Local Activation of CD8 T Cells and Systemic Tumor Eradication without Toxicity via Slow Release and Local Delivery of Agonistic CD40 Antibody

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Abstract

**Purpose:** Immunotherapy against tumors with anti-CD40 agonistic antibodies has been extensively studied in preclinical animal models and recently also in clinical trials. Although promising results have been obtained, antibody (Ab)-related toxicity has been a limiting factor. We reasoned that strict local activation of tumor-specific CD8 T cells through stimulation of CD40 on the dendritic cells (DC) in the tumor area while excluding systemic stimulation might be sufficient for effective tumor eradication and can limit systemic toxicity.

**Experimental Design:** Preclinical in vivo models for immunogenic tumors were used to investigate the potential of delivering a nontoxic dose of agonistic anti-CD40 Ab to the tumor region, including draining lymph node, in a slow-release formulation (montanide).

**Results:** The delivery of anti-CD40 monoclonal Ab, formulated in slow-release Montanide ISA-51, reprograms CTLs by inducing local but not systemic DC activation, resulting in effective tumor-specific CTL responses that eradicate local and distant tumors. Adverse side effects, assayed by organ histology and liver enzymes in the blood, were much lower after local anti-CD40 Ab delivery than systemic administration. The local delivery of anti-CD40 Ab activates only CTLs against antigens presented in the tumor-draining area, because unrelated distant tumors expressing different tumor antigens were not eradicated.

**Conclusions:** These results establish a novel therapeutic principle that local delivery and slow release of agonistic anti-CD40 Ab to the tumor-draining area effectively activates local tumor-specific CD8 T cells to become systemic effectors without causing systemic toxicity or nonspecific CTL activation. These findings have important implications for the use of anti-CD40 therapies in patients. Clin Cancer Res; 17(8); 2270–80. ©2011 AACR.

Introduction

CD8⁺ T cells (CTLs) recognize and kill specific target cells on the basis of their T-cell receptors that are selected to recognize antigens presented by MHC class I molecules (1). Since many tumors express aberrant antigens, CD8⁺ T cells have the potential to eradicate these tumors (2–4). The induction of antitumor T-cell priming, however, is often ineffective. One of the reasons for this phenomenon is that dendritic cells (DC), which cross-present the tumor antigens in tumor-draining lymph nodes (LN), are poorly activated because of a lack of danger signals and insufficient CD4⁺ T-cell help (5–8). Methods to effectively activate those DCs and thereby enhance the antitumor CTL response may thus provide significant improvement for the treatment of cancer.

The TNF receptor family member CD40 is a stimulatory molecule and constitutively expressed on a large variety of cells, including DCs, B cells, macrophages, and endothelial cells (9). Engagement of CD40 on DCs provides potent maturation signals leading to improved T-cell–mediated tumor rejection (10, 11). Several in vivo studies have shown that CD40 ligation by systemically delivered agonistic antibodies can induce robust antitumor immune responses, either as monotherapy or in combination with TLR ligands, cytokines, and chemotherapy, indicating clinical potential (12–17). However, serious side effects of treatment with agonistic CD40 antibodies in the clinic have been reported, which include cytokine release syndrome and liver function abnormalities (18). Adverse effects have also been reported for several other therapies that are based on antibodies that mediate systemic immune activation, such as CTLA-4 blocking antibodies and agonistic anti-CD28 antibodies (18–21).

Previously, we have shown that tumor antigens are predominantly presented by DCs in the tumor-draining...
Translational Relevance

Systemic delivery of agonistic anti-CD40 antibodies induces good antitumor immune responses in preclinical models, but dose-limiting toxicity hampers clinical success. We have used a novel delivery system based on the slow-release agent Montanide ISA-51 to distribute agonistic CD40 antibody (Ab) in the lymphoid drainage area of the tumor that stimulates local but not systemic dendritic cells (DC). Local DC activation results in a robust systemic antitumor CD8 T-cell response and local and distant tumor eradication without the side effects associated with the standard systemic administration of anti-CD40 Ab. These results indicate an important novel delivery platform for the use of anti-CD40 Ab and conceivably other immunostimulatory therapies in cancer patients.

