. The uPAR/uPA complex focuses the formation of plasmin and hence proteolytic activity to the vicinity of the tumor cell (20–22). Plasmin facilitates tumor cell migration, invasion, and metastasis by degrading fibrin and other matrix proteins directly and by activating several metalloproteinases that additionally degrade the extracellular matrix (17, 22).

Some tumor cell lines have been shown to up-regulate PAI-1 and uPAR when exposed to hypoxia in vitro (23–26). The hypoxia-induced up-regulation of uPAR is because of both increased transcriptional activation and mRNA stabilization (25), and has been shown to result in enhanced concentration of uPAR on the cell surface, elevated levels of cell-associated uPA, and increased cell invasiveness through...
a reconstituted basement membrane in vitro (26). These findings have led to the hypothesis that associations between tumor hypoxia and metastasis in vivo may be a result of efficient tumor cell invasion because of hypoxia-induced up-regulation of uPAR (26, 27). The human melanoma xenograft study reported here supports this hypothesis and suggests that hypoxia may promote spontaneous cancer metastasis. The R-18 melanoma was selected as a tumor model in the present study because preliminary screening experiments had revealed that R-18 cells show extensive uPAR up-regulation under hypoxic conditions. Moreover, the R-18 melanoma has retained essential biological features of the tumor from the donor patient and gives rise to organ-specific lymph node metastases when inoculated orthotopically in BALB/c-nu/nu mice (28).

MATERIALS AND METHODS

Cell Line. The R-18 cell line, established as described previously (28), was maintained in monolayer culture in RPMI 1640 (25 mM HEPES and l-glutamine) supplemented with 13% bovine calf serum, 250 mg/liter penicillin, and 50 mg/liter streptomycin. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 in air and subcultured twice a week. The cells were verified to be free from Mycoplasma contamination.

Hypoxia Treatment. Monolayer cultures were exposed to hypoxia by using the steel chamber method. The steel chamber was flushed with a humidified, highly purified gas mixture consisting of 95% N2 and 5% CO2 at a flow rate of 5 liter/min. Experiments were performed by using two highly different levels of hypoxia. The concentration of O2 in the medium was either ~500 ppm or <10 ppm after 30 min of flushing, depending on the O2 concentration of the gas mixture. Details of the procedure have been published elsewhere (29).

Western Blot Analysis. Cells were washed in PBS and boiled in Laemmli lysis buffer. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Membranes were incubated with antihuman tPA goat polyclonal antibody (Chemicon, Temecula, CA), antihuman uPA mouse monoclonal antibody (Calbiochem, San Diego, CA), antihuman PAI-1 mouse monoclonal antibody (Calbiochem), antihuman PAI-2 goat polyclonal antibody (American Diagnostica, Greenwich, CT), or antihuman uPAR mouse monoclonal antibody (American Diagnostica). Bound antibody was detected by using a biotin-streptavidin alkaline phosphatase staining procedure. Recombinant proteins were used as positive controls. The specificity of the antibody-antigen interactions was confirmed by peptide competition studies and by incubations of membranes in solutions without primary antibody. Blots were probed with anti-β-actin mouse monoclonal antibody (Sigma Chemical Co., St. Louis, MO) for loading and transfer control. Protein molecular weights were estimated by using prestained standards according to the instructions of the manufacturer (SDS-PAGE standards; Bio-Rad Laboratories, Hercules, CA).

Northern Blot Analysis. Total RNA was isolated from cells with TRIzol (Life Technologies, Inc., Grand Island, NY) according to the manufacturer’s protocol. Aliquots of 15 µg of RNA were separated by electrophoresis on 0.9% denaturing agarose/formaldehyde gels, blotted to Hybond nylon membranes (Amer sham, Arlington Heights, IL), and hybridized with a 1.1-kbp probe containing the entire human uPAR cDNA. The hybridization was performed for 2 h at 65°C in Quick-Hyb hybridization solution (Stratagene, La Jolla, CA). The probe was excised from the plasmid uPAR/Bluescript (American Type Culture Collection, Rockville, MD) with XbaI and EcoRI restriction enzymes and labeled with [32P]dCTP by using a random primer synthesis kit (A mer sham). Autoradiography was performed at ~80°C by using Kodak T-MAT-G films and Kodak Lanex X-OMAT intensifying screens (Kodak, Rochester, NY) after the membranes had been washed to a stringency of 0.1% SSC/0.1% SDS. The blots were stripped and reprobed with a 32P end-labeled oligonucleotide complementary to 18S rRNA to control for variation in sample loading and transfer.

