IMMUNOREGULATION OF INTRAOCULAR TUMOURS

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SUMMARY

Intraocular tumours reside within an organ that provides sanctuary from many immunological defence mechanisms. Antigens displayed on many intraocular tumours can elicit an aberrant systemic immune response in which systemic antigen-specific delayed-type hypersensitivity (DTH) is actively downregulated, thus denying the host one potential effector mechanism for controlling its intraocular tumour. Constituents within the aqueous humour inhibit the expression of DTH and natural killer cell effector mechanisms within the eye and thus protect intraocular tumours from immune-mediated rejection. Some experimental intraocular tumours in mice express potent tumour-specific antigens that stimulate the expansion of tumour-specific robust cytotoxic T lymphocyte (CTL) populations which enter the eye and mediate tumour rejection by piecemeal tumour necrosis. However, the presence of tumour-infiltrating lymphocytes (TIL) within an intraocular tumour does not inevitably lead to tumour resolution. In some cases CTL precursors infiltrate the intraocular tumour but fail to differentiate into mature cytolytic effector cells. Although uveal melanomas express melanoma-specific and melanoma-associated antigens that are capable of eliciting both humoral and cellular immunity, formidable barriers prevent the expression of tumour immunity. These barriers include: (a) anterior chamber-associated immune deviation; (b) *in situ* suppression of DTH effector cells; (c) suppression of natural killer cell activity *in oculi*; and (d) inactivation of the complement cascade by regulatory proteins expressed on uveal melanoma cells.

The unique immunological characteristics of the anterior chamber of the eye have been recognised for over a century.[1,2] Histoincompatible tissue grafts survive for extended periods within the anterior chamber of the eye, yet are promptly rejected when transplanted to extraocular sites. This curious exemption from the laws of transplantation immunology led to the concept of immunological privilege. The capacity of the anterior chamber to sustain the survival and growth of histoincompatible tissues did not escape the attention of tumour biologists, who used the anterior chamber of the rabbit eye to propagate human tumour grafts and assess their metastatic potential.[3] In the past 20 years there has been enormous progress in the characterisation and analysis of the immunological privilege of the anterior chamber at the cellular and molecular levels. Although it was previously believed that immunological privilege was restricted to the anterior chamber, recent evidence clearly indicates that the posterior compartments of the eye also possess immunological properties similar to the anterior chamber.[4,5] Thus, lessons learned regarding the immune regulation of tumours residing in the anterior segment of the eye may also apply to tumours of the uveal tract.

In an effort to understand whether the immune system perceives and influences the behaviour of intraocular tumours, investigators have followed two fundamental approaches. The first and more challenging approach has been the evaluation of the systemic immune responses to melanoma-associated antigens in uveal melanoma patients. The chief obstacle to this approach is the limited availability of appropriate assays and target cells for assessing cell-mediated immune responses to autologous melanoma-specific antigens and the inability to evaluate patients prospectively during the various stages of tumourigenesis. Moreover, by their very nature, clinical studies are handicapped by the inability to incorporate the various control groups needed for assessing specific parameters in a prospective setting.

A second approach for exploring the role of the immune system in intraocular tumours has been the utilisation of experimental animal models, in particular the orthotopic transplantation of syngeneic murine tumour cells and xenogeneic human uveal melanomas into the eyes of inbred mice and

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immunocompromised mice. The use of animal models has allowed for prospective experimental designs and strict control of crucial variables. However, since the murine tumours do not arise in the uveal tract, their relevance to the human counterpart is limited. Nonetheless, fundamental immunological principles can be derived from the prudent use of rodent intraocular tumour models. In this overview, I will attempt to integrate results from human and experimental animal studies to establish fundamental principles regarding the immunoregulation of intraocular tumours.

