Local Inhibition of Natural Killer Cell Activity Promotes the Progressive Growth of Intraocular Tumors

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Purpose. To study the effect of aqueous humor (AH)-mediated inhibition of natural killer (NK) cell activity on intraocular tumor progression.

Methods. Two NK-sensitive tumors, RMA-S lymphoma and OCM-3 uveal melanoma, were tested in vitro for susceptibility to NK cell-mediated lysis in the presence or absence of AH in conventional cytotoxicity assays. Various numbers of RMA-S and OCM-3 tumor cells were injected either subcutaneously or intracamerally into C57BL/6 severe combined immunodeficient mice and BALB/c nude mice respectively and tumor growth was monitored. The role of NK cell-mediated cytotoxicity in controlling tumor growth was confirmed by depleting NK cells in severe combined immunodeficiency mice by administering anti-asialo GM1 antibodies before subcutaneous tumor injection.

Results. AH significantly inhibited NK cell-mediated lysis of RMA-S and OCM-3 tumor cells in vitro and in vivo. NK sensitive RMA-S (1 × 10^6 cells) and OCM-3 tumors (1 × 10^6, 5 × 10^6 cells) were rejected after subcutaneous injection in C57BL/6 mice, whereas the same or even lower numbers of cells grew progressively in the eye. In vivo NK cell depletion resulted in progressive growth of subcutaneously injected RMA-S tumors at a dose rejected by mice with normal NK cell activity.


The intraocular microenvironment is an immunologically privileged site in which tissue and tumor allografts escape immunologic rejection and destruction. The intraocular milieu is endowed with a multitude of growth factors and immunosuppressive factors that promote the growth and survival of intraocular tumors.
of factors that contribute to ocular immune privilege by exerting immunomodulatory effects. An important contributor to immune privilege is transforming growth factor-β (TGF-β), a cytokine present within the anterior and posterior compartments of the eye. TGF-β is a pleiotropic molecule and its functions include suppression of T and B lymphocyte activity and in vitro inhibition of natural killer (NK) cell cytotoxicity. The posterior segment of the eye, especially the subtretinal space and vitreous, contains many immunomodulatory factors (including TGF-β) that maintain immune privilege at this site. The anterior segment of the eye is rich in immunosuppressive factors such as TGF-β, alpha-melanocyte stimulating hormone, glucocorticoids, vasoactive intestinal peptide, and many other molecules that inhibit cellular and humoral immune responses.

In addition to aqueous humor (AH)-borne immunosuppressive factors, dynamic immunoregulatory processes contribute to ocular immune privilege. Anterior chamber (AC) presentation of antigen evokes a unique deviant immune response in which delayed-type hypersensitivity is actively downregulated, whereas the antibody and cytotoxic T lymphocyte arms of the immune response are preserved. This selective diversion of the immune response away from delayed-type hypersensitivity has been termed AC-associated immune deviation and has been offered from delayed-type hypersensitivity is actively downregulated, whereas the antibody and cytotoxic T lymphocyte arms of the immune response are preserved.

Immune privilege represents a precarious balance between protective responses and unwitting tissue-damaging responses. The corneal endothelium that lines the AC is particularly vulnerable to immune-mediated injury because the cells that form this surface are terminally differentiated and cannot regenerate. According to the "missing self" hypothesis, NK cells recognize and lyse cells that express low levels of major histocompatibility complex (MHC) class I molecules. The paucity of MHC class I antigens on corneal endothelial cells, along with their inability to undergo mitosis, makes them especially vulnerable to NK-mediated injury. However, NK-mediated cytolysis is inhibited by at least two AH-borne factors, TGF-β and a 12-kDa protein. Both factors are found in murine, rabbit, and human AH (Apte RS, unpublished data, 1997).

Although AH-borne NK inhibitory factors are thought to protect the corneal endothelium from NK-mediated damage, this may become a liability in the case of intraocular tumors. Uveal melanomas with low surface class I expression are sensitive to NK cell-mediated lysis in vitro. Moreover, NK cell depletion in vivo results in increased incidence of hepatic metastases after intracameral injection of uveal melanomas in mice. NK cells can constitute a large percentage of the tumor-infiltrating lymphocyte population isolated from uveal melanomas. As described previously, AH contains at least two NK cell inhibitory factors. The ocular milieu may thus provide immunologic privilege for NK-sensitive tumors such as uveal melanomas. This hypothesis was tested prospectively using two NK-sensitive tumors.