Quantitation of Western and Northern Blots. Band densities in immunoblots and autoradiograms were quantitated by scanning laser densitometry using the ImageQuant software program (Molecular Dynamics, Sunnyvale, CA). All of the densitometric values were normalized to the values of β-actin (immunoblots) or 18S rRNA (autoradiograms).

Mice and Tumors. Adult (8–10 weeks of age) female BALB/c-nu/nu mice were used as host animals for xenografted tumors. The mice were bred at our research institute and maintained under specific pathogen-free conditions at constant temperature (24–26°C) and humidity (30–50%). Sterilized food and tap water were given ad libitum. Tumors were initiated from exponentially growing cell cultures. Approximately 3.5 × 106 cells suspended in 10 µl of Ca2+ and Mg2+-free HBSS were inoculated intradermally into the left mouse flank. The histological appearance of the tumors has been described previously (28). Animal experiments were approved by the Institutional Committee on Research Animal Care and were performed 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).

Detection of Tumor Hypoxia. Pimonidazole was used as a marker of tumor hypoxia. Hypoxic tumor regions were detected immunohistochemically as reported earlier by using a peroxidase-based indirect staining method (29). Pimonidazole hydrochloride, kindly supplied by Professor J. A. Raleigh, was dissolved into 0.9% NaCl and administered i.p. to tumor-bearing mice in doses of 30 mg/kg body weight. The tumors were dissected free from the mice 4 h after the pimonidazole administration and fixed in phosphate-buffered 4% paraformaldehyde. Slides with tumor tissue preparations were incubated with polyclonal rabbit antiserum to pimonidazole-protein adducts, a kind gift from Professor J. A. Raleigh. Visualization of the antibody complex was achieved with the 3,3-diaminobenzidine chromogen. Hematoxylin was used for counterstaining. Four cross-sections of each tumor were subjected to quantitative studies. Area fractions showing pimonidazole staining were determined by image analysis at a magnification of ×120 by using fields of view of 1.4 × 2.0 mm and at least 25 fields of view/tumor (29). The uncertainty associated with determining hypoxic fractions in R-18 tumors by this method has been examined by having 20 tumors analyzed independently by two investigators. The correlation between the hypoxic fractions determined by the two investigators was excellent (P < 0.0001; R2 = 0.98); the difference between their values never exceeded 2% (29).

Immunohistochemical Staining for tPA, uPA, PAI-1, PAI-2, and uPAR. Immunohistochemical staining of tumor tissue was performed by using an indirect immunoperoxidase method. Tumors were fixed in phosphate-buffered 4% paraformaldehyde or snap-frozen in liquid nitrogen. Antihuman tPA goat polyclonal antibody (Chemicon), antihuman uPA mouse monoclonal antibody (Calbiochem), antihuman PAI-1 goat polyclonal antibody (American Diagnostica), antihuman PAI-2 mouse monoclonal antibody (American Diagnostica), or antihuman uPAR mouse monoclonal antibody (American Diagnostica) was used as primary antibody. Controls included omission of the primary antibody, incubation with normal rabbit immunoglobulin or normal rabbit serum, and incubation with blocking peptides before staining. The sections were counterstained with hematoxylin. Quantitative studies of the expression of uPAR were based on four cross-sections of each tumor. An image analysis procedure, analogous to that applied to quantitate tumor hypoxia, was used to determine area fractions showing uPAR-positive staining.

Metastasis Assay. Primary tumors were initiated in the left mouse flank from cell cultures as described above. They were removed surgically when the largest diameter had attained a predetermined size (30). The mice were examined for the presence external lymph node metastases, i.e., enlarged lymph nodes, in the interscapular region, submandibular region, axillary region, and inguinal region twice a week. They were killed 3 months after the primary tumor was removed, when moribund, or when scored to be positive for external metastases. Mice that were killed 3 months after the primary tumor was removed or when moribund were examined for the presence of lymph node metastases in the abdomen and mediastinum. Lymph node metastases in the abdomen and/or mediastinum were always found in the moribund mice. Histological examinations confirmed that enlarged lymph nodes always contained metastatic deposits. Mice were scored to be metastasis negative only if enlarged lymph nodes could not be detected when the mice were killed and autopsied 3 months after the primary tumor was removed. This procedure is justified by previous long term experiments, which have shown that mice that appear healthy 3 months after the excision of the primary tumor also are free of lymph node metastases 3 months later (28).

