Effect of Renal Cell Carcinomas on the Development of Type 1 T-Cell Responses

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ABSTRACT

Purpose: We reported that in renal cell carcinoma patients with active disease, T-cell reactions to the tumor-associated antigens MAGE-6 and EphA2 are highly skewed toward Th2-type cytokine responses [interleukin (IL) 5]. Herein, we determined whether tumor-derived products, including gangliosides isolated from renal cell carcinoma patients, participate in the down-regulation of type 1 T-cell responses.

Experimental Design: T cells from healthy volunteers or renal cell carcinoma patients were cultured in the presence and absence of supernatants derived from renal cell carcinoma explants or with gangliosides isolated from those tumor supernatants. T cells were stimulated or not with either autologous dendritic cells pulsed with superantigen (Staphylococcus enterotoxin B) or with phorbol 12-myristate 13-acetate and ionomycin and then were assessed for type 1 or type 2 responses (cytokine production and gene expression) and apoptosis.

Results: Tumor supernatants efficiently inhibited the Th1-type responses [interferon (IFN) γ] of T cells stimulated with either S. enterotoxin B or phorbol 12-myristate 13-acetate and ionomycin but had no inhibitory effect on activated T-cell production of type 2 cytokines (IL-4, IL-5, and IL-10). Likewise, IFN-γ mRNA and protein production were inhibited when T cells were cocultured with either renal cell carcinoma supernatant-derived gangliosides or a commercial source of purified GD1a. It was also determined that gangliosides impair type 1 responses by inducing apoptosis of activated T cells.

Conclusions: We propose that renal cell carcinoma-derived tumor products such as gangliosides can induce a type 2 bias in antitumor immunity by initiating apoptosis in the IFN-γ-producing type 1 effector cells. This represents a relevant mechanism by which renal cell carcinoma can inhibit protective antitumor immunity.

INTRODUCTION

A number of studies indicate that a type 1 response (Th1/Th2) plays a critical role in the rejection of tumors (1–4). Th1-type CD4+ T cells secrete interferon (IFN) γ and interleukin (IL) 2 that promote cellular immunity, in part by providing helper signals for the cytotoxic CD8+ T lymphocytes that also have the capacity to produce IFN-γ in response to antigen (1–4). Th2-type cells produce IL-4 and IL-5 and typically promote a humoral immune response, whereas Th1/Th-reg cells produce immunosuppressive cytokines (IL-10 and transforming growth factor β) that can dampen both Th1- and Th2-type immune responses (5–8).

Several studies have examined the issue of type 1 versus type 2 polarization in renal cell carcinoma patients. Although one report suggests that a type 1 response predominates in renal cell carcinoma, most studies indicate that the cytokine profile observed is most consistent with a type 2 bias in situ (9–14). These studies mainly examined the cytokine profile of the infiltrating mononuclear cells, and none of the studies determined the type 1 and type 2 bias of T cells responding to tumor-associated antigens expressed on renal cell carcinoma.

