Hypoxia Increases Tumor Cell Shedding of MHC Class I Chain-Related Molecule: Role of Nitric Oxide

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Abstract

The MHC class I chain-related (MIC) molecules play important roles in tumor immune surveillance through their interaction with the NKG2D receptor on natural killer and cytotoxic T cells. Thus, shedding of the MIC molecules from the tumor cell membrane represents a potential mechanism of escape from NKG2D-mediated immune surveillance. Tumor hypoxia is associated with a poor clinical outcome for cancer patients. We show that hypoxia contributes to tumor cell shedding of MIC through a mechanism involving impaired nitric oxide (NO) signaling. Whereas hypoxia increased MIC shedding in human prostate cancer cells, activation of NO signaling inhibited hypoxia-mediated MIC shedding. Similar to incubation in hypoxia, pharmacologic inhibition of endogenous NO signaling increased MIC shedding. Parallel studies showed hypoxia-mediated tumor cell resistance to lysis by interleukin 2–activated peripheral blood lymphocytes (PBL) and NO-mediated attenuation of this resistance to lysis. Inhibition of NO production also led to resistance to PBL-mediated lysis. Interference of MIC-NKG2D interaction with a blocking anti-MIC antibody abrogated the effect of hypoxia and NO signaling on tumor cell sensitivity to PBL-mediated lysis. Finally, continuous transdermal delivery of the NO mimetic glyceryl trinitrate (7.3 μg/h) attenuated the growth of xenografted MIC-expressing human prostate tumors. These findings suggest that the hypoxic tumor microenvironment contributes to resistance to immune surveillance and that activation of NO signaling is of potential use in cancer immunotherapy. [Cancer Res 2008;68(12):4746–53]

Introduction

The MHC class I chain-related (MIC) molecules, MICA and MICB, as well as the UL16-binding proteins (ULBP), play important roles in tumor surveillance by natural killer (NK) cells, lymphokine-activated killer (LAK) cells, and cytotoxic T cells (1). Whereas MICA and MICB are absent from most normal tissues, they can be induced by cellular stresses, such as exposure to carcinogens and infection, and are expressed in a broad range of carcinomas and some hematopoietic malignancies (2). In humans, the interaction of cell surface MIC molecules with the C-type lectin-like NKG2D receptor on NK, LAK, and effector T cells leads to the activation of innate and adaptive immune responses with the subsequent lysis of the tumor cells (3). Thus, it has been proposed that MIC-NKG2D interactions are critical to the immune surveillance function of NK, LAK, and cytotoxic T cells.

There is evidence that the shedding of MIC ligands in a soluble form represents a mechanism of tumor cell escape from NKG2D-mediated immune surveillance (1, 3). Engagement of soluble MIC with NKG2D has been shown to down-regulate the expression of NKG2D on $CD8^+$ T cells and to suppress T-cell activation (3). Furthermore, high levels of soluble MIC were reported to decrease the levels of NKG2D and the homing receptor CXCR1 on NK cells (1). It was also shown that high levels of soluble MICA were present in the sera of patients with advanced prostate cancer and that serum MICA levels in these patients were inversely correlated with NK/LAK cell function (4). In that study, it was suggested that impaired immune surveillance may contribute to prostate cancer progression.

Hypoxia contributes to malignant progression in cancer through the stimulation of tumor cell phenotypes that include increased invasive and metastatic potential, as well as resistance to radiation and chemotherapy (5–8). Because these are survival traits selected by hypoxia, it is also possible that exposure to low levels of oxygen leads to adaptive responses that allow tumor cells to escape from immune surveillance. Although Fink and colleagues reported inhibition of NK cytotoxicity toward liver cell lines when the effector cells were cultured in hypoxic conditions (9), the effect of hypoxia on the ability of tumor cells to resist killing by immune effector cells is not well characterized.

Our research has shown that the development of hypoxia-induced malignant properties in cancer cells is secondary to inhibition of endogenous nitric oxide (NO) signaling. Specifically, we have shown that hypoxia-induced tumor cell invasiveness, metastatic ability, and resistance to chemotherapeutic agents are inhibited by molecules that activate the NO signaling pathway involving cyclic guanosine 3′,5′-monophosphate (cGMP) generation and that pharmacologic inhibition of NO signaling results in phenotypes similar to those induced by exposure to hypoxia (7, 10–12). In the present study, we provide evidence that hypoxia contributes to escape from immune surveillance in prostate cancer cells by increasing the shedding of MIC ligands. We also show that the hypoxia-mediated shedding of MIC, as well as the increased resistance to the cytolytic action of effector cells, occur through a mechanism involving impaired NO signaling.

Materials and Methods

Antibodies and reagents. Mouse anti-MICA/MICB (recognizing both MICA and MICB) and mouse anti-NKG2D monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. Negative control mouse IgG

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and goat anti-mouse second antibody conjugated with FITC was obtained from DakoCytomation. The NO synthase (NOS) inhibitor *N*-monomethyl-L-arginine (L-NMMA) and the protein kinase G (PKG) inhibitor KT5823 were from Calbiochem-Novobiochem. The nonhydrolysable analogue of cGMP, 8-bromo-cGMP, and human recombinant interleukin 2 (rhIL-2) were purchased from Sigma-Aldrich Canada Ltd. Glyceryl trinitrate (GTN) was obtained from Sabex. The nonselective matrix metalloproteinase (MMP) inhibitor Ilomastat (GM6001) was from Chemicon International, Inc. Duoset human MICA and MICB ELISA kits were from R&D Systems, 3,3′,5,5′-tetramethylbenzidine was purchased from Sigma-Aldrich Canada Ltd., [^{51}Cr] (sodium chromate) was purchased from PerkinElmer, and Ficoll-Paque Plus was obtained from Amersham Biosciences.

Cells and culture conditions. Human DU145 and PC-3 prostatic adenocarcinoma cells were obtained from the American Type Culture Collection. They were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Life Technologies Invitrogen Corp.). For incubations in standard conditions (20% O_2), cells were placed in a Thermo CO_2 incubator. For incubations in hypoxia, cells were placed in a chamber that was flushed with a gas mixture of 5% CO_2/95% N_2. Oxygen concentrations within the chamber were maintained for 24 h at 0.5% using a ProOx 110 oxygen regulator (Biospherix).

Peripheral blood was isolated from healthy male donors by venipuncture into heparinized vacutainer tubes (Becton Dickinson). Removal of blood was conducted after approval by the Queen's University Ethics Review Board. Mononuclear cells were separated by centrifugation of whole blood diluted with an equal amount of PBS layered over 3 mL Ficoll-Paque Plus for 20 min at 850 g. Cells were collected and washed with fresh PBS. To generate LAK cells, fresh peripheral blood lymphocytes (PBLs) were cultured in RPMI 1640 supplemented with 10% FBS and treated with 1,000 IU/mL rhIL-2 overnight in a regular CO_2 incubator at 37°C.

