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Adenovirus CD40 Ligand Gene Therapy Counteracts Immune Escape Mechanisms in the Tumor Microenvironment

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Tumors exhibit immune escape properties that promote their survival. These properties include modulation of Ag presentation, secretion of immunosuppressive factors, resistance to apoptosis, and induction of immune deviation, e.g., shifting from Th1- to Th2-type responses. These escape mechanisms have proven to hamper several immunotherapeutic strategies, and efforts need to be taken to revert this situation. We have studied the immunological effects of introducing CD40 ligand (CD40L), a potent dendritic cell activation molecule, into the tumor micromilieu by adenoviral gene transfer. For this purpose, a murine bladder cancer model (MB49) was used in C57BL/6 mice. The MB49 cells are known to induce IL-10 in the tumor environment. IL-10 potently inhibits the maturation of dendritic cells and thereby also the activation of CTLs. In this paper we show that CD40L immunogene therapy suppresses IL-10 and TGF-β production (2-fold decrease) and induces a typical Th1-type response in the tumor area (200-fold increase in IL-12 production). The antitumor responses obtained were MB49 cell specific, and the cytotoxicity driven by IL-12 and IFN-γ was not inhibited by IL-10. Adenovirus CD40L therapy was capable of regressing small tumors (five of six animals were tumor free) and inhibiting the progression of larger tumors even in the presence of other escape mechanisms, such as apoptosis resistance. Furthermore, CD40L-transduced MB49 cells promoted the maturation of dendritic cells (2-fold increase in IL-12) independently of IL-10. Our results argue for using adenovirus CD40L gene transfer, alone or in combination with other modalities, for the treatment of Th2-dominated tumors. The Journal of Immunology, 2004, 172: 7200–7205.

The immune system has the capacity to eradicate tumor cells in various ways. The most potent antitumor responses are generated along the so-called Th1 pathway driven by IL-12 and IFN-γ production (1). The main Th1 effector cell is the CTL (2). There are also other potent effector cells, such as NK cells, that can be activated to lyse tumor cells that present the correct set of stimulatory/inhibitory receptors (3).

Tumor cells can survive due to genetic or phenotypic selection of clones that escape from immune surveillance. Immune escape mechanisms can be divided into three distinct groups: 1) Ag processing and presentation may be impaired via mutations of the Ag-processing machinery or down-modulation of MHC class I molecules; 2) tumors may express or induce the expression of immunosuppressive molecules, such as IL-10, TGF-β, vascular endothelial growth factor, or mucin 1, which may suppress inflammatory danger signals in the tumor area (4, 5); and 3) tumor cells may exhibit dysfunctional apoptotic pathways with a mutated intracellular signaling, expression of CD95 decoy receptors, or over-expression of antiapoptotic molecules. For example, the molecule PI-9 (murine homologue, SPI-6) blocks the perforin/granzyme B pathway by binding irreversibly to granzyme B, thereby inhibiting its enzymatic activity (6). To develop effective immunotherapies for the treatment of cancer we need to understand, and learn how to control, the underlying mechanisms in the tumor milieu that direct the local immune responses.

In our previous studies we have shown that genetically modified murine bladder cancer, mouse bladder cancer-49 (MB49), cells stimulate protective immune responses against tumor formation (7). The dendritic cell (DC)3-activating molecule, CD40 ligand (CD40L), showed superior results compared with other immune stimulators. However, tumor escape mechanisms are obstacles in successful tumor immunotherapy, and human bladder cancer is associated with elevated IL-10 and TGF-β production as well as with dysfunctional CD95 apoptosis pathways (8–11). In this paper we clarify the mechanisms of action of CD40L in the MB49 tumor system, which is known to escape immune surveillance due to IL-10 induction (12).

The immunological basis of adenovirus CD40L (AdCD40L) immunogene therapy remains inadequately defined despite that the potential of this therapy has been proven in several models of cancer (13–15). The MB49 cells have been reported to induce IL-10 production in nearby cells. In that study IL-10 knockout mice showed a higher frequency of tumor rejection than wild-type mice (12). A later study showed that DCs from MB49-bearing mice had an impaired capacity to stimulate T cells in vitro (16). IL-10 is known to inhibit Th1-related immune responses while favoring Th2 or regulatory responses and is considered a common immune escape mediator of tumor cells (4, 5). Seo et al. (17) showed in a murine melanoma model (B16) that IL-10 expressed early in the tumor lesion was critical for the generation of CD4+ regulatory T cells that inhibited CTL activity. IL-10 also inhibits the maturation of DCs, which are essential for the activation and maintenance of tumor-specific CTL responses (18).