Treatment with Anti-uPAR Neutralizing Antibody. Anti-uPAR treatment was performed by using an antihuman uPAR mouse monoclonal antibody (R&D Systems, Abingdon, United Kingdom). The antibody shows no cross-reactivity with recombinant murine uPAR. The treatment consisted of 14
doses of 25 μg of antibody given to tumor-bearing mice in 24-h intervals. Control mice were treated at 24-h intervals with 14 doses of 25 μg of a mouse monoclonal antibody of the same isotype (IgG1) directed against the gp120 protein. Antibody solutions were diluted in PBS and administrated in volumes of 0.25 ml by i.p. injection.

Statistical Analysis. Results are presented as arithmetic mean ± SE. Correlations between two parameters were searched for by linear regression analysis. Statistical comparisons of data were performed by using the Student t test. The statistical analysis was performed by using SigmaStat statistical software (Jandel Scientific GmbH, Erkrath, Germany).

RESULTS

R-18 tumors showed high heterogeneous staining for pimonidazole, consistent with staining of hypoxic cells without staining of normoxic cells, as described in detail previously (29). Foci of hypoxic cells, differing substantially in size and shape, were seen throughout the tissue (Fig. 1A). Macroscopic necrotic regions could not be observed. However, a few necrotic or necrotizing cells could be seen in the middle of some of the largest hypoxic foci. The boundary line between stained and unstained cells was sharp, i.e., the cells showed either intense brown staining or no staining. Thus, hypoxic cells could easily be distinguished from normoxic cells.

R-18 tumors showed significant staining also for tPA, uPA, PAI-1, PAI-2, and uPAR. The expression of tPA, uPA, PAI-1, and PAI-2 was homogeneous within the tumors (data not shown). The uPAR expression, on the other hand, was highly heterogeneous (Fig. 1B). The staining pattern was similar to that for pimonidazole. Thus, foci of strongly uPAR-positive cells were seen throughout the tissue. The remaining tissue stained weakly for uPAR. The uPAR-positive foci were generally 1.3–1.5-fold larger than the hypoxic foci and could be defined easily, although the boundary line was not as sharp as for pimonidazole staining.

Tumors were stained for both pimonidazole and uPAR to investigate whether the uPAR-positive foci were a result of hypoxia-induced uPAR up-regulation. The uPAR-positive fraction, defined as the fractional tissue area being occupied by uPAR-positive foci, and the hypoxic fraction, defined as the fractional tissue area staining positive for pimonidazole, were measured in 50 tumors. The uPAR-positive fraction was strongly correlated to the hypoxic fraction (Fig. 1C; R² = 0.89; P < 0.00001). Moreover, examination of adjacent sections revealed a high degree of colocalization of uPAR and pimonidazole staining (Fig. 1, A and B). Quantitative colocalization studies were performed in 10 tumors. These studies showed that uPAR-positive foci covered 98.1 ± 1.7% of the area that stained positive for pimonidazole, and pimonidazole staining was seen in 71.9 ± 2.6% of the area occupied by uPAR-positive foci.

R-18 cells were grown under aerobic or hypoxic conditions in vitro.
and subjected to Western blot analysis to investigate whether the cells were capable of up-regulating tPA, uPA, PAI-1, PAI-2, and/or uPAR under hypoxic conditions (Fig. 1, D and E). Aerobic cells showed significant expression of tPA, uPA, PAI-1, PAI-2, and uPAR. Hypoxia-induced up-regulation of tPA, uPA, PAI-1, and PAI-2 could not be detected. The uPAR expression, on the other hand, was up-regulated significantly under hypoxic conditions. The level of uPAR protein increased gradually with time under hypoxic conditions and was enhanced by a factor of \( F \) (data not shown). Northern blot analysis of the expression of uPAR in R-18 cells in vitro gave results consistent with the results from the Western blot analysis. The level of uPAR mRNA was enhanced by a factor of \( \sim 10 \) after 16 h at an \( O_2 \) concentration of \( <10 \) ppm (Fig. 1F) and by a factor of \( \sim 8 \) after 16 h at an \( O_2 \) concentration of \( \sim 500 \) ppm (data not shown).