Assessment of surface MIC and NKG2D expression. Surface expression of MIC on tumor cells and NKG2D on human PBLs was analyzed with a Beckman Coulter EPICS Altra HSS flow cytometer (Beckman-Coulter). Tumor cells were seeded in 60-mm dishes and allowed to reach 80% confluence. Cells were then incubated in a standard CO_2 incubator (20% O_2) with or without L-NMMA or KT5823 or were placed in 0.5% O_2 with or without GTN or 8-bromo-cGMP for 24 h. Cells were then collected and incubated with mouse anti-human MICA/MICB monoclonal antibody for 1 h on ice. After washes, they were incubated with FITC-conjugated goat anti-mouse antibody for 1 h and then analyzed by flow cytometry. To determine the role of MMPs on MIC shedding, tumor cells were incubated with the MMP inhibitor Ilomastat (GM6001) in 20% or 0.5% O_2; they were then collected and immunolabeled for MIC as described above. To assess the effect of soluble factors released by DU145 cells on NKG2D expression by PBLs, the latter were isolated and stimulated with IL-2. They were then exposed to culture medium conditioned by DU145 cells incubated in 20% O_2 with or without L-NMMA or in 0.5% O_2 with or without GTN for 24 h. Expression of NKG2D on PBLs was determined by flow cytometry as described above.

Determination of MICA and MICB levels in the culture medium. Secretion of MICA and MICB by DU145 cells cultured under various conditions was assessed by ELISA using commercially available kits (R & D Systems) and following the manufacturer's instructions.

Cytotoxicity assays. Sensitivity of DU145 and PC-3 cells to lysis by IL-2–activated human PBLs was determined using a 4-h [^{51}Cr] release assay. After incubations under various conditions, DU145 cells (target cells) were labeled with 50 μCi sodium chromate and added to triplicate samples of serial 2-fold dilutions of PBLs (effector cells) in V-bottomed microtiter plates. Four effector/target ratios ranging from 25:1 to 3.125:1 were used in each experiment. For blocking studies, target cells were preincubated for 1 h at 4°C with anti-MICA/MICB monoclonal antibody. The anti-MICA/MICB antibody was also present during the coincubation of target and effector cells. After incubation for 4 h at 37°C in 20% O_2 [^{51}Cr] release from target cells was analyzed on a Beckman 5500 gamma counter, and the percentage of specific lysis was determined using the following formula: % lysis = 100 × [(mean experimental cpm − mean spontaneous cpm)/(mean maximum cpm − mean spontaneous cpm)]. All experiments were repeated at least thrice to ensure consistency of results.

Tumor growth studies. To determine the effect of low-dose NO mimetics on the growth of human prostate cancer cells *in vivo*, a xenograft murine model was used. Briefly, 6-wk-old to 8-wk-old male NIH Swiss nude mice (Taconic) were inoculated s.c. in the left hind flank with 2 million PC-3 cells in 0.2 mL of PBS. When the tumors reached a volume of ~150 mm^3, mice were divided in two groups of 10 to 12 each and were treated with either a 0.25 cm^2 slow-release transdermal patch (3M Pharmaceuticals) that delivers GTN at a rate of approximately 7.3 μg/h or with a placebo patch. To prevent mice from removing the GTN patches during treatment, mice were housed in individual cages, i.e., one mouse per cage. Also, patches were placed on the napes and were covered with a thin layer of New Skin Liquid Bandage (Medtech). Steady-state delivery of GTN was ensured by replacing the patches daily. Using a similar protocol we have previously shown the presence of GTN and GTN metabolites in plasma of treated mice, indicating effective transdermal delivery of the drug (7). Tumor growth was measured twice weekly using digital calipers and tumor volumes, as well as individual slopes of tumor growth curves were determined according to the formula (largest tumor diameter × smallest tumor diameter squared) × 0.5. Studies were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Analysis of MIC expression and hypoxia in prostate tumor xenografts. The expression of MICA/MICB in human PC-3 prostate tumors grown in nude mice was assessed by immunohistochemistry using a monoclonal anti-MICA/MICB antibody as described by Wu and colleagues (4). Regions of hypoxia in the tumor xenografts were determined immunohistochemically using a monoclonal antibody against the small molecule hypoxia marker pimonidazole hydrochloride (Hypoxyprobe-1, Chemicon) injected i.v. (60 mg/kg dissolved in PBS) 30 min before sacrifice. After injection, pimonidazole selectively binds to thiol groups in proteins of cells exposed to hypoxia (pO_2 < 10 mm Hg). Controls for both MICA/MICB and pimonidazole immunohistochemistry consisted of sections incubated with mouse IgG used at similar concentrations as those of the primary antibodies.

Statistical analysis. Groups involved in different oxygen treatments were mutually compared by a paired sample *t* test. In those with more than two samples, statistical significance was determined by use of one-way ANOVA followed by Fisher's post hoc analysis. For [^{51}Cr] release assay, the data from each series of experiments were fitted to the model. Interactions between these terms were considered and included where significant. Changes in cytolytic activity for the [^{51}Cr] release assay are expressed in odds ratios (OR) with 95% confidence intervals (95% CI). For *in vivo* studies, the slopes of the linear portion of the tumor growth curve for each mouse were calculated using linear regression. As the slopes did not assume a normal distribution, data were analyzed using the Kruskal Wallis test followed by Dunn's comparison post hoc test to determine significant differences. All statistical tests were two-sided, and differences considered statistically significant at $P < 0.05$. Computations were performed using S-plus 7.0 software for Windows (Insightful Corp.).