**MATERIALS AND METHODS.** Mice. C57BL/6 severe combined immunodeficiency (SCID) mice were obtained from the University of Texas Southwestern Medical Center at Dallas (Department of Microbiology Mouse Colony). BALB/c nu/nu mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Fas ligand (FasL) mutant gld mice (gld/gld) were provided by Dr. Rolf Yoho (Neuroscience Research Center, UT Southwestern Medical Center, Dallas, TX). All animals used in these experiments were housed and cared for in accordance with the guidelines of the University Committee for the Humane Care of Laboratory Animals, NIH Guidelines on Laboratory Animal Welfare, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Aqueous Humor.** Rabbit eyeballs (Pelfreeze Biologicals Division, Fayetteville, AR) were washed with a 1% iodine solution before AH collection. AH was collected from the AC by paracentesis under sterile conditions using a siliconized 25-gauge needle and syringe. Penicillin-streptomycin-fungizone (1%) were added to AH and stored at −70°C in siliconized vials.

**Natural Killer Cell Culture.** Spleen cells from C57BL/6 mice were cultured for 4 days at 37°C and 10% carbon dioxide atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 5 × 10^{-5} M 2-ME (Sigma, St. Louis, MO) and 1000 U/ml of recombinant human interleukin-2 (NCI; Biological Response Modifiers Program, Frederick, MD; Hoffman-La Roche, Nutley, NJ) and supplemented with 10% heat-inactivated fetal calf serum (Hyclone Research Products, Logan, UT), 1% MEM vitamins, 1% L-glutamine, 0.01 M Heps buffer, and 1% penicillin-streptomycin-fungizone (complete DMEM) to generate lymphokine-activated killer cells, which are highly enriched for NK cells. The cells then were layered over Histopaque-1078 (Sigma Chemical, St. Louis, MO), centrifuged at 3000 revolutions per minute for 20 minutes at room temperature, and the buffy coat was collected. These cells were used as effector cells in NK cell cytotoxicity assays.

**Tumor Cell Lines.** RMA-S is a C57BL/6-derived murine lymphoma cell line (provided by Dr. Michael Bennett, Department of Pathology, UT Southwestern Medical Center, Dallas, TX). The cells were maintained in RPMI-1640 (Hazleton, Denver, PA) supplemented with 10% heat-inactivated fetal calf serum, 1% nonessential amino acids, 1% L-glutamine, 0.01 M...
Hepes buffer, 1% sodium pyruvate, and 1% penicillin-streptomycin-fungizone solution (complete RPMI). RMA-S cells are characterized by a mutation in the TAP-2 transporter gene and consequently fail to process class I peptides and assemble MHC class I molecules. Thus, they fail to express stable class I molecules on the cell surface. OCM-1 and OCM-3 are human spindle and epithelioid uveal melanoma cell lines, respectively, and were provided by Dr. June Kan-Mitchell (University of California at San Diego, San Diego, CA). OCM-1 and OCM-3 cells were cultured in HAM's F12 medium containing 10% heat-inactivated fetal calf serum, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 0.01 M Hepes buffer, and 1% penicillin-streptomycin-fungizone solution.

Natural Killer Cell Cytotoxicity Assays. A 4-hour chromium release assay was used to determine NK cell cytotoxicity in vitro, as described previously. Briefly, RMA-S and OCM-3 tumor cells were labeled with $^{51}$Cr for 2 hours and washed three times with RPMI. NK cells and radiolabeled target cells then were coincubated at varying effector-to-target ratios in 96-well microtiter plates at 37°C for 4 hours after the plates were centrifuged at 500 revolutions per minute for 2 minutes. The plates were centrifuged at 500 revolutions per minute for 5 minutes after incubation, and the supernatants were harvested and counted on a gamma counter. Cytotoxicity was determined by the amount of $^{51}$Cr specifically released from target cells. Three replicates were used for each experimental group, and the percent-specific lysis was calculated using the formula: $\%$ cytolysis = [(cpm in experimental wells) — (cpm in wells with target cells alone)] / [(cpm incorporated into target cells) - (cpm in wells with target cells alone)] x 100. To assess the ability of AH to inhibit NK cell-mediated lysis, 4-day cultured NK cells were resuspended in medium or 50% AH and tested in conventional cytotoxicity assays.