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3 Abbreviations used in this paper: DC, dendritic cell; AdCD40L, adenovirus CD40L; CD40L, CD40 ligand.
In the present study we argue that the expression of the DC-activating molecule CD40L within the tumor milieu combats immune escape mechanisms such as IL-10 production, and this creates an avenue for effective Th1-related tumor eradication.

Materials and Methods

Cell culture

The murine cell lines MB49 (19) and Lewis lung cell carcinoma-1 (LLC-1; American Type Culture Collection, Manassas, VA) derived from C57BL/6 were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.1% sodium pyruvate. The NK cell-sensitive cell line YAC-1 (20) and primary cultures of splenocytes were cultured in RPMI medium (RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, 1% glutamine, 1% HEPES, 1% MEM, 1% 2-ME, and 0.1% sodium pyruvate). The murine bone marrow-derived DCs were differentiated and cultured in R10 medium, described above, with 25 ng/ml murine IL-4 and 50 ng/ml GM-CSF. The MB49 cell line was a gift from Dr. K. Esuvanathan (National University Hospital, Singapore), whereas YAC-1 cells were provided from Dr. J. Charo (Karolinska Institute, Stockholm, Sweden). Growth medium and supplements were purchased from Invitrogen (Paisley, U.K.). Murine cytokines were obtained from Nordic Biosite (Stockholm, Sweden).

Production of recombinant adenoviruses

Two replication-deficient, E1/E3-deleted human adenoviruses type 5 were produced using the AdEasys system (21). The AdCD40L virus code for the murine CD40L molecule, and AdID is a noncoding control virus. The pShuttle-CMV and pAdEasys vectors were gifts from Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD). Splenocytes were resected from C57BL/6 animals, and splenocytes were isolated by mechanical dissection. Cells were stimulated with 50 µg/ml ionomycin and 1 µg/ml PMA for 1 h. Total RNA was extracted with TRizol (Invitrogen), and first-strand cDNA was synthesized with oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen). Murine CD40L cDNA was amplified by using the forward (AGA GTC CTC GAG ATG ATA GAA ACA TAC AGC) and reverse (GGC GCG TCT AGA TCA GAG TTT GAG TAA GCC) primer pairs. The PCR product was XhoI/XbaI-directionally cloned into pShuttle-CMV and sequenced using BigDye Terminator (Applied Biosystems, Foster City, CA). Shuttle plasmids were homologously recombined with pAdEasys1 in BJ5183 bacteria to generate recombinant adenovirus DNA. Adenoviruses were produced by four rounds of transfection of 293 cells. Virus titer is determined by plaque assays.

Transduction of tumor cells

The MB49 tumor cells were transduced with 1000 PFU/cell under serum-free conditions. Briefly, the vector was admixed with tumor cells in a total volume of 200 µl of medium, incubated for 2 h at 37°C, and further cultured in six-well plates in 3 ml of serum-containing medium until analysis of transgene expression by flow cytometry.

Flow cytometry

Cells were analyzed by flow cytometry using mAbs directly conjugated with PE or FITC or secondary Abs (mouse anti-Armenian hamster IgG) conjugated with FITC. Abs against the following Ags were used: CD40L, CD40, MHC-I, MHC-II, CD80, CD83, and CD11c (BD Biosciences, San Diego, CA). The control Abs were hamster negative control FITC and hamster negative control PE (Serotec, Raleigh, NC). Single-cell suspensions of 1 × 10^6 cells were incubated with Abs for 10 min at room temperature, washed in 2 ml of PBS, and centrifuged at 200 × g. The supernatant was decanted. Cells were re suspended in 200 µl of 1% paraformaldehyde in PBS before analysis by flow cytometry (FACSCalibur; BD Biosciences, San Diego, CA).

Restimulations

Spleens were taken from naive and vaccinated mice and were prepared by mechanical dissection, followed by washing in PBS. The cells were either used as splenocytes or subjected to further isolation procedures. CD4^+ T cells were isolated by positive selection using anti-CD8^+ MACS beads (Miltenyi Biotec, Auburn, CA) according to company protocol. The different cell populations were then mixed with irradiated (60 Gy) MB49 tumor cells in a 5:1 ratio and cultured 3–7 days with R10 medium, described above, some cells in the presence of 200 pg/ml murine IL-10. For cytotoxic assays, 25 U/ml IL-2 was added to the cultures. DCs and MB49 cells were cocultured for 3 days at a 5:1 to 10:1 ratio in 2 ml of R10 medium. The DCs were then screened for the cell surface markers CD11c, CD80, MHC-I, MHC-II, CD40, and CD83.