Results

NO signaling modulates hypoxia-induced MICA shedding. Compared with incubation in standard conditions (20% O_2), exposure of DU145 prostate cancer cells to hypoxia (0.5% O_2) for 24 hours significantly increased MICA shedding into the culture supernatant (Fig. 1*A*; $P < 0.05$); whereas low concentrations of the NO mimetic GTN significantly attenuated this hypoxia-induced shedding of MICA (Fig. 1*A*; $P < 0.005$). Given our previous results implicating the role of inhibition of endogenous NO signaling in hypoxia-mediated induction of malignant phenotypes, DU145 cells were incubated with L-NMMA, a competitive inhibitor of NOS, in 20% O_2. Results revealed that, compared with control cells incubated in 20% O_2 without L-NMMA, incubation of DU145 cells with L-NMMA (5 μg/mL) also led to increased MICA shedding (Fig. 1*A*; $P < 0.005$). In contrast to MICA, the levels of MICB in the

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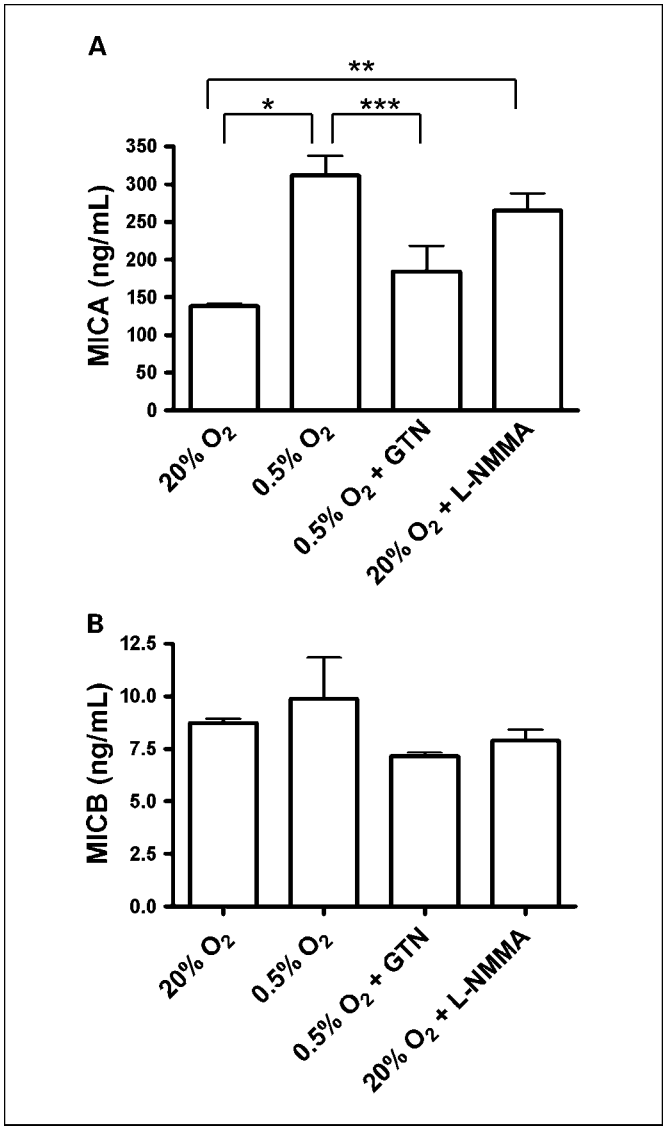

Figure 1. Effect of hypoxia and NO on MIC shedding. Results of ELISAs of conditioned medium from DU145 cell cultures revealed increased MICA shedding after 24-h incubation with 0.5% O_2 compared with standard (20% O_2) conditions (*A*; *, $P < 0.05$). Increased MICA shedding was also observed after a 24-h incubation with 5 μg/mL L-NMMA in standard (20% O_2) conditions (*A*; **, $P < 0.005$). Hypoxia-induced shedding of MICA was significantly attenuated by coincubation with 10 nmol/L GTN (*A*; ***, $P < 0.005$). In contrast to MICA, the levels of MICB in the culture medium were much lower and were not significantly affected by the above culture conditions (*B*).

culture medium were substantially lower and were not significantly affected by the above culture conditions (Fig. 1*B*).

NO signaling modulates hypoxia-induced MIC cell surface expression. To show that the observed hypoxia-induced MICA shedding also resulted in a measurable decrease in cell surface MIC expression, flow cytometry using a monoclonal antibody that recognizes both MICA and MICB was performed. Results revealed a decrease in surface MIC expression when DU145 cells were incubated for 24 hours in 0.5% O_2 (Fig. 2, *top*). Furthermore, restoration of NO signaling with low concentrations of GTN or with the nonhydrolysable analogue of cGMP, 8-bromo-cGMP, prevented the hypoxia-mediated decrease in surface MIC expression (Fig. 2, *second and third panels from top*); in a manner similar to incubation in hypoxia, incubation of cells with the NOS inhibitor L-NMMA also resulted in a decrease in surface MIC expression (Fig. 2, *fourth panel from top*). Further support for a role of the NO signaling pathway leading to generation of cGMP and subsequent activation of PKG in the hypoxia-mediated regulation of MIC shedding was provided by results showing that administration of the PKG inhibitor KT5823 decreased the expression of MIC on the DU145 cells after a 24-hour incubation in normoxic conditions (Fig. 2, *bottom*).

Given previous observations that MMPs play a role in tumor progression in part secondary to the shedding of cell surface receptors, including MIC (13–15), we investigated the effect of