To better define the polarization status of CD4+ T cells and CD8+ T cells, we examined the immune response to the renal cell carcinoma-associated antigens MAGE-6 and EphA2 using an enzyme-linked immunospot assay and MHC-peptide tetramers (15–17). MAGE-6, an antigen broadly expressed on different tumor types, is detected in >30% of renal cell carcinomas. Likewise, the tyrosine kinase receptor EphA2, an antigen that is overexpressed by a wide array of human tumor types, is present in a high percentage of renal cell carcinomas (90%; ref. 18). As we reported previously, peripheral blood CD4+ T cells derived from HLA-DR4+ patients with active disease (stage III/IV) demonstrated an impaired Th1-type response. MAGE-6–specific IFN-γ-producing CD4+ T cells were rarely detectable in the periphery, whereas IL-5 responses against that same antigen were frequently and readily apparent (15, 16). The CD4+ T-cell response to EphA2 epitopes was likewise skewed toward Th2-type reactivity in patients with more advanced disease (17). Interestingly, in patients where the tumor had been removed and there was no evidence of remaining disease, the cytokine response was predominately type 1 (IFN-γ), suggesting that the presence of tumor was responsible for the induction of the cytokine response.
of a type 2 response (IL-5) in patients with active disease (15–17). Additional findings now suggest that the type 2 bias detected in renal cell carcinoma patients with active disease may be attributable to an increased sensitivity of the MAGE-6 and EphA2 CD4^+ T cells to apoptosis. This notion was supported by the use of HLA-DR4 tetramers associated with MAGE-6 or EphA2 peptides (in collaboration with William Kwok, Benaroya Research Institute, Seattle, WA). Detectable binding of T cells to HLA-DR4-tumor peptide tetramers was noted in the peripheral blood of renal cell carcinoma patients, although the frequency was less than that for CD4^+ T cells binding to a viral peptide (FluM1; ref. 18). However, double staining with MHC tetramers and Annexin V demonstrated that a high percentage of the CD4^+ T-cell population displayed early signs of apoptosis. This was not true when evaluating influenza-specific T cells, suggesting that tumor-specific T cells were selectively undergoing apoptosis (18).

The finding that type 2 responses are characteristic of renal cell carcinoma patients with active disease, whereas type 1 responses are associated with disease-free status, indicates that the tumor environment may play a role in regulating the polarization of a T-cell response in those cancer patients (15–17). Data presented here suggest that tumor supernatants from renal cell carcinoma explants can similarly skew T cells from even healthy volunteers toward Th2 responsiveness, thus mimicking the phenotype observed in patients with active disease. Additional experiments suggest that gangliosides present in the tumor supernatant participate in the skewing to a type 2 response via an apoptotic mechanism.

MATERIALS AND METHODS

T-Cell Isolation. T cells were isolated from peripheral blood first with a Ficoll-Paque gradient (Amersham Pharmacia Biotech AB, Uppsala, Sweden), followed by negative selection using StemSep antibody mixture (StemCell Technologies, Vancouver, British Columbia, Canada). Blood and renal cell carcinoma tissue from patients as well as blood from normal volunteers was obtained through Institutional Review Board-approved protocols (CCF IRB4639 and 1382, and University of Pittsburgh IRB 020429).

Renal Cell Carcinoma Supernatants and Gangliosides Isolation. Renal cell carcinoma supernatant extracts were produced by cutting renal cell carcinoma tumor tissue into 3 × 3-mm pieces, washing off blood overnight, and then placing the pieces in RPMI 1640 (15 mL/g of tissue) for 3 or 4 days at 37°C in 5% carbon dioxide and 95% air. Supernatants were then removed, passed through a Nitex mess filter, centrifuged at 3000 rpm for 30 minutes, filtered through a 0.22-μm membrane; and stored at −20°C. Ganglioside extracts were isolated using a solvent partition method developed by McKallip et al. (19).

Th1 and Th2 Cytokine Determination. Isolated T cells were cultured with renal cell carcinoma supernatants or gangliosides for 48 hours with stimulation for 24 hours using phorbol 12-myristate 13-acetate (PMA; 10 ng/mL, Sigma, St. Louis, MO) and ionomycin (0.75 ng/mL, Sigma). Trypan blue exclusion of dead cells, and restimulated with PMA (10 ng/mL, Sigma) and ionomycin (5 ng/mL, Sigma) in the presence of brefeldin A (10 μg/mL, Sigma) in 96-well V-bottomed plates for 18 hours. Unstimulated cells cultured in the presence of brefeldin A were used as a control. Cells were then fixed in 4% paraformaldehyde and washed with 0.1% saponin in FACS flow buffer (0.2% bovine serum albumin and 0.02% NaN₃ in PBS).

