Successful Immunotherapy of an Intraocular Tumor in Mice

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ABSTRACT

Imune privilege in the eye is considered essential in the protection against local sight-threatening inflammatory responses. However, the deviant immune responses in the eye may also provide an ideal opportunity to uncontrolled growth of viruses or tumors by inhibiting intraocular immunological attack. To establish to what extent immune privilege interferes with T cell-mediated antitumor immunotherapy, we established a new ocular tumor model in the mouse and tested whether well-defined tumor-specific CTLs can eradicate an immunogenic intraocularly growing tumor. Tumor cells, transformed by human adenovirus type 5 early region 1 (Ad5E1), injected s.c. in a dose of 10^7 cells, did not induce s.c. tumor growth in C57BL/6 mice. However, an injection of 0.3 × 10^6 of these cells into the anterior chamber of the eye led to intraocular tumor growth in 95% of mice (n = 20). Tumor growth in the eye did not induce systemic tumor-specific tolerance, because 70% of the mice were able to eradicate the tumor spontaneously after 5 weeks. Mice vaccinated s.c. with irradiated tumor cells were protected against intraocular tumor challenge, indicating that preactivated memory T cells are able to protect against intraocular tumor growth. Moreover, an i.v. injection of an Ad5E1-specific CTL clone was able to eradicate established intraocularly transformed tumors, whereas the anatomy of the eye remained intact. These results demonstrate that tumor-specific, CTL-mediated immunity can be used successfully for the prevention and eradication of tumors growing in the immune-privileged anterior chamber of the eye, without detectable destruction of the eye.

INTRODUCTION

The presence of immunomodulators in the aqueous humor and the capacity of the eye to sustain the growth of foreign tissues indicate that the eye is an immune-privileged site (1–3). Nevertheless, several findings suggest that the immune system still plays an important role in the control of intraocular malignancies. For example, spontaneous regression of chorioidal melanomas and retinoblastomas, although rare, has been documented (4–6), and the expression of tumor-specific antigens on uveal melanomas is well established (7–9). These findings stimulated our interest in developing immunotherapeutic modalities for eradicating primary intraocular tumors and led us to investigate the potential problems that may be related to the local immune privilege.

In the past 20 years, various animal models of intraocular tumors have been used to study whether the immune system can be manipulated to prevent or eradicate ocular tumors. Tumor eradication by adoptive transfer of tumor-infiltrating lymphocytes could be induced in FVB/N mice (10). In this ocular tumor model, the mice needed to be sublethally irradiated to acquire extensively growing ocular tumors. In other studies, using the B16F10 ocular melanoma model (11) and the P91 mastocytoma model (12), only eradication of metastases and not the primary ocular tumor could be accomplished by adoptive T-cell transfer. However, although these studies applied adoptive transfer, they did not test whether adoptive transfer of T cells could lead to complete tumor eradication without damaging the surrounding ocular tissues in immunocompetent mice.

In the present study, we developed a syngeneic murine intraocular tumor model using Ad5E1-transformed tumor cells, which express well-defined CTL epitopes derived from the E1A and E1B oncoproteins (13, 14). Because these epitopes are recognized by available tumor-specific cytotoxic T cells, we determined whether therapeutic intervention in intraocular tumor-bearing mice would lead to tumor eradication without affecting the normal ocular tissue.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice (H-2^b, between 3 and 6 months of age) were obtained from Iffa Credo (Brussels, Belgium).

Tumor Cells. Ad5E1-transformed (C57BL/6 origin) and Ad5E1 + ras-transformed mouse embryo cells were generated and maintained as described previously (13, 14). Monocellular suspensions of Ad5E1-induced tumor cells were washed and resuspended in PBS for s.c. and intracameral injections.