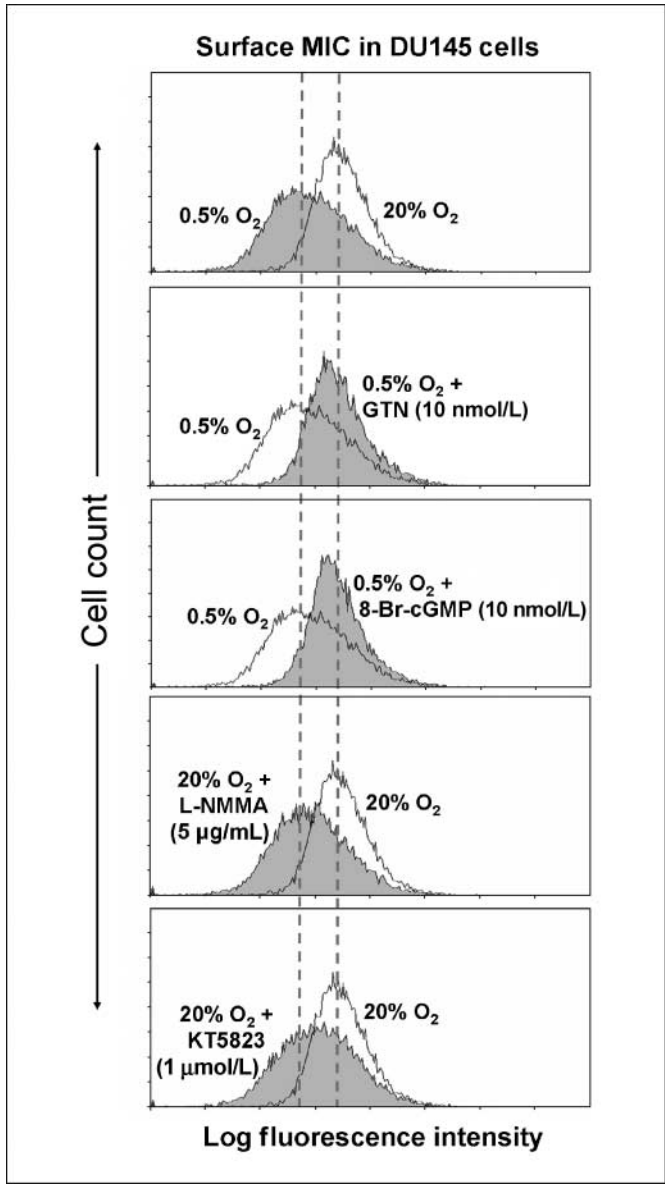

Figure 2. Effect of hypoxia and modulators of NO signaling on surface MIC expression. Flow cytometric analysis of MIC expression on DU145 cells revealed decreased expression when cells were incubated in 0.5% O_2 compared with 20% O_2 (*top*). The hypoxia-mediated decrease in surface MIC expression was prevented by coincubation with GTN (10 nmol/L; *second panel from top*) or 8-Br-cGMP (10 nmol/L; *third panel from top*). Similar decreases in cell surface MIC expression were observed when cells were incubated with L-NMMA (5 μg/mL; *fourth panel from top*) or with the PKG inhibitor KT5823 (10 μmol/L; *bottom*) in 20% O_2.

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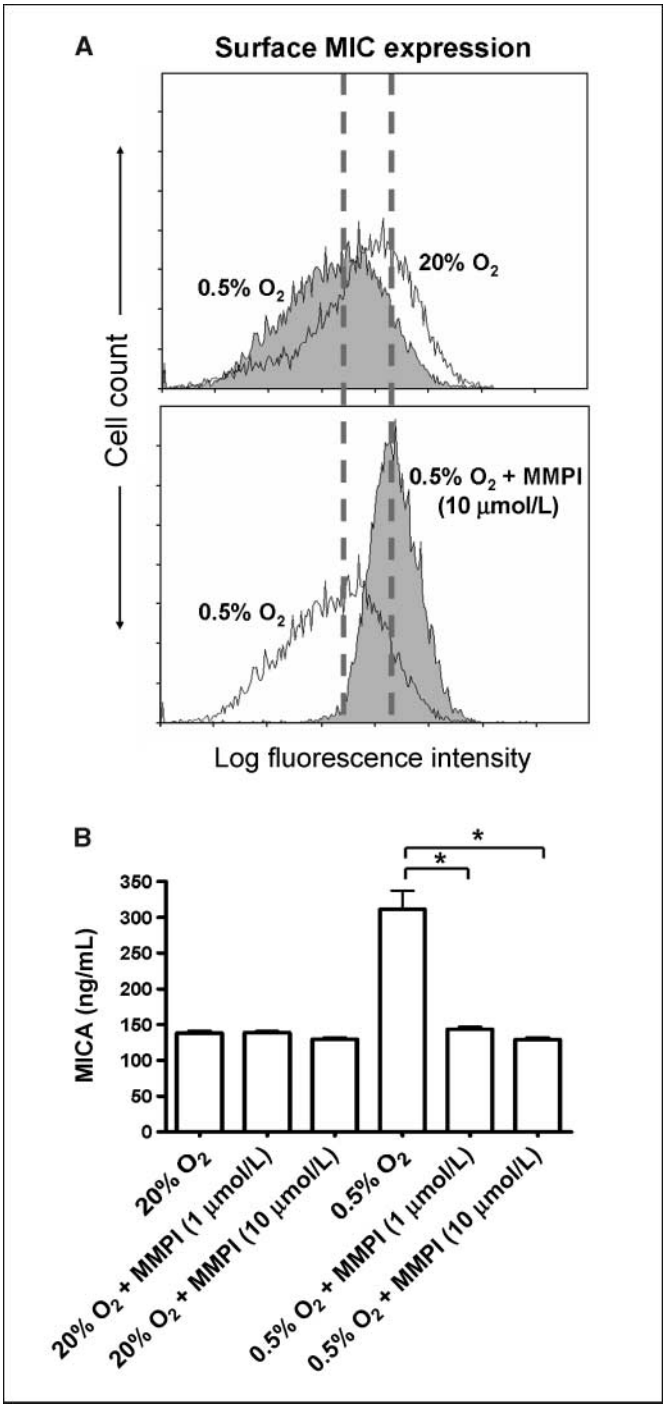

Figure 3. Role of MMPs in the hypoxia-mediated shedding of MICA. Flow cytometry showed attenuation of the hypoxia-mediated loss of surface MIC expression by the MMP inhibitor GM6001 (*MMPI*; 10 μmol/L; *A*). Results of ELISA of supernatants from cultures incubated with GM6001 (1 and 10 μmol/L) revealed no significant decrease of MICA shedding by DU145 cells incubated in 20% O_2 ($P = 0.06$) but a significant (*$P < 0.001$) decrease in hypoxia-induced MICA shedding (*B*).

MMPs in the observed hypoxia-induced shedding of MIC using the broad spectrum MMP inhibitor Ilomastat (GM6001). Incubation of DU145 cells in the presence of GM6001 attenuated the hypoxia induced loss of surface MIC expression (Fig. 3*A*). Furthermore, incubation with the MMP inhibitor did not lead to a significant inhibition of MICA shedding in cultures incubated in 20% O_2 but completely blocked MICA shedding in hypoxic cultures ($P < 0.001$; Fig. 3*B*).

Soluble MICA from hypoxic DU145 cell cultures down-regulates NKG2D expression on PBLs. To determine the role of the observed hypoxia-mediated shedding of MIC on NKG2D expression by effector cells, flow cytometry for NKG2D was performed on PBLs from healthy donors after a 24-hour incubation with supernatant obtained from DU145 cell cultures. No differences in NKG2D expression by PBLs were observed after incubation with either fresh culture medium or medium conditioned by DU145 cells incubated in 20% O_2 (Fig. 4, *top*). A decrease in NKG2D expression by PBLs was observed after a 24-hour incubation with conditioned medium from DU145 cell cultures incubated in 0.5% O_2 versus 20% O_2 (Fig. 4, *second panel from top*). This effect of hypoxic DU145 cell culture medium on NKG2D expression by PBLs was abolished with a blocking anti-MICA/MICB monoclonal antibody (Fig. 4, *third panel from top*) or by coincubating the hypoxic DU145 cells with low-dose GTN (Fig. 4, *fourth panel from top*). In a manner similar to hypoxic DU145 culture medium, a decrease in NKG2D expression occurred when PBLs were incubated with conditioned medium from DU145 cultures incubated with L-NMMA (Fig. 4, *bottom*).