Subcutaneous Tumor Growth. RMA-S, OCM-1, and OCM-3 tumor cells were resuspended in Hanks balanced salt solution, and 100 $\mu$l of the cell suspension was injected subcutaneously (SC) in the right flank of C57BL/6 SCID mice and BALB/c nude mice, respectively. RMA-S cells were injected at $1 \times 10^5$, $5 \times 10^4$, or $1 \times 10^4$ cells/mouse. OCM-1 and OCM-3 cells were injected at $1 \times 10^6$ or $5 \times 10^5$ cells/mouse. Tumor growth was monitored every 5 days after injection. Tumor size was measured with calipers, and the two largest diameters were scored in millimeters. Tumor size was calculated as square millimeters to represent the product of the two diameters measured.

Intraocular Tumor Inoculations. RMA-S and OCM-3 tumor cells were resuspended in Hanks balanced salt solution and injected intracameral (IC) as described previously. Briefly, a glass micropipette (approximately 80 $\mu$m in diameter) was fitted into a sterile 5-French infant-feeding tube, (Professional Medical Products, Ocala, FL), which was mounted onto a Hamilton automatic dispensing apparatus (Hamilton, Whittier, CA), and 5 $\mu$l tumor cell suspensions were dispensed into the AC of anesthetized mice. RMA-S cells were injected IC at either $5 \times 10^5$ or $1 \times 10^4$ cells/eye, whereas OCM3 cells were injected at $1 \times 10^5$ cells/eye. Using a dissecting microscope, anesthetized animals were observed, and tumor growth was recorded as percent of the AC occupied by the tumor.

Anti-Asialo GM1 Treatment. Anti-asialo GM1 antibody (Wako Chemicals, Dallas, TX) was used for in vivo depletion of NK cells. Mice were treated intraperitoneally (0.3 ml of a 1:40 dilution/mouse per day) with anti-asialo GM1 antibody on day −7 through day 0, and then every 5 days thereafter. RMA-S or OCM-3 tumors were transplanted SC on day 0. Tumor growth was monitored as described above.

RESULTS. Effect of Aqueous Humor on Natural Killer Cell-Mediated Cytolysis of Natural Killer-Sensitive Tumors. The effect of AH on NK cell-mediated lysis of two NK-sensitive tumors was tested in vitro. NK cells were resuspended in DMEM only or DMEM containing 50% AH. RMA-S lymphoma cells and OCM-3 uveal melanoma cells were evaluated for susceptibility to NK-mediated lysis in a conventional 4-hour $^{51}$Cr release assay. As shown in Figure 1, AH significantly inhibited NK cell-mediated lysis of both tumors.

Natural Killer-Mediated Rejection of RMA-S and OCM-3 Tumors at Extracocular Sites. The susceptibility of RMA-S lymphoma to NK cell-mediated rejection was assessed by SC transplanting various doses of tumor cells into T- and B-cell-deficient SCID mice, which are known to have a normal NK cell repertoire. As shown in Figure 2A, low doses of RMA-S cells (i.e., $1 \times 10^4$ and $5 \times 10^4$ cells/mouse) were rejected. However, 100-fold higher doses of RMA-S tumor cells were not rejected and developed into progressively growing subcutaneous tumors, presumably caused by tumor inocula exceeding the limits of NK cell effector capability in the SCID mouse.

As shown above, and by Ma et al., OCM-3 is a highly NK-sensitive tumor (>90% lysis in cytotoxicity assays in vitro) and OCM-1 is a relatively NK-insensitive tumor (<10% lysis). Additional experiments examined the susceptibility of OCM-3 human uveal melanoma cells to NK cell-mediated rejection. For these experiments, OCM-3 melanoma cells were transplanted SC into athymic nude mice to show that NK cell-mediated tumor rejection was not limited to the SCID mouse. The results showed that high doses of NK-sensitive OCM-3 cells failed to grow after SC transplantation into athymic nude mice (Fig. 2B). By contrast, the same doses of NK-insensitive OCM-1 melanoma cells grew progressively.
The rejection of NK-sensitive RMA-S and OCM-3 tumors presumably was mediated by NK cells because SCID and nude mice are devoid of normal T cells, but possess functional NK cells. This hypothesis was tested by depleting NK cells in SCID mice before SC challenge with RMA-S tumor inocula at a dose that was rejected consistently in SCID mice (i.e., $1 \times 10^4$ cells/mouse). Accordingly, SCID mice were depleted of NK cells by a series of intraperitoneal injections of anti-asialo GM1 antibody before SC challenge with $1 \times 10^4$ RMA-S tumor cells. As shown in Figure 3, $1 \times 10^4$ RMA-S tumor cells did not form palpable tumors in untreated SCID mice. By contrast, progressively growing subcutaneous RMA-S tumors developed in NK cell-depleted SCID mice after SC transplantation of $1 \times 10^4$ tumor cells.