RESULTS

Supernatants from Renal Cell Carcinoma Explants Inhibit Type 1 T-Cell Responses. To determine the effects of renal cell carcinoma supernatants on the autologous DC-mediated induction of Th1 responses, CD4^+ T cells from renal cell carcinoma patients were cultured with S. enteritoxin B-pulsed DCs in the presence or absence of various concentrations (0% to 25% v/v) of the renal cell carcinoma supernatants. On day 7, the resulting CD4^+ T cells were restimulated with PMA and ionomycin, and the Th1 cytokine profiles were assessed by intracellular cytokine flow cytometry. As shown in Fig. 1, the presence of renal cell carcinoma supernatant at concentrations exceeding 5% (v/v) reduced the absolute numbers of CD4^+ T cells pro-

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ducing the T H 1-type cytokine IFN-γ but had little or no effect on the absolute number of CD4+ responder T cells producing the T H 2-type cytokine IL-4. Thus, the supernatants from renal cell carcinoma explants appear to preferentially suppress the induction of type 1 CD4+ T-cell responses (versus type 2) against antigen-loaded DCs.

The inhibition of a type 1 response by renal cell carcinoma supernatants was also observed with peripheral blood T cells, containing a mixture of CD4+ and CD8+ cells, suggesting that both T H 1 and T C 1 are affected. As seen in Fig. 2, supernatants derived from multiple renal cell carcinoma explants (clear cell tumors) all inhibited IFN-γ production in response to PMA and ionomycin. Although not shown, we have also found that the renal cell carcinoma supernatants similarly inhibit the stimulus-dependent production of IL-2 by T lymphocytes. Interestingly, these supernatants did not inhibit the production of the suppressive cytokine, IL-10, by the activated T cells but rather caused a modest increase in IL-10 expression as determined by ELISA.

**Gangliosides within the Supernatants from Renal Cell Carcinoma Explants Promote T H 2-Type Biased T-Cell Responses.** To define the potential of shed tumor gangliosides to inhibit type 1 T-cell responses, we isolated gangliosides from tumor supernatants by a standard protocol and added them to peripheral blood T cells. After 48 hours of coculture, T cells were stimulated with PMA and ionomycin for 18 hours before isolating RNA and performing real-time PCR. There was minimal to no T-cell expression of mRNA for either IFN-γ or IL-5 in the absence of stimulation. The addition of gangliosides to the T-cell cultures did not inhibit the PMA and ionomycin-induced expression of IL-5 mRNA, although the isolated gangliosides caused almost complete inhibition of IFN-γ mRNA expression (Fig. 3). Similar results were observed with gangliosides isolated from an unrelated renal cell carcinoma supernatant (data not shown). Thus, these findings show that gangliosides present in the tumor supernatant induce type 2 biased immunity, similar to that promoted by the crude supernatant.

Prior studies by others using TLC have shown that when compared with normal adjacent tissue, renal cell carcinomas demonstrate increased expression of the gangliosides GM1, GM2, and GD1a (21). We have recently obtained similar results by using high-performance liquid chromatography to profile 12 different clear cell tumors and comparing those findings to the profile of gangliosides isolated from normal adjacent tissues.6

High-performance liquid chromatography analysis of gangliosides isolated from an renal cell carcinoma supernatant demonstrated the presence of multiple gangliosides, including those with retention times similar to the standard gangliosides GM1.

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and GD1a. Given that GD1a was increased in expression in renal cell carcinoma, we asked whether the purified bovine brain-derived ganglioside GD1a would alter the expression of type 1 and type 2 cytokines in T cells stimulated with PMA and ionomycin. As seen in Fig. 4, GD1a caused a significant inhibition in the induction of IFN-γ expression, while actually augmenting the expression of IL-5 induced by PMA and ionomycin. The concentration of GD1a used for these experiments was 200 μg/ml. The concentration of the mixture of gangliosides isolated from the tumor supernatants was estimated by quantifying the amount of lipid-bound sialic acid per milliliter of solution using a method published previously (22). With this assay the concentration of ganglioside (lipid-bound sialic acid) was between 4 and 14 μg/mL for the four different ganglioside preparations examined.