Hypoxia decreases the sensitivity of DU145 cells to PBL-mediated cytotoxicity via down-regulation of surface MICA expression. To determine the effect of the hypoxia-mediated decrease in surface MIC expression on the sensitivity of DU145 cells to the cytotoxic activity of IL-2–stimulated PBLs, [^{51}Cr] release assays were performed. As the target cells were repeatedly washed after exposure to hypoxia and during the [^{51}Cr] release assay, any decreased cytotoxic activity was secondary to decreased cell surface MIC expression and not because of down-regulation of NKG2D by soluble MIC. Compared with DU145 cells incubated in 20% O_2, DU145 cells preincubated in 0.5% O_2 for 24 hours were significantly more resistant to the cytotoxic activity of IL-2–stimulated PBLs (Fig. 5*A, top*). This hypoxia-mediated resistance to NK/LAK cytotoxicity was consistent at all effector-to-target cell ratios. The spontaneous release of [^{51}Cr] by the DU145 target cells in the absence of effector cells was consistently below 10% and did not differ between culture conditions. There was some variability in NK/LAK cell-mediated cytotoxicity between experiments and between different donors of the PBLs; however, within multiple experiments, the hypoxia-mediated resistance to killing was consistent. Furthermore, similar results were obtained using human PC-3 prostate cancer cells (results not shown).

Although the sensitivity to PBL-mediated cytotoxicity was unaffected by GTN in DU145 cells maintained in 20% O_2, administration of GTN (10^{-8} mol/L) at the beginning of a 24-hour incubation in 0.5% O_2 significantly attenuated the hypoxia-mediated resistance to killing in DU145 cells (Fig. 5*A, middle*) and PC-3 cells (not shown). In a similar manner to preincubation in hypoxia, incubation with the NOS inhibitor L-NMMA in 20% O_2 resulted in resistance of DU145 cells to PBL-mediated cytotoxicity (Fig. 5*A, bottom*).

When a blocking anti-MICA/MICB antibody was included in the [^{51}Cr] release assay (i.e., PBL-DU145 coincubations), the sensitivity to the cytolytic activity of donor PBLs was significantly decreased in DU145 cells preincubated in 20% O_2 (Fig. 5*B, top*); however, addition of anti-MICA/MICB antibody did not further decrease the sensitivity to PBL-mediated cytotoxicity in DU145 cells preincubated in 0.5% O_2 (Fig. 5*B, middle*). Furthermore, the presence of anti-MICA/MICB antibody significantly blunted the immunosensitizing effect of GTN on hypoxic DU145 cells (Fig. 5*B,*

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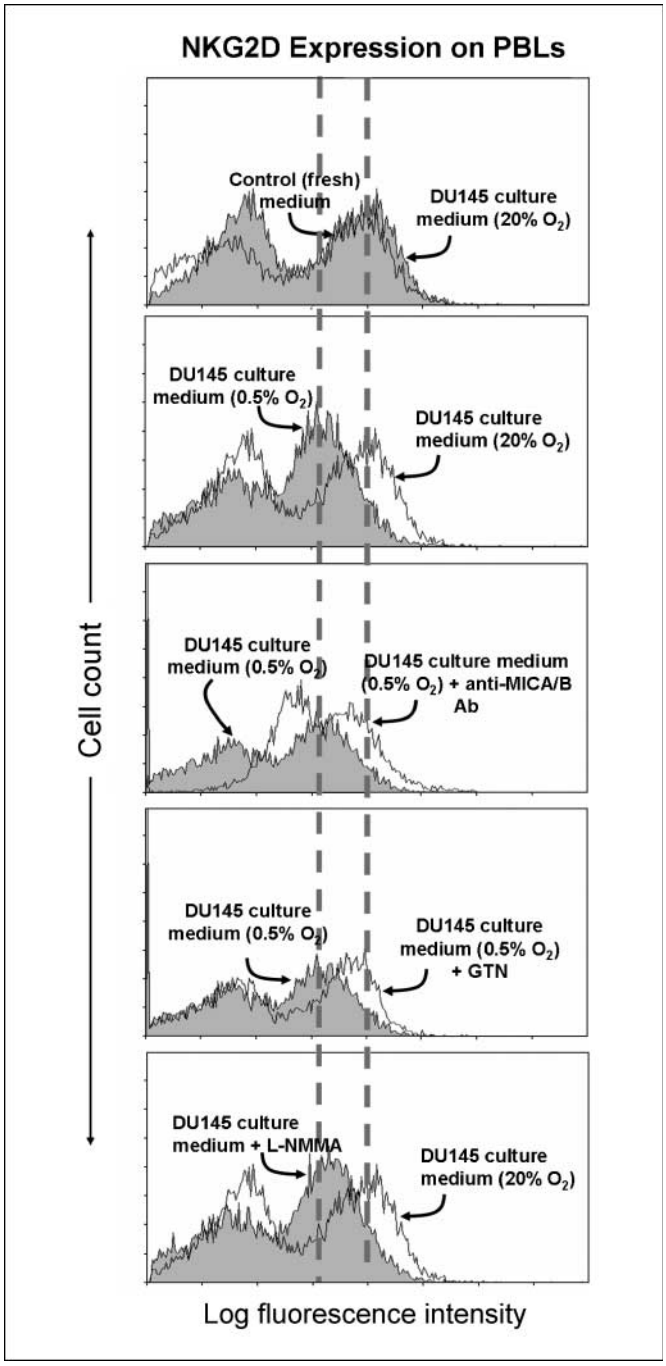

Figure 4. Effect of soluble MIC from hypoxic DU145 cell cultures on NKG2D expression by PBLs. Flow cytometric analysis revealed no differences in NKG2D expression in PBLs incubated with either fresh culture medium or medium conditioned by DU145 cells incubated in 20% O_2 (*top*); however, the expression of NKG2D in PBLs was inhibited after a 24-h exposure to medium conditioned by DU145 cells incubated in 0.5% O_2 (*second panel from top*). The decrease in NKG2D expression after exposure to conditioned medium was blocked by culturing PBLs with anti-MICA/MICB antibody (*third panel from top*) or by exposure to medium conditioned by hypoxic DU145 cells incubated with GTN (10 nmol/L; *fourth panel from top*), whereas incubation with medium conditioned by DU145 cells incubated with L-NMMA (0.5 μg/mL) resulted in decreased NKG2D (*bottom*).

bottom). Together, these observations provide evidence that the decreased sensitivity to PBL-mediated cytotoxicity in DU145 cells preincubated in hypoxia is at least in part due to decreased surface MIC expression.