Changes in hypoxic fraction, uPAR-positive fraction, and incidence of metastases with tumor size were studied to investigate whether metastasis was associated with hypoxia and/or uPAR expression. The hypoxic fraction (Fig. 2A) increased from \( \sim 2\% \) to \( \sim 11\% \) and the uPAR-positive fraction (Fig. 2B) from \( \sim 3\% \) to \( \sim 15\% \) when the longest tumor diameter increased from \( 5–7 \) mm to \( 11–13 \) mm. The mice did not develop metastases if the primary tumor was removed when the longest diameter was \( 5–7 \) mm or smaller. When the primary tumor was removed at larger sizes, the fraction of mice developing metastases increased with the size of the tumor and was \( 50–60\% \) when the longest diameter was \( 11–13 \) mm at tumor removal (Fig. 2C). The kinetics of the increases in hypoxic fraction, uPAR-positive fraction, and fraction of mice with metastases were similar; the increases were most pronounced when the longest tumor diameter increased from \( 7–9 \) mm to \( 9–11 \) mm, and sigmoid curves gave good fits to all three of the data sets.

The association between incidence of metastases, hypoxic fraction, and uPAR-positive fraction was additionally investigated by determining all three of the parameters in the same tumors. The experiment involved 50 mice. The primary tumors were removed when the longest diameter was \( 9–11 \) mm, and hypoxic fraction and uPAR-positive fraction were measured in each tumor and related to the metastatic status of the host. Twenty-three mice developed metastases whereas 27 mice did not. The number of involved lymph nodes in the metastasis-positive mice was usually one or two, but metastatic deposits were detected in up to six lymph nodes in a single mouse. The median hypoxic fraction was 9.35%, and the median uPAR-positive fraction was 12.95%. Fifteen of the 25 mice (60%) that had primary tumors with uPAR-positive fractions above the median value developed metastases, whereas 8 of the 25 mice (32%) that had primary tumors with uPAR-positive fractions below the median value were metastasis positive. Similarly, metastases were detected in 17 of the 25 mice (68%) that had primary tumors with hypoxic fractions above the median value and in 6 of the 25 mice (24%) that had hypoxic fractions below the median value. The latter proportions differ significantly (\( \chi^2 = 8.05; P = 0.005 \)). Moreover, the metastatic tumors showed \( \sim 1.5\)-fold higher hypoxic fraction than the nonmetastatic tumors (Fig. 3A; \( P = 0.0004 \)), and the uPAR-positive fraction was \( \sim 1.4\)-fold higher in the tumors that metastasized than in those that did not (Fig. 3B; \( P = 0.0003 \)). The uPAR-positive fraction was strongly correlated to the hypoxic fraction (Fig. 1C; \( P < 0.0001 \)).

The specific role of uPAR in the development of metastases was investigated by treating host mice with neutralizing antibody against uPAR. Three experiments were performed, each involving 10 treated mice and 10 control mice. The primary tumors were removed when the longest diameter was \( 9–11 \) mm. The mice were treated daily with
anti-uPAR antibody for 2 weeks, i.e., from when the longest tumor diameter was 5–7 mm to tumor removal. This treatment period was chosen because R-18 tumors do not normally metastasize before the longest diameter is 5–7 mm and have usually given rise to metastatic disease in 40–50% of the host mice when the longest diameter has attained 9–11 mm (Fig. 2C). The incidence of metastases was reduced substantially by the anti-uPAR treatment (Fig. 4; \( P = 0.004 \)). In fact, the anti-uPAR treatment blocked metastasis almost completely; only 1 of 30 treated mice developed metastatic disease, whereas 14 of 30 control mice were metastasis positive.

**DISCUSSION**

The experiments reported here demonstrate that spontaneous lymph node metastasis is associated with the hypoxic fraction of the primary tumor in R-18 human melanoma xenografts. First, the hypoxic fraction increased with tumor size and the incidence of metastases increased with the size of the primary tumor at tumor removal, and the kinetics of the increases were similar. Second, when the primary tumor was removed at a given size, the incidence of metastases was correlated to the hypoxic fraction at tumor removal, i.e., mice with primary tumors with hypoxic fractions above the median value developed metastatic disease more frequently than did mice with primary tumors with hypoxic fractions below the median value. Moreover, the hypoxic fraction was 1.5-fold higher in the primary tumors that gave rise to metastases than in those that did not metastasize. The difference in hypoxic fraction between the metastatic and nonmetastatic primary tumors was substantially larger than the experimental uncertainty involved in the assessment of the hypoxic fractions.