**Gangliosides Promote Type 2 Biased Immunity by Inducing Type 1 T-Cell Apoptosis.** We reported previously that gangliosides isolated from some renal cell carcinoma supernatants can induce T cells to undergo apoptosis (23). Here we examined the ability of tumor supernatant-derived gangliosides to inhibit IFN-γ production via the induced apoptotic death of type 1 T cells. This ganglioside preparation caused a significant inhibition in the production of IFN-γ protein, as demonstrated by ELISA, within 24 hours of stimulation with PMA and ionomycin (Fig. 5). The inhibition of IFN-γ expression coincided with the induction of T-cell death, which was also mediated by the tumor-derived ganglioside. Although activation with PMA and ionomycin alone did not induce any significant apoptosis, the presence of gangliosides induced 50% of the T cells to die. Although this cell death was assessed by the uptake of trypan blue, we also showed that this ganglioside preparation and others could induce DNA breaks as determined by the TUNEL assay (Fig. 6). The data in Fig. 6 also indicate that gangliosides appear to be most effective at inducing apoptosis in activated T cells. Although the ganglioside alone induced 35% of the resting T cells to undergo apoptosis, the number of apoptotic cells increased to >80% when activated with PMA and ionomycin.
Type 1 T-Cell Responses in Renal Cell Carcinoma

Fig. 5 Gangliosides isolated from supernatants of renal cell carcinoma explants are capable of inhibiting production of IFN-γ and inducing apoptosis in normal T cells. Peripheral blood T cells from healthy individuals were cultured with or without renal cell carcinoma-isolated gangliosides for 48 hours and were stimulated with PMA and ionomycin for the last 18 hours. The T-cell supernatants were then collected to measure protein production of the type 1 cytokine, IFN-γ, by ELISA (top) and the T cells counted with trypan blue exclusion to measure apoptosis (bottom). These findings show that renal cell carcinoma-derived gangliosides inhibited IFN-γ protein production and induce apoptosis in normal T cells.

DISCUSSION

There is evidence that immune responses to renal cell carcinoma protect patients against progression of the disease and in limited cases mediate tumor regression. This notion is supported by the infrequent although detectable occurrences of spontaneous regression in renal cell carcinoma patients and by the higher incidence of renal cell tumors in patients who are immunosuppressed after receiving kidney allografts (24–26). There is also a significant infiltrate of T lymphocytes in primary renal tumors (27), and in some patients, tumor-specific T-cell lines and clones have been expanded from their tumors (28–30). Furthermore, clonal expansion of T-cell receptor α/β T cells has been reported in renal cell tumors, most notably in those that are regressing (9, 31). Our previous findings show that CD4+ and CD8+ T cells specific for MAGE-6 and EphA2 antigens that are expressed on renal cell carcinoma are detectable in the peripheral blood and can mediate type 1 responses in patients who have no current evidence of disease. However, it is also evident that in patients with active renal cell carcinoma, the development of an effective type 1 T-cell response is impaired (15–17). A Th2-type CD4+ T cell bias to MAGE-6 antigen is not unique to renal cell carcinoma patients, because defective CD4+ IFN-γ response to MAGE-6 was reported in patients with melanoma (15, 16). Our findings of a Th1-type CD4+ deficiency to tumor-specific antigens is in agreement with a study that examined IL-4 and IFN-γ production by T cells from renal cell carcinoma patients in response to polyclonal activation, which showed that the proportion of CD4+ cells producing IL-4 was significantly higher and the Th1:Th2 ratio lower in patients with high-stage disease (32). Animal studies using the B16 melanoma model have demonstrated that there is a shift from a mixed Th1:Th2 type CD4+. T-cell response to a Th2:Tr-type dominated response during tumor progression and that administration of antibodies to IL-4, IL-10, and transforming growth factor-B1 blocked tumor-induced Th 2 bias (33–35). Whether the administration of cytokines, such as IFN-α, that have some clinical activity in renal cell carcinoma patients can cause a shift from a Th2 response to a Th1-type response is not known but is under investigation.