Low-dose GTN decreases the rate of prostate tumor growth *in vivo*. To determine the effect of low-dose NO mimetic treatment on the growth of prostate tumors *in vivo*, a xenogeneic heterotopic murine model was developed. Although mice do not express the human NKG2D ligands MICA/MICB, murine cancer cells express other ligands, including RAE-1, H60, and ULBP-like transcript-1, which act as ligands for NKG2D (16). Furthermore, it has been shown that human MICA/MICB on tumor cells can also bind murine NKG2D and act as potent activating ligands (16). Because of more favorable tumor growth compared with xenotransplanted DU145 cells, human PC-3 cells were used and implanted into the flanks of nude mice. The mice were concomitantly treated with a transdermal patch delivering low concentrations of GTN (7.3 μg/h) or with a placebo patch. Tumor volume measurements taken over a 7-week period indicated a significant decrease in the rate of growth of PC-3 prostate tumors in mice treated with low-dose GTN (Fig. 6*A* and *B*). Immunohistochemical analysis of PC-3 tumor sections using antibodies against reductively activated pimonidazole hydrochloride and MICA/MICB revealed extensive regions of hypoxia, as well as widespread areas of MIC immunolocalization (Fig. 6*C* and *D*).

Discussion

Interactions between tumor cells and their microenvironment play an important role in malignant progression and metastasis. In the present study, we provide evidence that hypoxia contributes to the ability of tumor cells to avoid destruction by effector cells through a mechanism that involves shedding of NKG2D ligands from the membrane. It has been reported that the strength of an antitumor immune response in mice is dependent on the levels of NKG2D ligands on the surface of tumor cells (17). Our study revealed that the hypoxia-mediated shedding of MIC is secondary to impaired NO signaling in the tumor cells. This conclusion is based on results showing that the shedding of MICA by tumor cells exposed to hypoxia can be blocked after administration of molecules that activate NO signaling. Also, the fact that pharmacologic inhibition of endogenous NO signaling in well-oxygenated cells resulted in adaptive responses similar to those induced by hypoxia, i.e., increased MICA shedding, supports the concept that hypoxia and decreased NO signaling are mechanistically linked.

There is evidence that exposure of cells to low levels of oxygen (1–3%) results in marked inhibition of NO production (18, 19). NO is produced from L-arginine in a reaction catalyzed by the enzyme synthases (NOS), with oxygen as a required cofactor (18–20). Hypoxia has also been shown to increase arginase activity in macrophages (21), thus diverting L-arginine metabolism away from the NO generation pathway and into the urea cycle. Thus, we propose that tumor cells can adapt to hypoxia via a mechanism that involves inhibition of NO signaling. Whereas NO signaling is multifaceted, the fact that low concentrations of GTN were able to inhibit hypoxia-induced MIC shedding suggests that the mechanism responsible for this effect requires the interaction of NO with transition metals (22). Classic NO signaling involves the NO-mediated activation of the iron-containing enzyme soluble guanylyl cyclase, which in turn catalyses the conversion of GTP into cGMP. The latter subsequently activates PKG, leading to the phosphorylation of various target molecules that regulate cell function and gene expression. In our study, support for a role of classic NO signaling in the inhibition of hypoxia-induced shedding of MIC

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was provided by the fact that activation of PKG with 8-bromo-cGMP led to inhibition of hypoxia-induced MIC shedding and that pharmacologic inhibition of PKG, with KT5823, resulted in increased MIC shedding.

Whereas our study focused primarily on the direct effect of hypoxia on the ability of tumor cells to evade recognition and lysis by effector cells, we also showed that hypoxia can indirectly decrease surface NKG2D levels and hence block the activation of effector cells. As previously reported by others using sera from colorectal cancer patients (1), our results revealed that effector cells exposed to soluble MIC present in the culture medium of DU145 cells preincubated in hypoxia or with L-NMMA displayed decreased levels of surface NKG2D. Interaction of soluble MIC with NKG2D on NK cells leads to internalization of NKG2D-MIC complexes and, consequently, to a decrease in cell surface expression of NKG2D (1). These decreases in surface NKG2D levels have been associated with defects in NK cell–mediated antitumor cytotoxicity (1). Soluble MIC present in the sera of colon cancer patients was also shown to inhibit the expression of the homing receptor CXCR1 in NK cells likely via a mechanism secondary to NKG2D down-regulation (1). It has also been shown by others that, regardless of NKG2D down-regulation, soluble MIC can contribute to immune escape by competitively inhibiting interactions between membrane-associated MIC and NKG2D (23).

Wu and colleagues recently showed that the shedding of MIC occurs in more advanced prostate cancers and that this shedding may be an important mechanism of tumor escape from NK/LAK cell-mediated lysis through the down-regulation of NKG2D expression (4). A significant correlation has been reported between soluble MICA levels and tumor stage and metastasis in patients with various malignancies (23, 24). Thus, the ability to shed MIC seems to be a selected trait linked to malignant progression. Based on our results, we propose that tumor hypoxia provides a key selection pressure driving MICA shedding. In the present study we did not observe a significant effect of hypoxia or L-NMMA on the shedding of MICB. However, the levels of MICB in the culture medium of DU145 cells were much lower than those of MICA. It is possible that the extent and efficiency of MIC shedding is dependent on the numbers of MIC molecules present on the cell surface. However, because the antibody used to for flow cytometry reacts with both MICA and MICB, it was not possible to determine the relative levels of these ligands on the cell surface.

An additional mechanism by which hypoxia could contribute to immune escape may involve the up-regulation of MHC class I antigens on the surface of tumor cells, as the binding of MHC class

