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*J Immunol* 1998; 161:1901-1907; 
http://www.jimmunol.org/content/161/4/1901

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Antitumor Effect of CD40 Ligand: Elicitation of Local and Systemic Antitumor Responses by IL-12 and B7

Atsuo Nakajima, Tomohiro Kodama, Shinji Morimoto, Miyuki Azuma, Kazuyoshi Takeda, Hideo Oshima, Shin-ichi Yoshino, Hideo Yagita, and Ko Okumura

The interaction between CD40 ligand (CD40L, CD154) and its receptor CD40 has been implicated in the establishment of cell-mediated immunity as well as humoral immune responses. To examine the role of CD40L in eliciting antitumor immunity, we introduced murine CD40L gene into P815 mastocytoma (CD40L-P815). CD40L-P815 cells underwent prompt rejection when inoculated s.c. into syngenic DBA/2 mice or athymic BALB/c nu/nu mice, which was mediated by NK cells and dependent on endogenous IL-12. The primary rejection of CD40L-P815 cells in DBA/2 mice elicited CD8+ T cell-mediated protective and systemic immunity against parental tumor cells, which was induced by CD4+ T cells and endogenous B7. These results indicated a potent antitumor effect of CD40L that is mediated by potentiation of host Ag-presenting cell functions, and introduction of CD40L will be useful as a new strategy of immuno-gene therapy against tumors.


Tumor cells evade host immune surveillance by several strategies. First, tumor cells arise from the self and thus are ignored by the immune system that is tolerant to the self, except for some virally induced tumors in which viral Ags serve as targets. However, recent studies have indicated that not only some mutant peptides from oncogenic gene products but also kinds of self peptides serve as target Ags of tumor-reactive T cells (1–4). Second, some tumor cells evade the recognition by tumor-reactive T cells by down-modulating the expression of MHC class I molecules that present the tumor Ag (5, 6). Third, some tumor cells inhibit the activation of tumor-reactive T cells by secreting suppressive cytokines (7). Lastly, recent studies have highlighted the lack of costimulatory molecules as a predominant reason for the inefficient stimulation of tumor-reactive T cells. It has been well established that efficient elicitation of T cell-mediated immune responses requires not only the TCR-mediated signal upon Ag recognition but also a costimulatory signal provided by the CD40-CD40L interaction as well as important roles in cellular immunity (20, 21). It has been reported that either CD40- or CD40L-deficient mice could not resolve the Leishmania major infection (22–24) and that administration of anti-CD40L mAb could prevent acute graft-vs-host disease, cardiac allograft rejection, and experimental allergic encephalomyelitis (25–27). The CD40 stimulation has been demonstrated to induce the production of proinflammatory cytokines such as TNF-α, IL-8, and IL-12 from macrophages and DC (28–35). It also augments the APC activity of B cells, macrophages, and DC by up-regulating the expression of B7 and other costimulatory molecules (36–38). A recent report demonstrated critical involvement of the CD40-CD40L interaction in induction of protective antitumor immunity by vaccination with irradiated tumor cells (39). In addition, it has also recently been demonstrated that transgenic expression of CD40L in some tumor cell lines reduced their tumorigenicity and elicited protective immunity, while the mechanism by which CD40L exerted the antitumor effect in vivo has not been elucidated (40, 41).

In the present study, we compared the costimulatory mode of CD40L with that of B7 by characterizing the host responses to CD40L- or B7-transduced tumor cells. CD40L exhibited a potent antitumor effect, which was distinct from B7 and predominantly mediated by potentiation of host APC functions. Our results indicated that introduction of CD40L into tumor elicits more efficient antitumor response than that of B7.

Materials and Methods

Mice

Six- to eight-week-old female DBA/2 mice and C57BL/6 mice were purchased from SLC (Shizuoka, Japan). BALB/c nu/nu mice were purchased from Japan Bioscience Laboratories (Shizuoka, Japan). ICR nu/nu mice were purchased from Charles River Japan Laboratories (Yokohama, Japan). BALB/c nu/nu mice were purchased from SLAC (Tokyo, Japan). All mice were maintained in our animal facility at the Juntendo University School of Medicine, with a 12-h light/dark cycle. Mice were inoculated s.c. with 104 P815 mastocytoma cells, 104 MC38 colon carcinoma cells, or 106 P1907 leukemia cells. The Journal of Immunology, 1998, 161: 1901–1907.

Received for publication February 6, 1998. Accepted for publication April 17, 1998.

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1 This work was supported by grants from the Ministry of Education, Science, Sports and Culture and the Ministry of Health, Japan.