Intracameral Inoculations. A modified quantitative technique for deposition of a definite number of tumor cells into the anterior chamber of the mouse eye was used (15). Mice were deeply anesthetized with a mixture (ratio, 1:1) of xylazine (Rompun 2%; Bayer, Leverkusen, Germany) and ketamine hydrochloride (Aescoket; Aesculaap bv, Boxtel, the Netherlands) given i.p. The eye was viewed under the low power (8 ×) of a dissecting microscope, and a sterile 30-gauge needle was used to puncture the cornea at the corneoscleral junction, parallel and anterior to the iris. A glass micropipette (~80 μm in diameter) was fitted into a sterile infant feeding tube, which was mounted onto a sterile 0.1-m Hamilton syringe (Hamilton Co., Inc., Whittier, CA). The pipette, loaded with Ad5E1 cell suspension (0.3 × 10^6 cells/4 μl), was introduced through the puncture site of the cornea, and 4 μl of the Ad5E1 cell suspension was delivered into the anterior chamber. The eyes were examined three times per week with a dissecting microscope to observe and document tumor growth.

s.c. Inoculations. Ad5E1-transformed tumor cells or Ad5E1 + ras-transformed cells (10^3) were suspended in 250 μl of PBS and inoculated s.c. in the right flank.

Cell-mediated Lymphocyte Cytotoxicity. Cell-mediated cytotoxicity was measured by using a Eu³⁺ release assay as described previously (16). The mean percentage of specific lysis in triplicate wells was calculated as follows: % specific lysis = [(cpm experimental release – cpm spontaneous release)/ (cpm maximum release – cpm spontaneous release)] × 100.

Peptide Immunization and Challenge with Ad5E1 + ras-transformed Tumor Cells. Peptide immunizations were performed as described previously (17, 18). Peptides dissolved in 100 μl of PBS were mixed extensively with 100 μl of incomplete Freund’s adjuvant and 0.5% BSA. The 200-μl mixture was injected s.c. in C57BL/6 mice. Two weeks later, mice received a s.c. challenge with 10^6 Ad5E1 + ras-transformed tumor cells (clone SR2,3B) suspended in 250 μl of PBS.
In Vivo Administration of C57BL/6 Ad5E1-specific CTL Clone 0.1C2.

In vivo administration of tumor-specific CTL clones was performed as described previously (13, 14). In short, C57BL/6 mice with growing Ad5E1-induced tumors in the anterior chamber were assigned randomly to one of the following three treatments: (a) i.v. injection of Ad5E1-specific CTL clone 0.1C2 (1.5 × 10^7) in combination with a s.c. injection of 10^5 Cetus units of recombinant interleukin 2; (b) i.v. injection of HPV-16-specific CTL clone 9.5 (control), in combination with a s.c. injection of 10^5 Cetus units of recombinant interleukin 2; and (c) no treatment.

RESULTS

To establish a system in which we could address the question of whether T cell-mediated antitumor responses can prevent or eradicate ocular tumors, we injected C57BL/6 mice s.c. and intracamerally with 10^7, respectively, 0.3 × 10^6 Ad5E1-transformed tumor cells. These tumor cells express at least two H-2b epitopes recognized by Ad5E1-specific CTL. We obtained a successful inoculation of the syngeneic Ad5E1-induced tumor cells in the anterior chamber of immunocompetent C57BL/6 mice (95%; n = 20), whereas no tumor growth was observed when 30 times more tumor cells were injected s.c. Intraocular tumor masses in the eye grew slowly but consistently (Fig. 1) and occupied the anterior chamber almost completely by day 35 (Fig. 2). Sometimes tumors regressed spontaneously after day 27. These results show that Ad5E1-transformed tumor cells can form tumors in the anterior chamber of the eye, despite the fact that they express potent CTL epitopes. This emphasizes the unique immunological-privilege of the eye.