Cytotoxic assay

The cytotoxic assays were performed according to standard 51Cr release protocols. Briefly, target cells (MB49, LLC-1, and YAC-1) were labeled with 111Cr and seeded into 96-well plates. Effector cells (splenocytes and CD40L^+ T cells) were added to target cells in various ratios and incubated for 4–6 h. Supernatants were collected, and the 51Cr released was detected using a gamma counter (Wallac, Turku, Finland). The percentage of lysed target cells was calculated by the following formula: ([sample release– spontaneous release]/maximum release – spontaneous release) × 100.

ELISA

Supernatants from restimulations were collected and analyzed for protein levels of IL-10, IL-12, and IFN-γ using ELISA kits (Nordic BioSite) according to the manufacturer’s protocols.

Quantitative-PCR (TaqMan)

Biopsies from tumors and lymph nodes were snap-frozen in liquid nitrogen before storage at –80°C. The biopsies were mechanically pulverized and dissolved in extraction buffer (100 mM Tris (pH 8), 10 mM EDTA, 5 nM DTT, 1% lithium dodecyl sulfate, and 500 mM LiCl). The tissue extract was used for cDNA synthesis with oligo(dT)–coated manifold supports as described previously (22, 23). Quantitative PCR was performed using the TaqMan system (iCycler; Bio-Rad, Hercules, CA). The cDNA was mixed with PCR Gold buffer, Taq polymerase AmpliTag Gold (Applied Biosystems), MgCl2 in varying concentrations ranging from 1.5 to 3 mM, 0.2 mM dNTPs (Invitrogen) probes, and primer pairs in a final volume of 25 µl. Probes and primer pairs for IL-10, TGF-β, TNF-α, and β-actin were designed according to described sequences (24) (Genset Oligos, Paris, France), with an exception for the IL-10 forward primer, which was designed as follows: 5′-TGACAAATAGAACGACAGGATGTCG-3′. Detection of IL-12 was performed using Pre-Developed TaqMan Assay Reagents for murine IL-12p40 (Applied Biosystems). To minimize the effects of different cDNA content in the samples, the copy numbers were correlated with β-actin levels by multiplying each sample value with a calculated correlation factor. The correlation factor was obtained by division of the highest β-actin copy number by the β-actin copy number of the sample.

Animal experiments

For in vitro restimulation studies, female C57BL/6 mice were vaccinated s.c. four times weekly with 10^4 AdCD40L–transduced MB49 cells before challenge with wild-type MB49 tumor cells. For intratumoral vector treatment the mice were inoculated with either 1 × 10^6 or 2 × 10^6 wild-type MB49 tumor cells. Tumors were grown to a minimum size of 2.5 or 5 mm in diameter before immunogene therapy of the tumor nodule was initiated (10^5 PFU/mouse). All animal experiments were approved by the local animal ethics committee.

Results

AdCD40L immunogene therapy shrinks growing tumors

The murine bladder tumor cell line MB49 rapidly forms aggressively growing tumors in C57BL/6 mice. However, four injections of adenoviral vectors expressing the CD40L transgene in small tumor nodules (~2.5 mm) inhibited growth and led to the regression of tumors (Fig. 1). On day 15 the experiment was interrupted due to the large tumors in control mice. Biopsies were taken from all groups for evaluation of cytokine profiles. Cured mice remained tumor free on day 30 after tumor inoculation. Even progression of larger tumors (~5 mm) could be inhibited by AdCD40L therapy (Fig. 2).