Differences in hypoxic fraction among individual tumors of the same size initiated in identical hosts from the same cell culture are generally a result of stochastic development of the vasculature rather than genetic differences among the tumors (31). This is most likely true also for R-18 tumors, because it has been shown that long term culturing of R-18 cells under hypoxic conditions *in vitro* does not induce or select for hypoxia-resistant cell variants. Therefore, the association between metastasis and hypoxia reported here is unlikely to be a result of genetic differences among the primary tumors but reflects rather that a stochastic development of hypoxic regions increased the probability of metastasis. Consequently, the present study strongly suggests that hypoxia may promote the development of metastatic disease in human cancer.

The plasminogen activation system plays a critical role in tumor growth, invasion, and metastasis (17–19). Two of its components, PAI-1 and uPAR, have been shown to be up-regulated in some tumor cells exposed to hypoxia *in vitro* (23–26). The hypoxia-induced metastasis in R-18 tumors was most likely mediated by uPAR. First, Western and Northern blot analyses of cells in culture showed that R-18 cells were capable of up-regulating uPAR under hypoxic conditions. Second, examination of immunohistochemical preparations demonstrated that all components of the plasminogen activation system were expressed in normoxic tumor regions and that uPAR was up-regulated in hypoxic tumor regions, i.e., the uPAR-positive fraction was strongly correlated to the hypoxic fraction in individual tumors, and there was a high degree of colocalization of uPAR and pimonidazole staining. Third, metastatic tumors showed 1.4-fold higher uPAR-positive fraction than nonmetastatic tumors of the same size, and the kinetics of the increase in incidence of metastases with the size of the primary tumor at tumor removal was similar to the kinetics of the increase in uPAR-positive fraction with tumor size.

Finally, treatment with neutralizing antibody against uPAR blocked metastasis almost completely, suggesting that functional uPAR is a prerequisite for the development of metastases in R-18 tumors. Hypoxia probably promoted metastasis in R-18 tumors by up-regulating uPAR, enhancing the cell surface concentration of uPAR/uPA, increasing the local concentration of plasmin and thereby the local degradation of extracellular protein structures, and hence facilitating tumor cell migration and intravasation. The hypoxia-induced up-regulation of uPAR protein in R-18 cells could be because of transcripational activation of the gene encoding uPAR and/or posttranslational effects of hypoxia. Both increased transcription and mRNA stability were shown to contribute to the hypoxia-induced up-regulation of uPAR in MCF7 human breast carcinoma cells (25).

However, it should be noted that hypoxia is probably not an essential condition for the development of metastases in R-18 tumors. The expression of uPAR in normoxic tumor regions may well be sufficiently high that normoxic cells also have a certain probability of giving rise to metastases. R-18 cells showed significant uPAR expression under aerobic conditions *in vitro*, and the tissue surrounding the uPAR-positive foci in R-18 tumors stained weakly for uPAR. Moreover, the uPAR-positive foci were generally 1.3–1.5-fold larger than the pimonidazole-positive foci. Studies of R-18 tumors have shown that pimonidazole staining appears only in cells having oxygen tensions <7–10 mm Hg (29). The threshold oxygen tension for hypoxia-induced uPAR up-regulation may be higher than that for pimonidazole staining, causing the uPAR-positive foci to be larger than the pimonidazole-positive foci. An alternative explanation is that some normoxic tumor cells also may show strong uPAR expression, perhaps because of glucose starvation or low tissue pH (2–6).

Hypoxia is probably not a sufficient condition for the development of metastases in R-18 tumors either. Although the hypoxic fraction differed significantly between metastatic and nonmetastatic primary tumors, a substantial percentage of the nonmetastatic tumors showed hypoxic fractions within the same range as those of the metastatic tumors.