Material and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. C57BL/6 Kh (B6, H-2b) mice were bred at the Leiden University Medical Center (LUMC) animal facility. The experiments were approved by the animal experimental committee of the University of Leiden.

Tumor experiments

Mouse embryonic cells transformed by the early region 1A of human adenovirus type 5 (Ad5E1A) plus E1a (AR6; ref. 24) were cultured in Iscove’s modified Dulbecco’s medium (IMDM; BioWhittaker) supplemented with 4% fetal calf serum (FCS), 50 μmol/L 2-ME (β-mercaptoethanol), and 100 IU/ml penicillin/streptavidin. EG7 tumor cells expressing the full-length OVA antigen were cultured in IMDM supplemented with 8% FCS, 50 μmol/L 2-ME (β-mercaptoethanol), 2 mmol/L glutamine, and 100 IU/ml penicillin supplemented with 400 μg/ml G418 (Gibco; ref. 25). AR6 tumor cells do not express CD40, and EG7 tumor cells express low levels of CD40 on their cell surface.

The AR6 (E1A expressing) tumor cells (7.5 × 10⁶) were injected subcutaneously into 7- to 13-week-old male mice in 200 μL of PRS. Treatment was started 8 to 14 days after tumor inoculation when palpable tumors were present. EG7 tumor cells (1 × 10⁶) in 200 μL of PBS were injected subcutaneously into 7- to 13-week-old male mice. Treatment was started 3 days later. Secondary tumors were injected 1 day before start of treatment. Tumor size was measured twice weekly in 3 dimensions, and mice were killed when tumor size exceeded 1 cm³.

Flow cytometry

Single-cell suspension of blood and spleens, after erythrocyte lysis, and LNs were stained with anti-CD8α (clone 53-6.7), anti-CD62L (clone MEL14), anti-CD11c (clone H13), anti-CD70 (clone FR70), CD90.1 (Thy1.1; clone OX-7), CD19 (clone 1D3), and CD3ε (clone 145-2C11) all from BD Biosciences, anti-KLRG-1 (clone 2F1) from Southern Biotech, and E1A234-243-loaded H-2Db tetramers and OVA257-264-loaded H-2Kb tetramers.

Agonistic CD40 Ab treatment

The FGK-45 hybridoma cells producing an agonistic anti-CD40 Ab were provided by A. Rolink (Basel Institute for Immunology, Basel, Switzerland; ref. 26). Hybridomas were cultured in Protein-Free Hybridoma Medium (Gibco), and monoclonal Abs (mAb) were purified by a Protein G column. FGK-45 Ab contained less than 2 IU/mg endotoxin. Mice with high-dose systemic treatment were intravenously administered with 100 μg of Ab in PBS on 3 consecutive days. Montanide/Ab emulsions were made by mixing different dosages of Ab in PBS 1:1 with montanide (Montanide ISA-51; Seppic) and vortexing for 30 minutes.

Serum analyses

Serum samples were taken from mice at several time points after treatment. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) analyses were performed by the Department of Clinical Chemistry, LUMC Hospital, according to standard protocols. Anti-CD40 Ab concentrations were analyzed by ELISA with anti-rat antibodies (BD Bioscience).

Histology

Liver, lung, and kidney were isolated from mice at several time points after treatment. Tissues were fixed with formalin and embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin. Images were captured using a Zeiss Axioskop 40 microscope and processed using AxioVision AC software.

In vivo cytotoxicity assay

In vivo cytotoxicity was determined using target cells Thy1.1+ splenocytes. Target cells were labeled with 5 μmol/L carboxyfluorescein succinimidyl ester (CFSE) and pulsed with E1A234-243, SGPSNP1PEI peptide (0.5 μg/ml for 90
minutes at 37°C, or labeled with 0.5 μmol/L CFSE and pulsed with control peptide. Target cells were mixed in a 1:1 ratio of E1A pulsed to control cells and injected intravenously (5 x 10^6 cells of each population) into naive and tumor-bearing mice 9 days after being treated with low-dose anti-CD40 in montanide, or high-dose systemic anti-CD40, or receiving no treatment. Three days after injection of the target cells, spleens were isolated and the number of CFSEhi and control CFSElo Thy1.1+ target cells was determined by flow cytometry. The percentage of specific killing is calculated as follows: [1 – (ratio tumor bearing/ratio naive)] x 100%. Ratio is defined as the number of E1A CFSEhi target cells/number of control CFSElo target cells.