SYSTEMIC IMMUNE RESPONSES TO INTRAOCULAR TUMOURS

Uveal melanomas are frequently perceived by the systemic immune apparatus, as demonstrated by the appearance of serum antibodies directed against melanoma-associated antigens in over 70% of the uveal melanoma patients.[6] In spite of this high incidence of humoral antibody, no studies to date have found either a positive or negative correlation between the presence of antibody and host survival. A recent report by Goslings and co-workers[7] may help to explain the absence of a beneficial effect of antibody directed against melanoma-associated antigens in uveal melanoma patients. Immunohistochemical examination of tissue sections of human uveal melanomas revealed the presence of complement decay accelerating factor on 9 of the 10 uveal melanomas examined. Moreover, the complement regulatory protein, CD59, which inhibits complement membrane complex formation, was expressed on all 10 specimens. Based on these findings, one would predict that uveal melanomas would be highly resistant to antibody-mediated lysis and that, as a result, antibody would play a minor role in controlling uveal melanomas.

Uveal melanomas can also sensitise the cellular immune response. Char *et al.*[8] demonstrated positive cutaneous hypersensitivity responses to melanoma-associated antigens in uveal melanoma patients. Others have found *in vitro* evidence of cellular immunity in the form of lymphoproliferative responses to melanoma antigens, indicating that the uveal melanoma patient's cellular immune apparatus was sensitised.[6] Kan-Mitchell and co-workers[9,10] have reported the development of major histocompatibility complex (MHC)-restricted and MHC-unrestricted cytotoxic T lymphocyte (CTL) activity in the peripheral blood of uveal melanoma patients. However, none of these studies was able to provide an unequivocal correlation between the host's tumour-specific immune responses and prognosis.

Studies using transplantable murine tumours have revealed the presence of an unusual immunological response elicited by antigens introduced into the anterior segment of the eye.[11,12] Allogeneic tumours that were promptly rejected following subcutaneous transplantation grew progressively in the eye.[11,12] Moreover, analysis of the systemic immune response to intraocular tumour allografts revealed the presence of normal antibody and CTL responses but a profound and persistent suppression of delayed type hypersensitivity (DTH).[13] This aberrant immune response to intraocular antigens is termed anterior chamber-associated immune deviation (ACAID)[14] and has been demonstrated with a wide range of antigens including melanoma-associated antigens expressed on syngeneic murine melanomas.[15] In each of the intraocular murine tumour models studied to date, the development of ACAID is intimately correlated with progressive tumour growth. However, not all tumours induce ACAID. Tumours expressing potent tumour-specific antigens fail to induce ACAID and instead elicit robust DTH and CTL responses following intracameral transplantation.[16–20] The acquisition of systemic cellular immunity directed against the tumour-specific antigens coincides with intraocular tumour rejection and suggests that tumour destruction occurs via cell-mediated immune mechanisms.

ROLE OF INTRAOCULAR TUMOUR INFILTRATING LYMPHOCYTES

Tumour infiltrating lymphocytes (TIL) have been detected in 5–12% of the uveal melanomas examined.[21] Phenotypic analysis of TIL from uveal melanoma patients has produced variable results. In one study TIL were predominantly $CD8^+$ T cells,[22] in another study $CD4^+$ and $CD8^+$ cells were present in equal amounts,[23] and in yet another report $CD16^+$ natural killer (NK) cells were the dominant lymphocyte population, accounting for 41% of the TIL.[24] The prognostic significance of TIL in uveal melanomas is also unclear. Some investigators have reported that the presence of TIL was favourable for survival,[25,26] while others have found the opposite.[27] Likewise, analysis of the T cell receptor (TCR) utilisation of uveal melanoma TIL has been equivocal. Nitta *et al.*[28] detected oligoclonality of TCR expression in uveal melanoma TIL which suggested the presence of an antigen-specific T-cell-mediated immune response. By contrast, Durie *et al.*[29] failed to find evidence of restricted TCR utilisation in the TIL isolated from six uveal melanomas. Thus, it appears that in some circumstances antigen-specific T cells can enter the eye and presumably act to control uveal melanoma progression while in other cases TIL are incapable of controlling the intraocular neoplasm.