Does Placement of Ad5E1-transformed Tumor Cells in the Anterior Chamber Induce Systemic Tolerance? Because intraocular tumor growth has been reported to induce specific peripheral T cell tolerance (1, 19), we studied whether mice with a growing intraocular Ad5E1-transformed tumor were able to reject a s.c. challenge with Ad5E1 + ras-transformed tumor cells. Some Ad5E1 + ras tumors form small but detectable tumors in naive mice that regress after 2–3 weeks (20). The rejection of the s.c. challenge has been shown to be crucially dependent on CD8⁺ T cells directed against an E1B-encoded peptide. However, s.c. injection with this E1B peptide in C57BL/6 mice leads to Ad5E1B-specific CTL tolerance, resulting in the inability of vaccinated animals to control the outgrowth of E1B-expressing tumors (21). Thus, outgrowth of Ad5E1 + ras tumors can be used as a readout for systemic epitope-specific CTL tolerance induction, as well as for the induction of protective immune responses, because mice vaccinated with irradiated tumor cells resist tumor challenge. To study whether a growing Ad5E1-induced tumor in the anterior chamber is ignored by tumor-specific CTL, induces peripheral CTL tolerance, or induces systemic protective immunity, mice (n = 8) with an intraocular tumor that occupied ≥50% of the anterior chamber were challenged s.c. with Ad5E1 + ras-transformed tumor cells. These mice, in contrast to animals (n = 8) vaccinated s.c. with irradiated Ad5E1-induced tumor cells, developed a small s.c. tumor that disappeared after 10 days. Mice receiving the tolerizing E1B peptide were not able to control tumor growth (Fig. 3), confirming the tolerizing potential of this CTL epitope (21). These results indicate that the ocular tumor did not induce systemic tolerance or immunity.

Effect of s.c. Immunization on Primary Intraocular Tumor Growth. Because the Ad5E1-intraocular tumor did not induce systemic tumor-specific T-cell tolerance, we addressed the question of whether memory T cells, induced by vaccination with irradiated tumor

![Fig. 1. Growth pattern of Ad5E1-induced tumor after injection of 0.3 × 10^6 tumor cells intracamerally in C57BL/6 mice (n = 20).](cancerres.aacrjournals.org)
cells, could prevent a challenge of tumor cells given intracamerally. On day 0, mice \((n = 10)\) were vaccinated with irradiated Ad5E1-induced tumor cells s.c. in the right flank. After 2 weeks, the mice were inoculated with Ad5E1-induced tumor cells \(\left(0.3 \times 10^6\right)\) in the anterior chamber. Vaccinated mice did not show any intraocular tumor growth, whereas 9 of 10 mice in the control group showed tumor growth in the anterior chamber (data not shown). These observations indicate that vaccination with irradiated tumor cells can lead to protective immunity against intraocular tumor growth.

**Adoptive Transfer of Ad5E1-specific CTL Clone 0.1C2.** The results described above indicate that preactivated CTLs can control the outgrowth of intraocular tumors. To study whether a tumor-specific CTL clone is able to eradicate established tumors, we treated intraocular tumor-bearing animals by adoptive transfer with E1B-specific CTL clones. Mice with a tumor mass filling 50–75% of the anterior chamber received an i.v. injection of \(1.5 \times 10^7\) E1B-specific CTL clone 0.1C2 (\(\bullet\)). A control group \((n = 5)\) received an HPV-16 specific CTL, clone 9.5 (\(\circ\)); one group did not receive any treatment (\(\triangle\)). Tumors in mice receiving the E1B-specific CTL had disappeared within 3 days of adoptive transfer.

Fig. 3. Mice were vaccinated s.c. with E1B peptide in incomplete Freund’s adjuvant (\(\square\)), immunized s.c. with irradiated Ad5E1-induced tumor cells in PBS (\(\triangle\)), or inoculated with Ad5E1-induced tumor cells in the anterior chamber (\(\Delta\)). Two weeks later when the intraocular tumors filled >50% of the anterior chamber, all groups of mice including a naive group of mice (\(\bigcirc\)) were challenged s.c. with live Ad5E1 + ras-transformed cells in the right flank. Mean tumor volumes \(\pm\) SE \((n = 7)\) are shown in cubic millimeters. No enhanced outgrowth of Ad5E1 + ras-transformed tumor in eye tumor-bearing animals (\(\triangle\)) was detected.