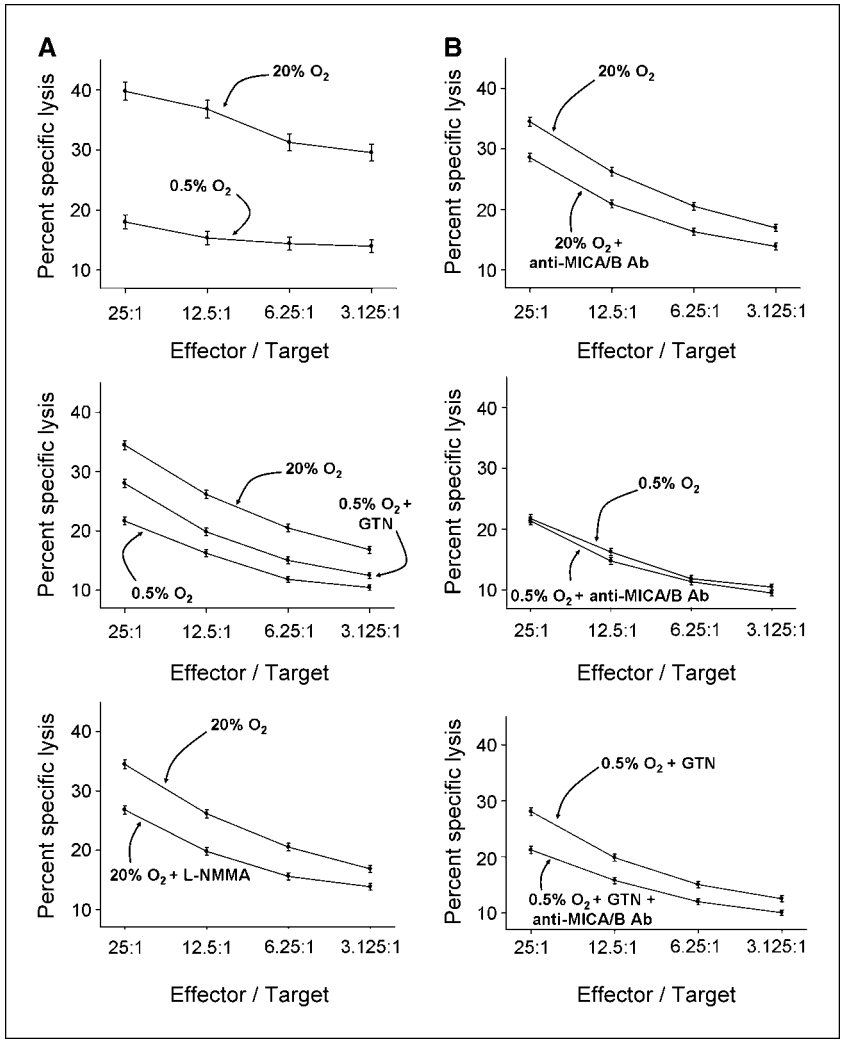

Figure 5. Resistance of DU145 cells to the cytolytic activity of IL-2–stimulated PBLs. DU145 cells were preexposed to various culture conditions for 24 h before a 4-h [^{51}Cr] release assay with fresh PBLs stimulated overnight with 1,000 IU IL-2. Results revealed increased resistance to PBL-mediated lysis in DU145 cells preincubated in 0.5% O_2 versus 20% O_2 (OR of 20% O_2 and 0.5% O_2, 1.89; 95% CI, 1.82–2.0; *A, top*). Resistance to PBL-mediated lysis was significantly inhibited in hypoxic DU145 cells by administration of low concentrations of GTN (10 nmol/L; OR of 0.5% O_2 + GTN and 0.5% O_2, 1.41; 95% CI, 1.33–1.48; *A, middle*). Resistance to PBL-mediated cytotoxicity was observed in DU145 cells preincubated with L-NMMA (0.5 μg/mL) at 20% O_2 (20% O_2 and 20% O_2 + L-NMMA OR, 1.27; 95% CI, 1.19–1.35; *A, bottom*). Incubation of DU145 cells in 20% O_2 with a blocking MICA/MICB antibody resulted in increased resistance to lysis by PBLs (20% O_2 and 20% O_2 + MICA/MICB antibody OR, 1.315; 95% CI, 1.282–1.351; *B, top*). Incubation of DU145 cells in 0.5% O_2 with a blocking MICA/MICB antibody resulted in no difference in cytolytic activity of PBLs (0.5% O_2 and 0.5% O_2 + MICA/MICB antibody OR, 1.064; 95% CI, 1.031–1.099; *B, middle*). Incubation with the blocking MICA/MICB antibody attenuated the immunosensitizing effect of GTN (0.5% O_2 + GTN and 0.5% O_2 + GTN + MIC antibody OR, 1.35; 95% CI, 1.31–1.39; *B, bottom*).

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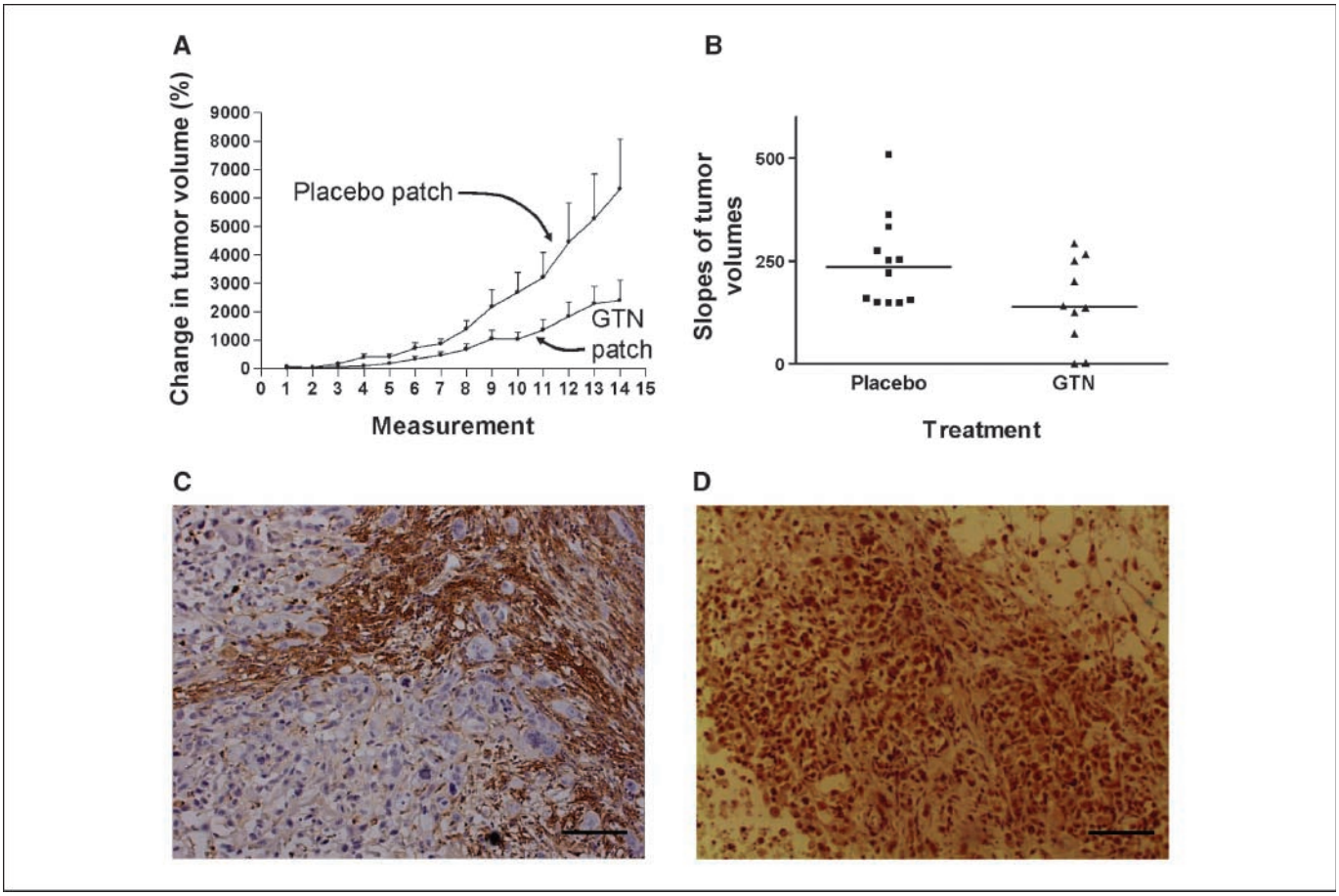