Progressive Tumor Growth After Intraocular Inoculation. In contrast to tumors injected SC, low doses of RMA-S ($1 \times 10^4, 5 \times 10^3$) and OCM-3 ($1 \times 10^5$) tumor cells grew progressively after IC inoculation (Figs. 4A and 4B, respectively). In both cases, the tumors grew rapidly within the eye and eventually occupied 100% of the AC. These results suggest that AH-
mediated inhibition of NK cell activity in the AC of the eye permits the growth of tumor cells at doses 10 to 20 times lower than those that are normally rejected by NK cells at extraocular sites.

It recently has been shown that the expression of FasL within the eye induces apoptotic death of Fas+ leukocytes that enter the AC. It is possible that the ability of NK-sensitive tumors to escape rejection in the eye is because of FasL-mediated inactivation of infiltrating NK cells. This hypothesis was tested by transplanting $1 \times 10^5$ RMA-S cells IC into gld/gld mice that lack functional FasL. The results showed that IC inocula of RMA-S cells produced progressively growing intraocular tumors that were indistinguishable from those in SCID mice (data not shown).

DISCUSSION. In this study, we have shown that AH inhibits NK cell-mediated lysis of tumor cells that express reduced amounts of MHC class I molecules and are susceptible to NK cell-mediated lysis. The results also show that AH significantly reduces the susceptibility of syngeneic (RMA-S) and xenogeneic (OCM-3) tumor cells to NK-mediated lysis in vitro. Low doses of RMA-S and OCM-3 tumor cells are rejected at subcutaneous sites, but tumor inocula containing 10 to 20 times less tumor cells escape NK-mediated lysis and grow progressively within the eye. Given the high susceptibility of these tumors to NK cell-mediated lysis, the failure of the immune system to mount an effective antitumor response in the eye probably is because of the inhibition of NK cell activity in the ocular milieu. This is supported by the finding that depletion of NK cells in vivo with anti-asialo GM1 antibody treatment before tumor challenge leads to progressive subcutaneous growth of RMA-S at doses that do not produce tumors in mice with normal NK cell activity.

The current study illustrates that ocular immune privilege is a double-edge sword. Inhibition of overzealous immune responses in the eye protects the delicate ocular tissues from immunologic destruction. This is a teleologically significant protective mechanism for preserving vision. Conversely, immune privilege may be detrimental to the host because a disabled immune system would be unable to launch effective antitumor
immune responses. The privileged environment in the anterior segment, maintained in part by the immunosuppressive properties of AH, might facilitate unhampered growth and proliferation of uveal melanomas that are otherwise susceptible to NK cell-mediated lysis. This is especially significant because NK cells have been shown in tumor-infiltrating lymphocyte populations isolated from human uveal melanomas.

NK cells that enter the eye are potentially important mediators of tumor immunity. However, as shown here, the intraocular milieu inhibits NK function and permits the progressive growth of NK-sensitive intraocular tumors. The inhibition of NK function might be because of the FasL-induced inactivation of NK cells within the eye. However, unrestricted growth of RMA-S tumors in the eyes of mice lacking FasL (i.e., gld/gld mice) argues against FasL-induced apoptosis as a mechanism for the tumor to escape NK-mediated rejection in the eye. By contrast, the capacity of AH to produce immediate inhibition of NK activity in vitro strongly suggests that NK-sensitive intraocular tumors escape elimination because of the NK inhibitory effects of AH-borne factors.

One can only speculate whether the inhibitory effects of AH favor the survival of NK-sensitive human uveal melanomas. However, one recent study has shown a significant correlation between low MHC class I antigen expression on primary uveal melanomas and prolonged patient survival. It is tempting to speculate that uveal melanomas expressing low levels of MHC class I antigens escape NK-mediated rejection within the eye, but are rejected by the potent NK cell population within the liver, the most common site for uveal melanoma metastases. Spindle cell uveal melanomas display the least malignant behavior and, coincidentally, express only low levels of MHC class I antigen.

The profound suppression of NK cell-mediated surveillance would seem to favor the progressive growth of tumors arising in the anterior segment of the eye, such as iris melanomas. However, the relatively benign behavior of iris melanomas indicates that nonimmunologic factors can influence the malignancy of intraocular tumors.

Further exploration of the unique immunologic and nonimmunologic characteristics of the intraocular milieu should provide a better understanding of the pathophysiology of uveal melanoma and offer important possibilities for immunotherapy.

**Key Words**

anterior chamber, aqueous humor, natural killer, ocular melanoma

**References**