Fig. 4. Intraocular Ad5E1-induced tumors were successfully eradicated by adoptively transferred Ad5E1-specific CTL. Mice \((n = 5)\) with intraocular Ad5E1-tumors that filled 50–70% of the anterior chamber were treated by i.v. injection of \(1.5 \times 10^7\) Ad5E1-specific CTL clone 0.1C2 (\(\bullet\)). A control group \((n = 5)\) received an HPV-16 specific CTL clone 9.5 (\(\triangle\)); one group did not receive any treatment (\(\triangle\)). Tumors in mice receiving the E1B-specific CTL had disappeared within 3 days of adoptive transfer.

Fig. 5. A–C, eye with a progressively growing tumor (day 26). No signs of infiltration by lymphocytes are present. D–F, tumor that already is undergoing spontaneous regression (day 35). Small remnants of tumor can be observed on the iris. G–I, anterior segment of a murine eye, in which a large tumor was eradicated by adoptive transfer of CTLs 7 days earlier. No visible signs of damage remain. All sections are H&E stained; original magnifications: A, D, and G, \(\times 6\); B, E, and H, \(\times 100\); C, F, and I, \(\times 200\).

Fig. 6. A–C, eye with a progressing tumor (day 26). No signs of infiltration by lymphocytes are present. D–F, tumor that already is undergoing spontaneous regression (day 35). Small remnants of tumor can be observed on the iris. G–I, anterior segment of a murine eye, in which a large tumor was eradicated by adoptive transfer of CTLs 7 days earlier. No visible signs of damage remain. All sections are H&E stained; original magnifications: A, D, and G, \(\times 6\); B, E, and H, \(\times 100\); C, F, and I, \(\times 200\).

Fig. 7. A–C, eye with a progressing tumor (day 26). No signs of infiltration by lymphocytes are present. D–F, tumor that already is undergoing spontaneous regression (day 35). Small remnants of tumor can be observed on the iris. G–I, anterior segment of a murine eye, in which a large tumor was eradicated by adoptive transfer of CTLs 7 days earlier. No visible signs of damage remain. All sections are H&E stained; original magnifications: A, D, and G, \(\times 6\); B, E, and H, \(\times 100\); C, F, and I, \(\times 200\).
were observed. After injection of CTLs, areas of inflammation were also absent (Fig. 5).

**CTL Reactivity Directed Against Ad5E1-induced Tumor Cells Growing in the Anterior Chamber.** Because the anterior chamber is an immune-privileged site, it may sustain growth of a tumor, which would be rejected in another location. Surprisingly, after we followed tumor growth for longer periods, the tumor in the anterior chamber was ultimately rejected. To study whether this was associated with the induction of Ad5E1-reactive CTLs, we determined Ad5E1-specific CTL reactivity. Twelve days after inoculation of Ad5E1-induced cells into the anterior chamber, mice had a tumor that filled ~50–75% of the anterior chamber. Spleens were collected, and bulk CTL cultures were tested for their reactivity against the E1B epitope. Although tumor-specific CTLs were present in mice immunized with Ad5E1-induced tumor cells, no specific CTL reactivity could be measured at this time in the cultures derived from intraocular tumor-bearing animals. These results confirm the observation that the intraocular tumor is initially ignored by the immune system (Fig. 6A). However, in mice that rejected their intraocular tumor at approximately week 6, E1B-specific CTLs could readily be detected. These results show that, despite immune privilege, the immune system is able to respond spontaneously against intraocularly growing tumors (Fig. 6B).