Results from animal models have shed some light on the role of TIL in intraocular tumours. Using a highly immunogenic ultraviolet (UV)-light-induced

fibrosarcoma model, Knisely and co-workers[16] demonstrated that syngeneic intraocular UV-induced tumours underwent rejection following lymphocytic infiltration. Tumour resolution culminated in the complete disappearance of the intraocular tumour without evidence of damage to juxtaposed normal ocular tissues. Tumour resolution was characterised by piecemeal necrosis with no evidence of ischaemia or vascular injury. Functional analysis of the tumour-rejectors indicated that the hosts developed strong DTH and CTL reactivities to the tumour antigens. Fluorescence activated cell sorter (FACS) analysis of the TIL revealed that the TIL were composed of $CD8^+$, $CD4^+$ and $B220^+$ lymphocytes.[16] *In vitro* analyses indicated that the TIL produced direct tumour cytolytic activity. Proof that the TIL were the mediators of intraocular tumour rejection was demonstrated in experiments in which the TIL were adoptively transferred into the eyes of immuno-incompetent recipients. In these hosts, intraocular tumour rejection demonstrated the same clinical and histopathological features as tumour rejection in the original immunocompetent hosts.[17] Thus, a rough equivalent of Koch's postulates was satisfied: (a) TIL were present in all cases of intraocular tumour resolution; (b) TIL demonstrated direct, tumour-specific cytolytic activity *in vitro*; and (c) adoptive transfer of TIL to immunoincompetent hosts produced tumour rejection that bore the same clinical and histopathological characteristics that were found in the original immunocompetent counterparts. The histopathological nature of the intraocular tumour resolution was consistent with a CTL-mediated mechanism. Even though the hosts developed vibrant DTH responses as measured in conventional footpad swelling assays, there was no evidence of DTH lesions in the affected eyes before, during or after intraocular tumour rejection. There was a conspicuous absence of vascular endothelial injury, ischaemia, haemostasis, fibrin deposition or innocent bystander cell damage within the eyes of immuno-competent hosts.

The absence of compelling evidence for the expression of DTH lesions in the resolving tumours suggested that effector cells that mediate DTH were either absent or functionally silenced *in oculi*. FACS analysis of the TIL indicated that 9% of the TIL were comprised of $CD4^+$ T cells, a phenotype normally associated with DTH. Previous studies have demonstrated that as few as 10 T cells can mediate a detectable DTH response in mice.[18] Therefore, we suspected that there were more than adequate numbers of DTH effector cells within the TIL population to mediate DTH. This was tested by a local adoptive transfer of DTH assay in which the TIL were removed, mixed with X-irradiated tissue-cultured tumour cells, and injected into the pinnae of the ears of normal mice that had never been previously exposed to the UV tumour. The results clearly demonstrated that the TIL produced significant DTH reactivity in the ears of normal mice. Moreover, histopathological examination of the ears revealed the presence of lesions characteristic of DTH lesions in the footpads of extraocularly immunised hosts.[19] Thus, TIL contain a subpopulation of lymphocytes capable of producing DTH lesions outside the eye but which are functionally silenced *in oculi*.

In similar studies Ma and co-workers[20] used a murine melanoma cell line derived from uveal tumours arising in transgenic FVB/n mice carrying the SV40 oncogene. The uveal tumours underwent immune-mediated rejection following transplantation to the anterior segments of non-transgenic FVB/n hosts. As in the UV tumour model, the transgenic uveal melanomas became infiltrated with TIL prior to undergoing piecemeal necrosis. Rejection culminated in complete elimination of the intraocular tumour without evidence of injury to normal innocent bystander ocular tissues. Even though the hosts developed strong systemic DTH and CTL reactivity to the SV40 large T antigen expressed on the tumours, there was no evidence of DTH lesions before or after intraocular tumour rejection. TIL consisted of 71–73% Thy 1.2^+ lymphocytes on days 7 through 21. However, there was a conspicuous shift in the dominant T cell population that coincided with tumour rejection. During the early stage of tumour growth (day 7), $CD4^+$ cells were the dominant T cell population and comprised 39% of the T cells, while $CD8^+$ cells represented 23% of the TIL population. However, by day 21 these two populations were reversed such that only 19% of the T cells were $CD4^+$ while 44% were $CD8^+$.[20] The predominance of $CD8^+$ cells in the TIL population coincided with the development of potent cytolytic activity. TIL isolated from eyes on day 21 demonstrated direct cytolytic activity against the transgenic uveal melanoma cells but no activity against cutaneous B16 murine melanoma target cells.