2 Address correspondence and reprint requests to Dr. Atsuo Nakajima, Department of Immunology, National Children’s Medical Research Center; CREST, Japan Science and Technology Corporation (JST), Tokyo, Japan; and Department of First Surgery, Faculty of Medicine, University of Tokyo, Tokyo, Japan.

3 Abbreviations used in this paper: CD40L, CD40 ligand; DC, dendritic cell; CTLA4Ig, murine CTLA-4 human Ig fusion protein.
from Charles River Japan (Atsugi, Japan). These mice were used in accordance with the guidelines of the Committee on Animals of the Juntendo University School of Medicine.

Tumor cells and transfectants

Murine CD40L cDNA was prepared by RT-PCR from total RNA of anti-CD3-activated D10.G4 T cell clone by using an oligonucleotide corresponding to the first six codons as the 5′ primer and to the last six codons as the 3′ primer, according to the published sequence (42). The 5′ and 3′ primers were tagged with XhoI or NotI site, respectively. After XhoI and NotI digestion, the PCR product was subcloned into pBluescript II SK+ (Stratagene, La Jolla, CA), and nucleotide sequence was confirmed by sequencing. The CD40L cDNA was then transferred into XhoI and NotI sites of the BCMGSneo expression vector. A murine mastocytoma cell line P815 of DBA/2 origin and a murine melanoma cell line B16 of C57BL/6 origin were transfected with murine CD40L cDNA in BCMGSneo by electroporation as described (43). Stable transfectants were obtained by selection in 1 mg/ml G418 (Sigma, St. Louis, MO), and the cells expressing CD40L were isolated by flow cytometry. The mock P815 or mock B16 cells were derived similarly but transfected with the vector without insert. B7-1-P815 cells were prepared as described previously (43).

Immunofluorescence and flow cytometry

Cells were first preincubated with anti-FcγR mAb (2.4G2, PharMingen, San Diego, CA) to block nonspecific binding of mAb to FcγR and then incubated with either phycoerythrin-conjugated or biotinylated mAbs for 30 min at 4°C. Biotinylated mAbs were detected with phycoerythrin-conjugated streptavidin (Becton Dickinson, San Jose, CA). mAb against the following Ags were obtained from PharnMingen: CD40L (MR1), CD40 (HM40–3), B7-1 (1G10), B7-2 (GL1), ICAM-1 (3E2), MHC class II (M5/114), and MHC class I (34–2–12). Murine CTLA-4 human Ig fusion protein (CTLA4Ig) was generously provided by Dr. Peter Lane (Basel Institute for Immunology, Basel, Switzerland). FITC-conjugated goat anti-human IgG Fc (absorbed with mouse Ig) was purchased from CalTag (South San Francisco, CA). The stained cells were analyzed on FACSscan (Becton Dickinson).

In vitro assay and ELISA

Peritoneal cells were isolated from DBA/2 mice and plated at 2 x 10^6 cells/well in RPMI 1640 containing 10% FBS, 1 mM l-glutamine, 0.05 mM 2-ME, and antibiotics. After a 2-h incubation at 37°C, plates were washed three times to remove nonadherent cells. Adherent cells (0.8–1.2 x 10^6/well, >90% CD11b^- as estimated by flow cytometry) were cocultured with mitomycin C-treated P815 or CD40L-P815 cells at the indicated numbers in the presence of 1 μg/ml anti-IL-10 (JES5–2A5) and 2 μg/ml indomethacin. After 20 h, cell-free culture supernatants were subjected to IL-12 p70-specific ELISA according to the protocol recommended by the manufacturer (PharMingen). In some experiments, adherent peritoneal cells were cultured with 2 x 10^6 cells of P815 or CD40L-P815 for 24 h and stained with CTLA4Ig followed by FITC-conjugated anti-human IgG Fc to evaluate B7 levels.

Tumor growth and in vivo Ab treatment

P815, CD40L-P815, or B7-1-P815 cells (2 x 10^3/mouse) were s.c. inoculated into the shaved back of DBA/2 or BALB/c nu/nu mice in a 0.1-ml volume of PBS. In some experiments, CD40L-P815 (2 x 10^3 cells) were preincubated with 100 μg of anti-CD40L mAb in vitro before inoculation. Similarly, 1 x 10^6 cells of B16 or CD40L-B16 were inoculated into C57BL/6 mice as described above. The mice were monitored for tumor growth weekly, and tumor size was determined by measuring two perpendicular diameters with a caliper. Some mice that had rejected the primary inoculation of CD40L or B7-1 transfectants were then challenged with 2 x 10^3 parental P815 cells or 1 x 10^6 L5178Y cells at the distant sites on day 30 after the primary inoculation. In some experiments, 2 x 10^5 cells each of CD40L-P815 and parental P815 were simultaneously inoculated at the same or distant sites. The results are expressed as mean ± SD of five to ten mice in each group.