Th2-dominated immune environment in tumors is reversed by AdCD40L therapy

It is known that MB49 cells induce IL-10 production in the tumor site and that IL-10 knockout mice reject MB49 tumor cells more efficiently than wild-type mice (12). As IL-10 inhibits the maturation of DCs, we speculated that introduction of the DC-activating molecule, CD40L, in the tumor microenvironment might circumvent the effects of IL-10. To investigate this further, tumor biopsies...
were screened for cytokine mRNA copy levels by quantitative PCR. To compare cytokine expression among the groups, mRNA levels were correlated with the housekeeping gene β-actin. The tumor microenvironment was dominated by increasing levels of Th2/suppressor cytokines, such as IL-10 and TGF-β, as well as a rather stable expression of TNF-α. The Th1-promoting cytokine IL-12 was not detectable in untreated mice. Upon AdCD40L treatment, IL-10 and TGF-β levels declined, whereas TNF-α remained stable after an initial decrease (Fig. 3A). However, IL-12 expression could be detected after a single AdCD40L treatment and was still detectable after three treatments. Injection of control vector also decreased the IL-10 and TGF-β expression, but did not induce IL-12 expression. Of note, the control vector did not affect the growth of the tumors (Fig. 2). Hence, the combination of Th2/suppressor cytokine inhibition together with Th1 cytokine induction seemed pivotal for the regression of tumors. As shown, AdCD40L therapy modified the immune hostile milieu, particularly in the tumor environment. Next, systemic effects of CD40L therapy were investigated by analysis of cytokines in the periphery of the tumor. The positive effects of intratumor CD40L expression, but did not induce IL-12 expression. Of note, the control vector did not affect the growth of the tumors (Fig. 2). Hence, the combination of Th2/suppressor cytokine inhibition together with Th1 cytokine induction seemed pivotal for the regression of tumors. As shown, AdCD40L therapy modified the immune hostile milieu, particularly in the tumor environment. Next, systemic effects of CD40L therapy were investigated by analysis of cytokines in the periphery of the tumor. The positive effects of intratumor CD40L treatment on local cytokine mRNA levels were not reflected in the draining lymph nodes (data not shown). IL-10, TGF-β, and IL-12 remained unaltered during the treatments, although TNF-α expression was higher in CD40L-treated mice.

**Down-regulation of serum IL-10 in AdCD40L-treated mice**

Tumor-bearing mice had elevated levels of IL-10 in serum compared with normal counterparts (Fig. 3B). Thus, the effect of CD40L on IL-10 expression does not seem to be restricted only to the tumor area.

**IL-10 participates in the inactivation of tumor-specific T cells**

MB49-specific CTLs are efficiently generated by repeated in vivo injections of AdCD40L-transduced MB49 cells, followed by 1-wk stimulation in vitro of CD8+ cells with irradiated MB49 cells (7). This method is preferred to in vitro generation of murine CTLs with peptide/tumor lysate-pulsed mature DCs because of the short life span of mature DCs. These CTLs lysed MB49 cells without killing the irrelevant syngeneic target LLC1 or the NK cell-sensitive target YAC-1 (Fig. 4). Interestingly, the cytotoxicity against MB49 was lost if rIL-10 was added during in vitro stimulations of CD8+ cells or if unsorted splenocytes were restimulated with MB49 cells.

Cytokine analysis of the supernatants from CTL/MB49 in vitro stimulations revealed that IL-10 was absent when sorted CD8+ cells were stimulated. In contrast, when unsorted splenocytes were stimulated, IL-10 was present at high levels. Stimulation of unsorted splenocytes with CD40L-expressing tumor cells abolished IL-10 production, but increased both IFN-γ and IL-12 secretion (Fig. 5). Note that splenocytes/CD8+ cells (which include CD8+ DCs) from either vaccinated or naive mice respond to AdCD40L with IL-12 production, because the number of immature DCs should be equal in both groups. These data further demonstrate the potency of IL-10 as an immune escape mechanism by tumors and show that CD40L therapy may reverse the effects of this cytokine.

**MB49 cells inhibit maturation of DCs in vitro**

Yang and Latimie (16) published data suggesting that MB49 tumor-induced IL-10 suppresses the ability of DCs to stimulate CD4+ and CD8+ T cell responses. In this study we demonstrate that MB49 cells inhibit the spontaneous maturation of murine DCs in vitro (Fig. 6), which may be an explanation for their findings. The maturation markers CD83 and MHC class II were expressed at low levels typical of the immature DC phenotype when DCs were mixed with MB49 cells (Fig. 6, A and B). Introduction of CD40L via coculture with transduced MB49 cells did not affect
IL-10 production at this time point (Fig. 6C), but restored DC maturation and stimulated DCs to express elevated levels of CD83, MHC class II, and IL-12 (Fig. 6A, B, and D). Control vector by itself

FIGURE 3. AdCD40L therapy inhibits IL-10 and TGF-β induction. Biopsies were taken 3 days post-treatment 1 (1X; □) and 3 days post-treatment 3 (3X; ■). The cytokine mRNA levels in the tumors (A) were measured by quantitative PCR, whereas IL-10 levels in serum (B) were measured by ELISA. ∗, Tumors that had significantly higher or lower levels of Th1 and Th2suppressor cytokines than controls are indicated (α = 0.05; p < 0.05, by independent t test).