Results

Local treatment with a low dose of agonistic anti-CD40 Ab in a slow-release formulation combines effective treatment and decreased toxicity

To determine the most optimal antitumor treatment with agonistic anti-CD40 antibodies, we compared the effects of different administration methods and Ab dosage on both tumor eradication efficacy and toxicity in organs such as liver. Mice were inoculated subcutaneously with adenovirus protein E1A expressing tumor cells (AR6), previously described to be eradicated by CD8+ T-cells (24), which grew into palpable tumors over 10 days. Subsequently, these tumor-bearing mice were treated with: (i) a standard dose (100 μg) of agonistic anti-CD40 Ab delivered systemically during 3 consecutive days; (ii) a low dose (30 μg) delivered systemically (intravenously); (iii) a low dose delivered locally (subcutaneously) in saline; or (iv) a low dose delivered locally in a slow-release formulation (Montanide ISA-51; 27). The subcutaneous injections were administered in the area between the tumor and the tumor-draining inguinal LN. As shown in Figure 1A, mice receiving local treatment either in montanide or in saline displayed an equal percentage of tumor eradication and survival, and both of these treatments are equally effective in clearing the tumor as the standard systemic intravenous treatment. The low-dose intravenously injected group, however, showed no evidence of tumor growth reduction, and as a consequence, most mice in this group did not survive. These results indicate that a low dose of anti-CD40 can be equally effective as a high dose to eradicate tumors, provided that it is injected closely to the tumor and/or tumor-draining LN.

We assessed the toxicity caused by the aforementioned treatments by analyzing the liver enzymes ALAT and ASAT levels between the low-dose plus slow-release treatment and the standard high-dose systemic treatment showed that these liver enzymes were elevated especially during the first days after systemic treatment and suggest that tissue damage follows (Fig. 1C). The administration of a high dose (150 μg) of anti-CD40 Ab in a slow-release formulation also causes liver inflammation as determined by liver enzyme elevation (data not shown). Histologic analysis performed at several time points after treatment showed that, indeed, damage to the liver was evident (Fig. 1D). At day 3 posttreatment, the livers from mice that received high-dose systemic anti-CD40 treatment were severely affected. Livers displayed lobular foci with a minor component of portal inflammation. The lobular foci of inflammation consisted of lymphocytes (predominantly CD3+), plasma cells, and histiocytes (including F4/80+ macrophages; data not shown). At day 7 posttreatment, lobular inflammations had mostly subsided but portal inflammation (with lymphocytes) was enhanced. In contrast, livers from mice receiving a low-dose anti-CD40 Ab in montanide displayed much less extensive signs of liver damage on day 3 post-treatment, and, on day 7, only remnant signs of inflammation were visible. The lungs and kidneys in mice that were treated with a high-dose systemic anti-CD40 also showed more severe damage, as evidenced by lymphocytic infiltration, than these organs in mice that had received low-dose local treatment (data not shown). Together, these data show that a lower dose and local subcutaneous injection of CD40 agonistic Ab are sufficient for tumor eradication and that this treatment, if applied in a slow-release formulation, caused the least immune-mediated toxicity.