For in vivo depletion of T cells, mice were i.p. administered with 1 mg each of anti-CD4 (GK1.5) and/or anti-CD8 (53.6.72) mAbs 1 day before the tumor inoculation. For depleting NK cells, 500 μg of anti-asialo GM1 Ab (Yamasu, Chiba, Japan) was administered i.v. 1 day before the tumor inoculation. In preliminary experiments, depletion of the respective populations was confirmed up to 7 days after the treatment. To block the B7-CD28 interaction, 250 μg each of anti-B7-1 (RM80) and anti-B7-2 (P03) mAbs (43, 44) were i.p. administered twice a week for 2 weeks starting at 1 day before the tumor inoculation. Endogenous IL-12 or IL-4 were neutralized by i.p. administration of 1 mg anti-IL-12 (C17.8, generously provided by Dr. G. Trinchieri, The Wistar Institute of Anatomy and Biology, Philadelphia, PA) or anti-IL-4 (1B11, PharMingen), respectively, twice a week for 2 weeks starting at day –1. In each experiment, equivalent an amount of rat IgG (Sigma) was administered as control and did not affect the tumor growth.

Results

Expression of surface molecules on CD40L-P815

We transfected the murine CD40L cDNA into murine mastocytoma P815 cells that had not expressed CD40L. Stable transfectants expressing high levels of murine CD40L (CD40L-P815) were identified by FACS analysis (Fig. 1). CD40L-P815 and parental P815 cells expressed comparable levels of MHC class I and ICAM-1 but did not express CD40, B7-1, or B7-2 (Fig. 1). We also analyzed the expression of these molecules on B7-1-P815 cells and obtained similar results except for the high expression of B7-1 (Fig. 1, and data not shown). No difference in the growth rate in vitro was observed between CD40L-P815 or B7-1-P815 and the parental P815 cells (data not shown).

CD40L-P815 stimulates IL-12 production and up-regulation of B7 by macrophages.

It has been known that the stimulation of APC such as macrophages or DC with CD40L leads to production of IL-12 and up-regulation of B7 molecules (28–38). To verify whether CD40L-P815 cells have such abilities, we stimulated peritoneal macrophages from DBA/2 mice with CD40L-P815 cells in vitro. As shown in Figure 2A, peritoneal macrophages produced IL-12 p70 when cocultured with CD40L-P815 but not with wild-type macrophages.
P815 cells. Up-regulation of B7 molecules by CD40L-P815 cells was also observed when cocultured with CD40L-P815 cells. After 20 h, cell-free culture supernatants were subjected to IL-12 p70-specific ELISA. Peritoneal macrophages were cocultured with the indicated numbers of P815 (empty bars) or CD40L-P815 cells. Up-regulation of B7 molecules by CD40L-P815 cells was functional in activating stimulatory macrophages.

Tumor rejection induced by CD40L

To examine the effect of CD40L on tumor growth in vivo, P815 or CD40L-P815 cells were inoculated s.c. into syngeneic DBA/2 mice. B7-1-P815 cells were also inoculated for comparison. Although either the parental P815 or mock P815 cells grew progressively and killed the mice within 50 days, the CD40L-P815 cells were completely rejected without forming a palpable tumor (Fig. 3A). CD40L-B16 cells, but not parental B16 cells, were also rejected without transient growth in all mice, similar to the rejection of CD40L-P815 cells (data not shown). The rejection of CD40L-P815 was due to the local expression of CD40L, since the CD40L-P815 cells inoculated on day 0 were s.c. challenged with parental P815 cells or DBA/2-derived T-cell lymphoma L5178Y cells on day 30. As shown in Figure 4A, parental P815 cells, but not L5178Y cells, were rejected by these recipients, indicating the development of P815-specific protective immunity. In contrast, such a protective immunity was not developed in BALB/c nu/nu mice even though they still rejected the secondary challenge with CD40L-P815 (Fig. 4B). Depletion of CD4+ but not CD8+ T cells at the time of primary CD40L-P815 inoculation consistently abrogated the protection, indicating that CD4+ T cells were required for the induction of protective immunity (Fig. 4C). Since the immunized CD40L-P815 did not express MHC class II (Fig. 1), this implied the contribution of host APC expressing MHC class II to the development of protective immunity. We also performed the cell depletion study at the time of secondary challenge with P815. Depletion of CD8+ T cells, but not CD4+ T cells or NK cells, abrogated the rejection of P815 cells (Fig. 4D), indicating that the effector cells were CD8+ T cells, as expected from the lack of MHC class II on P815 cells.