The presence of TIL in intraocular neoplasms does not guarantee a favourable outcome. Ksander and co-workers[30] utilised the murine P815 mastocytoma cell line which arose in the DBA/2 mouse and MHC class I antigens and multiple minor histocompatibility (H) antigens of the DBA/2 mouse strain. Intraocularly transplanted P815 mastocytoma cells grow progressively in the eyes of BALB/c mice, which share the same MHC genotype as the DBA/2 mouse strain but differ at multiple minor *H* gene loci. Examination of the TIL in progressively growing P815 mastocytomas in allogeneic BALB/c mice revealed the presence of CTL precursors which failed to differentiate into functional cytotoxic

T cells.[30] By contrast, intraocularly transplanted P815 cells are promptly rejected in allogeneic C57BL/6 mice, which recognise and respond to both MHC and multiple minor DBA/2 alloantigens. In these hosts, the P815 tumours become infiltrated with lymphocytes that undergo complete differentiation into functional cytolytic cells.[31] Rejection of intraocular P815 tumour occurs in a manner similar to that for the murine regressor tumours mentioned above. The C57BL/6 host perceives a larger array of antigens (i.e. MHC plus minor antigens) on the P815 cells than the BALB/c host (i.e. minor H antigens only). Thus, the nature of the antigens expressed on the intraocular tumour greatly influences whether the TIL will differentiate into functional cytolytic cells and whether the tumour will grow progressively or undergo immune-mediated rejection.

Studies on TIL detected in biopsies of human uveal melanomas have produced variable results. Whelchel and co-workers[27] reported that the presence of TIL was associated with an unfavourable prognosis while others have found the opposite association.[25,26] The variable results in the prognosis of patients demonstrating TIL in their uveal melanomas may be analogous to the experimental mouse studies described above and attributable to differences in the nature of the antigens expressed by the uveal melanomas.

Intraocular Regulation of Natural Killer Cell Activity

Natural killer (NK) cells are a subset of lymphocytes found in blood and lymphoid tissues and are believed to play an important role in the immune surveillance of virus infections and neoplasms.

Recently, we have discovered a small peptide present in the aqueous humour (AH) that produces an immediate and profound inhibition of NK-mediated lysis of tumour cells *in vitro*.[32] The natural killer inhibitory factor (NKIF) does not appear to act by altering the expression of activation molecules (e.g. NK 1.1 and 2B4) or inhibitory molecules (e.g. 5E6) on the NK cell membrane. Moreover, NKIF does not inhibit the binding of NK cells to tumour target cells. However, the inhibitory molecule prevents tyrosine phosphorylation of NK cells and the exocytosis of cytolytic granules (Apte and Niederkorn, submitted for publication). Although transforming growth factor-beta (TGF-β) is present in the aqueous humour[33,34] in concentrations (i.e. 1–5 ng/ml) that are known to suppress NK-mediated cytolysis *in vitro*,[35] the immediate inhibitory effect of aqueous humour is not due to TGF-β since antibody against TGF-β does not neutralise NKIF activity and because the inhibitory effects of aqueous humour are immediate, while TGF-β-mediated inhibition requires 20 hours of *in vitro* incubation for it to be detected.[35]

The presence of NKIF and TGF-β in the aqueous humour has important implications for the fate of intraocular tumours, especially those of the anterior segment. Uveal melanomas are susceptible to NK-cell-mediated lysis *in vitro*.[36] Moreover, TGF-β is capable of downregulating the expression of MHC class I antigens on human uveal melanoma cells and thereby increasing their susceptibility to NK-cell-mediated lysis.[36] However, since TGF-β and NKIF are both present in the aqueous humour and TGF-β is also produced by cells in the posterior segment,[37,38] the possibility exists that intraocular tumours reside in a milieu that protects tumours that might otherwise be highly susceptible to elimination by NK cells. It is likely that intraocular tumours encounter NK cells, since immunohistochemical studies on human uveal melanomas have demonstrated the presence of NK cells among the TIL populations.[23,24] Moreover, one study found CD16+ lymphocytes (i.e. NK cells) to be the predominant lymphocyte, comprising 41% of the TIL in a human uveal melanoma.[24]