Local delivery of low-dose anti-CD40 in montanide is essential for therapeutic efficacy

To examine whether a low-dose anti-CD40 Ab therapy in a slow-release formulation (montanide) is effective to induce only an antitumor response when delivered close to the tumor, we tested whether contralateral injection could also induce tumor eradication. As shown in Figure 2, tumor growth and survival of mice treated with low-dose anti-CD40 Ab in montanide close to the tumor were comparable with those of mice treated with high-dose systemic anti-CD40 Ab. Mice treated with low-dose montanide-formulated Ab in the contralateral flank, however, did not eradicate the tumor (Fig. 2A and B). When tumor-bearing mice were treated with a high dose of anti-CD40 Ab in montanide (150 μg), either close to the tumor or in the opposite flank (contralateral flank), the antitumor effects were similar, indicating that only a low dose is confined in its therapeutic action to the tumor-draining area (Supplementary Fig. S2A). Mice locally injected with a montanide depot lacking anti-CD40 Ab did not show any antitumor effect (Supplementary Fig. S2B). These data show that the therapeutic effect of low-dose agonistic CD40 Ab treatment in montanide was strictly dependent on local delivery in the vicinity of the tumor and/or tumor-draining LN.
Figure 1. Low-dose local treatment with anti-CD40 Ab gives lower toxicity than high-dose systemic treatment. Tumor-bearing mice were treated with different dosages and administration methods of anti-CD40 agonist Ab 8 days after tumor inoculation. Tumor survival after administration of 30 µg of Ab either intravenously or subcutaneously in saline, or montanide was compared with the standard protocol of 3 subsequent high-dose systemic injection of Ab. A, 8 mice per group, representative of 3 experiments. Liver enzyme concentrations in serum, 24 hours after treatment with different administration methods of 30 µg of Ab. Student’s t-test revealed significant differences between groups. B, 4 mice per group, representative of 2 experiments (i.v. vs. montanide, \( P = 0.002 \) and 0.001 for ALAT and ASAT, respectively; montanide vs. saline, \( P = 0.004 \) and 0.09 for ALAT and ASAT, respectively). Liver enzymes in serum in time after treatment with 30 µg of Ab subcutaneously in montanide compared with standard protocol. C, 7 mice per group, representative of 2 experiments. Histologic sections of liver at days 1, 3, and 7 after treatment with 30 µg of Ab subcutaneously in montanide compared with standard protocol. D, representative sections of groups of 3 mice.
Local treatment with low-dose CD40 agonist causes systemic CTL responses equal to high-dose systemic treatment

The induction of tumor eradication by local treatment with anti-CD40 Ab in the vicinity of a tumor could be explained by the activation of local CD8 T cells to become systemic effector CTLs. To examine this possibility, we analyzed tumor-specific CD8^+ T cells in secondary lymphoid organs of treated and untreated mice. The antitumor CTL response was clearly detectable systemically in blood at day 8 posttreatment with both the standard high-dose intravenous protocol and the locally administered low-dose treatment in montanide, in comparison with nontreated mice, indicating that the local treatment is capable of inducing a potent systemic CD8 T-cell response (Fig. 3A). To determine the functionality of these tumor-specific CTLs, we carried out an in vivo cytotoxicity assay. Specific lysis of target cells in vivo correlated with the presence of tumor-specific (tetramer-positive) CD8^+ T cells in blood. Both treated groups of mice specifically killed target cells loaded with the tumor antigen to a similar extent in contrast to the nontreated group (Fig. 3B).

We next investigated phenotypic effects of these treatments on the cell surface of tumor-specific CTLs in spleen and LN. By examining effector T-cell markers CD62L and KLRG-1 (killer cell lectin–like receptor G1) on tetramer-positive T cells, we could determine whether the different administration routes activated tumor-specific T cells in a qualitatively different manner. The downmodulation of the homing receptor CD62L and the upregulation of the KLRG-1 (marker for effector cells) were similar in tumor-draining LNs and spleens after either high-dose systemic or low-dose local treatments (Fig. 3C and D). In nontreated mice, however, the KLRG-1 expression on tetramer-positive CD8^+ T cells was lower in the draining LNs. The tumor-specific CTL response in this group could not be detected systemically in blood and spleen.