Clinical studies have shown that tumor hypoxia is associated with invasive growth and metastasis (9–11), and may be an independent prognostic factor adversely influencing survival in patients with squamous cell carcinoma of the uterine cervix (32–35) or the head and neck (36–38). The mechanisms behind these observations have not been identified. However, it is not inconceivable that the plasminogen activation system may be involved. A large body of clinical data has demonstrated an association between high levels of uPA, uPAR, or PAI-1 and either aggressive tumor characteristics or a poor patient prognosis in multiple types of solid tumors (20, 39), including cervical and head and neck squamous cell carcinomas (40–43). Particularly interesting is the observation of an association between tumor hypoxia and the expression of uPA in patients with squamous cell carcinoma of the head and neck (44).
The mechanism of hypoxia-induced metastasis suggested here for R-18 tumors is by no means the only mechanism by which tumor hypoxia may promote metastasis. A recent review, based on novel insights from studies of experimental tumors and cells in culture, and recent advances in gene regulation and signal transduction, has identified several possible mechanisms of hypoxia-induced cancer metastasis (4). Thus, hypoxia may induce point mutations and DNA strand breakage leading to deletions, amplifications, and genomic instability. Hypoxia may also provide a physiological pressure in tumors selecting for metastatic cell phenotypes. Moreover, hypoxia may induce a temporary increase in the expression of gene products involved in the metastatic cascade, either through gene amplifications or, as suggested for R-18 tumors, through a natural physiological process by activating oxygen sensors, hypoxia signal transduction pathways, and DNA transcription factors (4).

Hypoxia-induced activation of HIF-1 and other transcription factors may lead to increased expression of many gene products (2–4). The possibility that gene products other than uPAR were up-regulated in hypoxic regions in R-18 tumors and played a significant role in the metastatic process can, therefore, not be excluded. Several angiogenesis factors have been shown to be up-regulated under hypoxic conditions (2, 4, 7, 8), and there are experimental data suggesting that hypoxia-induced expression of VEGF may lead to increased neovascularization and, hence, increased metastasis (14). However, angiogenesis factors were probably not involved in hypoxia-induced metastasis in R-18 tumors. Analysis of immunohistochemical preparations has revealed that there is no colocalization of pimonidazole staining and staining indicating up-regulation of VEGF, interleukin 8, angiogenin, or platelet-derived endothelial cell growth factor, and there is no association between hypoxia and mean or hot spot microvascular density.5

Moreover, many solid tumors produce VEGF-C and VEGF-D, and recent studies have shown that VEGF-C and VEGF-D may promote lymphangiogenesis and, hence, lymph node metastasis (45–47). However, tumor-induced lymphangiogenesis was probably not involved in the development of lymph node metastases in R-18 tumors. R-18 tumors do not show significant expression of VEGF-C or VEGF-D, and functional lymphatics cannot be detected in the interior of R-18 tumors by fluorescence or ferritin microlymphography.6

Components of the plasminogen activation system may be important targets for inhibiting cancer metastasis. Studies of xenografted tumors, particularly breast carcinoma and colon carcinoma, have suggested that inhibition of uPA activity or uPAR/uPA interaction may result in a reduced number of metastatic foci (20, 48, 49). The present study showed that treatment of R-18 melanomas with neutralizing antibody against uPAR prevented lymph node metastasis almost completely. Therefore, functional uPAR may be an essential condition for the development of metastases in these tumors. This observation suggests that the plasminogen activation system may be an important target also for the treatment of malignant melanoma, a suggestion that is consistent with earlier experimental and clinical studies of this tumor type. Thus, Quax et al. (50) studied xenografts of six human melanoma cell lines and found that only two of the lines were capable of forming spontaneous pulmonary metastases after s.c. inoculation, and these two lines were also the only ones showing uPA-mediated matrix degradation in vitro and uPA expression in vivo. Moreover, studies of primary tumors in man have shown that the expression of components of the plasminogen activation system, particularly uPA and uPAR, is associated with melanoma progression (51–53).

R-18 melanomas show an organ-specific metastatic pattern in BALB/c-כ/nu mice that reflects the pattern of lymph node metastasis in the donor patient (28) and are, therefore, excellent models for studying molecular mechanisms of lymph node metastasis. The present study has shown that hypoxia promotes lymph node metastasis in R-18 melanoma xenografts by up-regulating uPAR and that treatment with neutralizing antibody against uPAR prevents metastasis almost completely. Consequently, the plasminogen activation system may be an important target for the treatment of malignant melanoma.

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4 Unpublished observations.


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