**DISCUSSION**

Ad5E1-transformed tumor cells inoculated into the anterior chamber of C57BL/6 mice grew to significant masses, whereas even 30 times more of these cells injected s.c. were rapidly rejected. This observation indicates that an immunocompetent C57BL/6 mouse is able to recognize and induce an immune response against Ad5E1-tumor cells, but that the local immune privilege of the eye inhibits or postpones the development of an appropriate intraocular antitumor response. Our results show that tumor-specific CTLs induced by vaccination or adoptively transferred into immunocompetent C57BL/6 mice are able to prevent and eradicate intraocular tumors without damaging the normal ocular host tissue. This important finding suggests that T-cell immunotherapy might be of significance as an immunotherapeutic option against intraocular tumors. To the best of our knowledge only one earlier successful attempt of adoptive transfer experiments performed in an intraocular tumor model has been reported. FVB/N mice bearing intraocular SV40-induced tumors were successfully treated by adoptive transfer of tumor-infiltrating lymphocytes. However these mice had to be sublethally irradiated before adoptive transfer, thereby affecting possible immunoregulatory circuits operative in the host (10).

The anterior chamber is a known immune-privileged site with the capacity to interfere with antitumor responses (22, 23). Placement of tumor cells in the anterior chamber might therefore even lead to the induction of T-cell tolerance (22). To study the interference of the intraocular environment with the development of antitumor responses in vivo, we tested whether Ad5E1-tumor cells injected into the anterior chamber induced T-cell tolerance. We obtained no evidence of the development of tumor-specific tolerance. Instead, our results indicate that intraocular tumors were initially ignored by the immune system but eventually induced systemic T cell-mediated immunity. The reason why these tumors, despite progressive growth, were ultimately able to induce E1B-specific CTL responses is as yet unclear but might be explained by the immunogenic characteristics of the antigens expressed on the tumor cells or the necessity of reaching a critical antigen density in the anterior chamber (24).

Remarkably, as for the adoptive transfer experiments, no discernible scarring was observed after spontaneous eradication of the intraocular tumor. The results of these experiments showed that growing intraocular tumors can induce systemic T cell-mediated immunity, although with a delayed appearance.

In another syngeneic intraocular murine tumor model, Niederkorn and Meunier (25) used a P91 mastocytoma (DBA/2 origin) tumor cell line. This cell line expressed strong tumor-specific antigens. Similarly to our Ad5E1-induced tumor cell line, P91 tumors grew transiently in the anterior chamber and were then spontaneously rejected (12). However, rejection of the P91 intraocular tumor was accompanied by ischemic necrosis and complete atrophy of the affected eye. This rejection was considered to be consistent with a delayed-type hypersensitivity-mediated process (26). The Ad5E1-induced tumor in our model is also rejected, but the eye seems to be completely unaffected. This suggests that tumor-specific CTLs, not delayed-type hypersensitivity, are responsible for the tumor eradication in our model, as suggested by our results with passive transfer of cloned CTLs. This mode of tumor rejection is very similar to that described in a UV5C25 ocular tumor model (27). In a histopathological analysis, Niederkorn (26) described this way of spontaneous tumor rejection as piecemeal necrosis and strongly suggested that this type of rejection was mediated by direct cytolyis by tumor-specific cytotoxic T cells. It confirms the idea that the immunogenic strength of antigens expressed by inoculated tumor cells may influence the outcome of immunological privilege of the anterior chamber (28). We hypothesize that the E1B oncoprotein, which is expressed by Ad5E1-induced tumor cells, is a potent antigenic stimulus that finally breaks the putative state of immune deviation (29) and induces a strong tumor-specific CTL reaction. This is also the case with strongly immunogenic (such as MHC-incompatible) tumors, like P815 tumors in the eye of C57BL/6 mice. The local inhibiting circuits are overcome, and conversion of precursor CTLs into CTLs takes place, leading to tumor rejection (30).

Complete protection against intraocularly growing Ad5E1-in-
duced tumors could be achieved by s.c. vaccination with tumor cells. Such induced T cells were able to prevent intraocular tumor growth. This result contributes to the reflection that manipulation of the immune system can lead to tumor eradication and even to protection against intraocular tumor growth, despite local immune privilege.

Although the AdSe1-induced tumor is based on a murine cell line, its unique pattern of rejection bears an interesting similarity to the behavior of uveal melanoma and retinoblastoma, which are known to its unique pattern of rejection bears an interesting similarity to the behavior of uveal melanoma and retinoblastoma, which are known to

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