Our findings suggest that soluble mediators present in the tumor microenvironment represent one possible mechanism to explain the type 2 bias observed in renal cell carcinoma patients with progressing disease. It is known that tumors exhibit augmented synthesis of select gangliosides, which are shed into the tumor microenvironment (36, 37). Malignant melanomas and neuroblastomas overexpress GD3, GD2, and GM2 (38), whereas renal cell carcinoma demonstrates increased expression of GD1a, GM1, and GM2 compared with normal kidney tissue (21). Gangliosides isolated from different tumors are known to inhibit immune responses (39, 40). In vitro data presented herein show that gangliosides present in supernatants from renal cell carcinoma explants can polarize T-cell responses toward a dominant type 2 functional phenotype after mitogenic activation with either the superantigen S. enterotoxin B or PMA and ionomycin. Our findings are consistent with those of others (41), which demonstrated that a mixture of bovine brain-derived gangliosides inhibited IFN-γ production but not IL-4 after the T-cell stimulation. Herein, we show that GD1a (bovine derived), a ganglioside overexpressed in renal cell carcinoma, can also

Fig. 6 Gangliosides isolated from renal cell carcinoma supernatants can induce apoptosis in normal T cells. T cells from healthy volunteers were incubated with gangliosides isolated from renal cell carcinoma supernatants, with or without stimulation with PMA and ionomycin. The T cells were then assessed for apoptosis by TUNEL analysis. The isolated gangliosides from renal cell carcinoma did induce apoptosis of T cells after 48 hours of coculture, and the level of ganglioside-induced apoptosis was increased by simultaneous stimulation with PMA and ionomycin.
inhibit the development of type 1 T-cell responses. It is currently not known which of the gangliosides expressed by renal cell carcinoma and present in supernatants from renal cell carcinoma explants are responsible for the selective suppression of the type 1 response, although this is an active area of our ongoing investigations. It is also possible that the impaired Th CD4+ response to tumor-associated antigens in renal cell carcinoma patients is due in part to circulating gangliosides derived from the tumor. Others have shown that GD2 levels are detectable in the peripheral blood of neuroblastoma patients and that rapid disease progress and low survival rate was associated with high circulation of GD2 in these patients (42, 43). We are currently testing whether renal cell carcinoma patients display elevated levels of gangliosides in their sera and whether gangliosides levels correlate with the reported Th2 bias.

In addition, other soluble mediators besides gangliosides are likely to contribute to the induction of a Th2-type T-cell response bias in renal cell carcinoma patients. We noted previously in a subset of patients with stage IV disease (3 of 8) that transforming growth factor beta but not IL-10 was produced in vitro after EphiA2 peptide stimulation of responder CD4+ T cells. Interestingly, these same patients displayed both weak Th1 (IFN-gamma) and weak type 2 (IL-5) CD4+ T-cell reactivities against EphA2 peptides (17). Thus, it is possible that Th3/T-reg CD4+ T cells may play a role in inhibiting the development of Th1-type T-cell responses against the EphA2 tumor-associated antigen in at least some renal cell carcinoma patients.

Our data also suggest that the induction of apoptosis in the type 1 T cells is a major mechanism that potentially explains the type 2 bias that is observed in peripheral blood T cells responding to tumor antigens (MAGE-6 and EphA2) in vivo and to mitogens in vitro. Furthermore, tumor-derived products, including gangliosides, may be involved in promoting the apoptosis of type 1 T cells. This idea is supported by our observations that gangliosides isolated from tumor supernatants that are apoptotic, may be involved in promoting the apoptosis of EphA2 tumor-associated antigen in at least some renal cell carcinoma patients.

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