To determine the effects of CD40-mediated DC maturation in tumor-draining versus nondraining LNs of tumor-bearing mice, we measured the expression of the TNF ligand family member CD70 on the cell surface of DCs (29–31). In mice that were treated intravenously, DCs in both tumor-draining and nondraining LNs showed high expression of cell surface CD70, which indicates systemic activation of DCs (Fig. 4). In contrast, the CD70 expression on the cell surface of DCs in mice that received local treatment in montanide formulation was strongly upregulated in tumor-draining but not in nondraining LNs. Similar results were obtained with staining of the costimulatory molecule CD80 (B7.1). Treating mice with montanide alone or montanide containing control Ab did not mature DCs in the LN (data not shown). Thus, predominantly, the...
DCs in the draining LN of mice treated with the low dose of anti-CD40 in montanide are activated whereas the DCs in lymphoid organs distant from the tumor-draining area remain immature.

**Local treatment can eradicate a distant tumor**

We hypothesized that even though an Ab treatment is delivered locally, it could still be effective in eradicating metastasized (secondary) related (presenting the same tumor antigens) tumors due to the induction of a systemic CTL response. To test this hypothesis, we used a model for metastasis in which we inoculated groups of mice subcutaneously with tumor cells in the right flank (first tumor) except for 1 group, which was not inoculated. Eight days later, when tumors in the right flank were palpable, we inoculated all groups subcutaneously with tumor cells in...
the left flank (second tumor). Mice were treated the next day, either with high-dose anti-CD40 Ab intravenously or with low-dose anti-CD40 Ab in montanide, either injected locally close to the palpable primary tumor in the right flank or injected in the right flank where no tumor cells were inoculated. Tumor growth of tumors on both flanks was measured regularly, and the antitumor CTL response was analyzed in blood. As depicted in Figure 5, tumor-specific CD8+ T cells were observed in the blood of the systemically treated group and of the group that was treated locally near the right flank tumor. Tumor-specific CTLs were not demonstrable in the blood of mice that were treated subcutaneously in the right flank and were not inoculated with tumor cells at this site. Consistent with these findings, the growth of the secondary tumor on the left flank is strongly inhibited in the groups treated with high-dose systemic anti-CD40 Ab and the group treated with the low-dose anti-CD40 Ab in montanide, which was administered close to the tumor in the right flank (Fig. 5C and D; groups II and III). Mice that received no primary tumor and were injected with the low-dose anti-CD40 Ab in the right flank succumbed to rapid outgrowth of the secondary tumor (Fig. 5B and C; group IV). Taken together, these studies show that local treatment with anti-CD40 Ab in a slow-release formulation is effective in prompting systemic tumor-specific CTL expansion and eradication of distant related tumors.

Local treatment of primary tumors is capable of eradicating distant secondary tumors but not unrelated tumors

To substantiate that local treatment activates exclusively specific antitumor CTL responses that eradicate related (but not unrelated) distant tumors, thereby avoiding systemic T-cell activation, which may cause unwanted side effects such as immunopathology and autoimmunity, we decided to employ a combination of 2 unrelated tumors (AR6, mouse embryonic cell line expressing adenovirus protein E1A and EG7, lymphoma expressing OVA protein) in 1 experimental model. By utilizing these tumor cells as first and/or second tumors, we could investigate the tumor-clearing potential of CTLs activated by local anti-CD40 treatment and the role of tumor antigens presented at the local injection site (see scheme in Fig. 6). First, we established that local anti-CD40 treatment was successful in generating tumor-specific CTL responses and tumor eradication following EG7 tumor challenge (data not shown). Next, we challenged mice with 2 unrelated tumors according to scheme (Fig. 6B). We found that the related secondary tumor was completely eradicated when a specific CTL response was activated by the primary tumor (Fig. 6; group I) but grew out when unrelated tumors (expressing tumor antigens that were different from that of the second tumor) were employed as a first tumor (Fig. 6; group II). In mice that received EG7 as a primary tumor and were injected with both AR6 and EG7 tumor cells as distant tumors (Fig. 6; group III), the outgrowing tumor was a solitary AR6 tumor as determined by histology (Supplementary Fig. S3). In all cases, tumor eradication was accompanied by a systemic CTL response specific for the antigen present at the treatment site (Fig. 6C). These results indicate that local treatment with anti-CD40 Ab activates CTLs specific for tumor antigens that are present in the vicinity of the local treatment area and, in case of metastasis of related tumors, these CTLs possess potent systemic antitumor cytotoxicity.