FIGURE 4. Specific lysis of MB49 tumor cells can be inhibited by IL-10. Cytotoxicity assays (51Cr release) were performed with splenocytes from naive (A) or immunized (B) mice without success. By using purified CD8+ cells, IL-10 production was avoided, and MB49 targets could be lysed (C). Note that addition of rIL-10 to the restimulations of CD8+ cells suppressed cytotoxicity. The different target cells used are indicated in A, B, and D, whereas the different effector cells against the MB49 target are listed in C.
slightly up-regulated CD83, MHC class II, and IL-12 production, which may be due to adenoviral protein stimulation of DCs.

Discussion

The most potent immunotherapy strategies for tumor treatment engage activation of tumor-specific CTLs. Tumor-specific CTLs are detectable in cancer patients, but they are often sparse, anergic, and unable to control the growing tumor. Anergy or different stages of unresponsiveness are induced by several mechanisms, including Ag presentation in the absence of costimulation (5). Immature DCs and regulatory T cells present in the tumor area may be responsible for inactivation of CTLs (6). It has been shown that IL-10 and TGF-β inhibit DC maturation and may thereby indirectly eliminate CTL responses (25).

In the present study we demonstrate that the IL-10-inducing murine bladder tumor MB49 can be efficiently treated with AdCD40L therapy. Small, but aggressively growing, tumors could be completely eradicated in most mice (five of six). Also, the progression of larger tumors could be inhibited. Introduction of CD40L into the tumor microenvironment inhibited IL-10 and TGF-β expression and stimulated effective IL-12 production. The pre-existing Th2 milieu was thereby tilted toward a Th1 pattern, which apparently supported the activation of tumor-specific CTLs. In experiments not shown in this report we demonstrated that interruption of AdCD40L treatment of large tumors eventually leads to tumor progression. Hence, the balance needs to be continuously shifted toward Th1 until the tumor has been defeated.

By performing cytotoxicity assays, we showed that the presence of IL-10 during stimulation of CD8+ cells from MB49-immunized mice eradicated the CTL lytic capacity. In the absence of IL-10, the lytic capacity was restored, and MB49 target cells could be efficiently killed. This could be due to development of immature/regulatory DCs within the CD8+ cell population during the in vitro stimulation, but needs to be formally proven.

Murine bone marrow-derived DCs may spontaneously mature when cultured in vitro (26). By coculturing DCs with MB49 cells, the spontaneous maturation was abolished. When using CD40L-expressing MB49 cells instead, the DCs matured and increased their IL-12 production 2-fold. IL-10 could be detected in all cultures, but its inhibitory effect on DC maturation was reversed by the presence of CD40L. These results emphasize that IL-10 induction in the tumor area is a potent immune escape mechanism that may be overcome by CD40L treatment. However, we cannot rule out other participants in this process.

As mentioned, many tumors are associated with IL-10 production. However, the role of IL-10 is dual. In some models IL-10 has been used to activate antitumor responses (27). It has been suggested that the local concentration of this cytokine may be pivotal for the outcome. IL-10 expression may also have an important role for inhibiting an “overheating” of immune responses. IL-10 can be produced by most immune cells, and there are also reports that other cell types may be able to produce this Th2-associated cytokine.

IL-10 exerts its function on both APCs and lymphocytes and therefore has a major impact on regulation of the Th1/Th2 balance. IL-10 is capable of suppressing the expression of proinflammatory cytokines, chemokines, adhesion molecules, and costimulatory molecules via inhibition of the NF-κB pathway (28). Despite our efforts, we have been unable to date to identify the IL-10 producing cell(s) in our model. Preliminary results indicate that it may involve a mixture of different cell types. It remains to be shown whether the IL-10 production is due to direct signaling from the MB49 tumor cells to a specific cell type or represents an inflammatory response to tumor growth and necrosis. However, the antitumor effect in our model is not dependent on the decreased IL-10 levels alone, as shown by control vector treatments. A broad activation occurs during CD40L stimulation. One important feature is the release of IL-12 by matured DCs. With previously published data we have demonstrated that NK cell killing as a result of released IL-12 may play a role in the initial response, but T cells are...
needed for curing the tumor (29). Further, the AdCD40L-treated tumor is early infiltrated with both CD4+ and CD8+ T cells (7).

The generation of potent immunotherapies that overcome tumor cell strategies to escape immune surveillance is a challenge. We demonstrate in this study that biologically relevant concentrations of IL-10 and/or TGF-β appear to be instrumental in the immune escape of MB49 mouse bladder cancer cells. However, this mechanism is circumvented by AdCD40L immunogene therapy.

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