We recently tested the hypothesis that aqueous-humour-mediated inhibition of NK cell activity promotes the progressive growth of intraocular tumours (manuscript in preparation). Two models were used. The first model utilised a pair of well-characterised murine lymphomas: RMA and RMA-S. The RMA lymphoma expresses normal levels of murine MHC class I antigens and is resistant to NK-cell-mediated lysis, while the RMA-S mutant fails to assemble and express MHC class I antigens and, as a result, is highly susceptible to NK-cell-mediated cytolysis. NK-sensitive RMA-S cells (10^5 cells/mouse) were promptly rejected following subcutaneous transplantation into severe combined immune deficient (SCID) mice which lack both T and B cells but express normal NK activity. Rejection was mediated by NK cells because *in vivo* elimination of NK cells by intraperitoneal injection of anti-asialo GM1 antibody prevented tumour rejection and resulted in the progressive growth of the subcutaneous tumours. By contrast, RMA-S tumour cells transplanted into the eyes of SCID mice grew progressively, even at doses 20-fold lower than those which were rejected following subcutaneous transplantation. As expected, NK-resistant RMA tumours grew progressively following either intracameral or subcutaneous transplantation, even when the tumour inoculum was reduced 10-fold. Similar results were found in experiments using NK-sensitive and NK-resistant human uveal melanoma cells. That is, NK-sensitive OCM-3 human uveal melanoma cells were rejected following subcutaneous transplantation, yet grew progressively when the same number or even 10-fold fewer cells were transplanted intracamerally. Thus, tumours which can be rejected by NK cells at extraocular sites enjoy immunological sanctuary

within the eye and escape NK-cell-mediated elimination. Whether this scenario occurs in human uveal melanoma patients is unknown.

Barriers to the Immune Surveillance of Intraocular Tumours

On the surface one might predict that the immune privilege of the eye is an absolute and insurmountable barrier that shields intraocular tumours from the immune surveillance apparatus. However, under certain conditions the immune system is capable of perceiving and eliminating intraocular tumours. Intraocular tumours that express highly immunogenic tumour-specific antigens can abort the immune privilege of the eye and provoke a robust immune response that culminates in the complete eradication of the intraocular neoplasm.

Uveal melanomas display melanoma-specific and melanoma-associated antigens which can elicit both humoral and cellular immune responses. Uveal melanomas can elicit antibody responses which can be detected in as many as 78% of the uveal melanoma patients tested.[6] Likewise, MHC class I-restricted CTL have been isolated from uveal melanoma patients and are able to lyse autologous uveal melanoma cells *in vitro*, yet are unable to control the primary intraocular tumour.[9,10] Since the immune system can respond to uveal melanomas, why is the incidence of spontaneous resolution so low?

There are numerous barriers that thwart immunological responses to uveal melanomas. The induction of ACAID would certainly favour the survival and progressive growth of intraocular tumours. Tumours that fail to induce ACAID invariably undergo immune-mediated rejection.[12,15] Likewise, abrogation of ACAID by splenectomy results in spontaneous resolution of intraocular tumour allografts.[39]

As mentioned earlier, the intraocular milieu prevents the expression of DTH and NK effector mechanisms. Although $CD8^+$ T cells can mediate the rejection of some highly immunogenic tumours,[16,17,20,31] they fail to fully differentiate into cytolytic effector cells in weakly immunogenic intraocular tumours.[30]

Uveal melanomas also contribute to their evasion of immunological rejection through their expression of the complement regulatory proteins which render them resistant to lysis by complement fixing antibodies.[7] Thus, the immunological privilege of the eye and the expression of immunoregulatory molecules on the uveal melanoma surface conspire to reduce the likelihood of immunological rejection of intraocular melanomas. Overcoming these barriers is a challenging prerequisite for the development of rational therapeutic strategies for managing intraocular neoplasms.

Key words: Uveal melanoma, Immune regulation, Intraocular tumour, Cytotoxic T lymphocytes, Natural killer cells.

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