Discussion

We here report a novel administration method for anti-CD40 agonistic Ab as a monotherapy against immunogenic tumors. By using a slow-release agent (montanide) to deliver the Ab close to the tumor-draining LN, we could induce the activation of tumor-specific CTLs as defined by expansion and differentiation into potent effectors and effusion to the systemic circulation. Importantly, we could also reduce the adverse side effects, including liver toxicity as observed with intravenous injections. This treatment is effective only when injected close to the tumor but is capable of activating a systemic CTL response that can eradicate tumors at distant locations. The systemic CTL response is specific for tumor antigens present at the site of treatment, which avoids unwanted systemic T-cell activation and its associated detrimental side effects such as immunopathology and autoimmunity.

Previously, we reported that both natural killer cells and CD4+ T cells are not critically involved in the antitumor effect of anti-CD40 Ab treatment against our tumor model but that cytotoxic CD8+ T cells play a crucial role (10, 22). Together with the knowledge that the tumor antigen was presented mainly by CD11c+ cells in the tumor-draining LN (22), led us to focus on targeting the CD40-activating
Ab to these areas in order to activate the tumor antigen-presenting APCs, which, in turn, activate the tumor-specific CTL. We hypothesize that activation of DCs in the LN is instrumental in the systemic antitumor response caused by our local treatment. Our finding that DCs in the tumor-draining LN after local treatment express CD70, whereas the DCs in nondraining LN do not, underlines the local induction of immune responses, which nevertheless induce a systemic antitumor CTL response that can eradicate distant related tumors.

Considering the presence of CD40 molecules on numerous cell types distributed through the body and their role in various processes, it is not unexpected that toxicity caused by systemic treatment with an agonist Ab against CD40 can occur, but thus far, this has been a largely ignored phenomenon in preclinical animal models. In addition, some groups reported that very high dosages of anti-CD40 induce deleterious effects on CD4 and CD8 T-cell responses (32, 33).

Several studies have been published on the effects of targeting the tumor or the tumor-draining LN, using tumor-specific antibodies or cellular vaccines producing cytokines and antibodies (34–37). Although these studies have successful outcomes, they are all technically challenging and require knowledge about tumor specificity. The method of administration as reported here is relatively straightforward and applicable to different types of immunogenic tumors. Jackaman and colleagues and we have previously shown that intratumoral injections of anti-CD40 led to eradication of local and distal tumors. However, these studies have not addressed the toxicity as described here (38).
using a slow-release delivery method to target anti-CD40 agonistic Ab to the tumor-draining area, we could drastically decrease the dose needed for effective antitumor CTL activation and significantly reduce Ab-mediated side effects. We found that toxicity, as measured by serum liver enzyme levels, in mice after systemic injection reaches plateau levels already at an injected dose of 10 μg whereas antitumor CTL activation is not effective at this dose, or even at 30 μg. We therefore conclude that systemic injection of agonistic CD40 Ab has no proper therapeutic window (unpublished observations). Our findings have important implications for the use of anti-CD40 Ab in the clinic, as CD40 agonist Ab therapy in a clinical trial was found to be associated with biological and antitumor activity but was hampered by dose-limiting toxicity (18). We agree that anti-CD40 Ab monotherapy, as described in this study, is not always capable of tumor eradication and that the use of combinatorial treatments will be beneficial, and propose that our superior administration method (i.e., delivery of a low-dose agonistic CD40 Ab in a slow-release formulation in the tumor-draining area) is also valuable when combining anti-CD40 Ab treatment with other agents.

In conclusion, this study shows that precision guiding of tumor-specific CTL by local delivery of immunostimulants to DCs cross-presenting tumor antigen constitutes a novel way to elicit systemic therapeutic CTL responses. This approach lends itself without difficulty to clinical exploratory trials because Montanide ISA-51 delivery is safe in human individuals (40) and because appropriate agonistic antibodies against human CD40 are available (18).
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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