Figure 6. PC-3 tumor growth in a xenogeneic nude mouse model. Significant decrease of tumor growth (*A*) and slope of tumor volume (*B*) over a 7-week period in mice treated with low-dose GTN as a skin patch after 2 million PC-3 cells injected s.c. Immunoperoxidase staining for regions of hypoxia (Hypoxyprobe-1 adducts, Chemicon) in human PC-3 prostate tumors in the xenogeneic model (*C*). Tumor-bearing mice were injected with 60 mg/kg bioreductive pimonidazole hydrochloride i.v. 30 min before sacrifice. The presence of hypoxic regions was confirmed by dark brown staining in contrast with well-oxygenated, nonhypoxic areas. Immunoperoxidase staining of MIC protein in PC-3 tumors (*D*). Control sections incubated with mouse IgG at the same concentrations as those used for the primary antibodies revealed lack of staining (not shown). *Bar,* 50 μm.

I to inhibitory receptors on NK cells is known to prevent the activation of the latter. Our preliminary flow cytometry results using an antibody that reacts with a monomorphic epitope on the α-chain polypeptide of human class I MHC molecules revealed that, whereas DU145 cells do indeed express MHC class I antigen on the surface, exposure of these cells to hypoxia did not result in significantly higher levels of MHC class I expression.[5] It is interesting that despite the presence of MHC class I antigens on the surface of DU145 cells, IL-2–activated PBLs were still able to lyse the tumor cells. It has been shown that MIC binding to NKG2D receptors on NK cells can override negative signals generated after engagement of inhibitory MHC class I receptors (25). Thus, hypoxia-mediated decreases in surface MIC levels likely lead to enhanced inhibitory signals in the effector cells. We are currently investigating whether hypoxia causes the shedding of other NKG2D ligands, such as the ULBPs. In a manner similar to the shedding of MICA and MICB, the shedding of ULBP2 from the surface of tumor cells has been shown to involve MMP activity (14).

In addition to inducing the shedding of MIC from the surface of tumor cells, it is now well established that hypoxia up-regulates the expression of other malignant phenotypes in tumor cells, such as resistance to chemotherapeutic agents (7, 26–28), as well as invasive and metastatic potential (6, 11, 29, 30). Furthermore, the extent of hypoxia in the tumor mass has been shown to be an independent marker of a poor prognosis for patients with various types of cancers and a correlation has been shown between tumor grade and the presence of hypoxic regions within the tumor mass (31–33). Our previous studies revealed that the hypoxia-mediated acquisition of tumor cell invasiveness, metastatic potential, and resistance to chemotherapeutic agents can be inhibited by activating NO signaling (7, 10–12). The results of the present study indicate that another important aspect of malignant progression, i.e., escape from immune surveillance, may also be regulated in a similar way.

As previously shown by others (3, 13), the results of our study support a role for an MMP(s) in the shedding of MICA from the surface of tumor cells. Although our results did not show a significant inhibition of MICA shedding with the MMP inhibitor GM6001 in cells incubated in 20% O_2 (P = 0.06), there was a significant attenuation of the hypoxia-induced MICA shedding. Whereas the identity of the MMP responsible for MIC shedding is presently unknown, the fact that in the presence of the MMP inhibitor hypoxia-induced shedding of MICA was abolished

[5] N. Hu, D.R. Siemens, and C.H. Graham, unpublished observation.

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suggests the involvement of hypoxia-inducible MMP activity in MIC shedding.

In our study, results of [^{51}Cr] release assays revealed an association between hypoxia-induced MIC shedding in DU145 cells and resistance to lysis. As for MIC shedding, hypoxia-induced resistance to PBL-mediated lysis was prevented by incubation with low concentrations of GTN. A causal relationship between hypoxia-induced MIC shedding and resistance to PBL-mediated lysis was confirmed by results showing that, in the presence of an anti-MICA/MICB antibody which prevents MIC-NKG2D interactions, overall sensitivity to lysis was decreased and the effects of hypoxia and GTN on tumor cell resistance to lysis were abolished.

MIC shedding seems to facilitate the ability of several tumors, particularly prostate cancer, to evade host immune surveillance and allow ongoing progression and metastases. Given our *in vitro* results on hypoxia-induced MIC shedding and its observed attenuation by reestablishing NO signaling, we performed *in vivo* tumor growth studies in mice treated with low-dose GTN. We previously showed that treatment of mice with a similar low-dose GTN transdermal patch results in detectable levels of GTN and metabolites in the plasma compared with placebo-treated mice (7). Treatment of mice with low-dose GTN resulted in a significant reduction in the rate of tumor growth. Whereas nude mice are athymic and therefore unable to mount cytotoxic T-cell responses, they are capable of mounting NK cell–mediated antitumor responses (34). Immunohistochemical studies confirmed the presence of hypoxia in the xenografted tumors, although no correlation of MIC expression could be made with the treatment groups. It is possible that the observed GTN-mediated inhibition of tumor growth is secondary to multiple factors. It is unlikely, however, that the antitumor action of low-dose GTN is due to direct cytotoxic or cytostatic effects on the tumor cells, as we have previously shown the absence of such effects on cancer cells *in vitro* (11).

Although the expression of surface MIC at early stages of malignant progression is thought to lead to activation of effector cells and destruction of the tumor cells, the development of hypoxia within the microenvironment of advanced tumors may lead to the shedding of MIC and, subsequently, to immune escape. Our results provide evidence that this hypoxic adaptation of tumor cells may be linked to impaired NO signaling. Therefore, reactivation of NO signaling through administration of NO mimetic agents may be a useful approach to enhance immune surveillance, as well as immunotherapy of cancer.

Disclosure of Potential Conflicts of Interest

D.R. Siemens: commercial research grant, Abbott Merck; member of clinical advisory board of Nometics.

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