Induction of systemic protective immunity is B7 dependent

We next verified the systemic therapeutic effect of the CD40L vaccination by inoculating CD40L-P815 and P815 cells simultaneously at the distant sites on the back of a DBA/2 mouse. As shown in Figure 4E, P815 cells regressed after transient formation of tumor, and this inhibition was abrogated by the depletion of CD4+ T cells. Such an inhibition on the distant P815 was not observed in BALB/c nu/nu mice (Fig. 3G). These results indicated that the T cell-mediated systemic immunity elicited by CD40L-P815 was efficient for eradicating the wild-type tumors at distant sites. The transient growth and subsequent regression of P815 cells observed in Figure 4E was very similar to those observed with B7-1-P815 (Fig. 3A), both of which were mediated by T cells.
Elicitation of T cell immune responses generally requires a co-stimulation by APC, but the CD40L-P815 cells lacked B7 (Fig. 1). We then examined the contribution of B7 on host APC. Administration of the anti-B7-1 and B7-2 mAbs did not affect the primary rejection of CD40L-P815 (Fig. 3A), but it did abrogate the elicitation of protective immunity against subsequent challenge with P815 (Fig. 4C). The anti-B7-1 and anti-B7-2 treatment also abrogated the systemic inhibition of P815 cells, while sparing the rejection of CD40L-P815 (Fig. 4E). These results indicated that the elicitation of T cell-mediated systemic immunity by CD40L was dependent on B7 molecules that were potentially up-regulated on host APC by CD40L.

**Discussion**

In the present study, we demonstrated that s.c. inoculation of CD40L-transduced tumor cells led to both local and systemic effects. The primary rejection of CD40L-P815 did not require T cells but was mediated by NK cells and dependent on IL-12. In contrast, the development of CD8\(^+\) T cell-mediated systemic protection against wild-type P815 required CD4\(^+\) T cells and was dependent on B7. Since the CD40L-P815 cells did neither produce IL-12 nor express B7 and MHC class II, a critical contribution of host APC was suggested.

In vivo depletion of lymphocyte subpopulations indicated that NK cells were essential for the primary rejection of CD40L-P815 cells. However, a direct activation of NK cells by CD40L was unlikely, since NK cells did not express CD40, as estimated by flow cytometry, and the CD40L-P815 cells did not exhibit increased susceptibility to NK cells in vitro (data not shown). We then assumed an indirect mechanism for the NK cell activation and found a critical contribution of endogenous IL-12. This was also confirmed by the result that depletion of NK cells or administration...
of anti-IL-12 mAb also abrogated the rejection of CD40L-B16 cells (data not shown). It has been shown that CD40L-stimulated macrophages and DC secrete not only IL-12 but also TNF-α, IL-8, and macrophage inflammatory protein (MIP)-1α (28–35), which might be responsible for NK cell infiltration at the site of CD40L-P815 inoculation. It seems most likely that CD40L expressed on tumor cells acted on dermal macrophages and/or epidermal Langerhans cells to produce IL-12, which activated infiltrating NK cells to lyse the tumor cells. This indirect activation scenario is consistent with our observation that the NK cell-mediated effect was locally limited but could also eliminate bystander tumor cells that did not express CD40L (Fig. 2G). In our preliminary experiments, CD40L-P815 and P815 cells were equally susceptible to IL-12-activated NK cells in vitro (data not shown). The bystander effect exerted by the CD40L transfectants would be beneficial for eradicating a tumor mass by CD40L gene transduction since only a part of tumor cells must be transduced. A similar bystander effect has recently been reported by Grossmann et al. (41). They demonstrated that expression of CD40L by less than 1.5% of neuro-2a neuroblastoma cells suppressed the tumor growth, although they did not address the contribution of NK cells or IL-12.

The primary rejection of CD40L-P815 cells was T cell independent, but it left CD8+ T cell-mediated protective immunity against subsequent challenge with wild-type P815 cells, which required CD4+ T cells and B7 for development. Although CD4+ T cells were required for the induction of protective immunity, P815 cells did not express class II MHC molecules, suggesting the importance of CD8+ T cells as effector cells. In fact, the protection was abrogated when the CD40L-P815-primed mice were depleted of CD8+ T cells at the time of challenge with wild-type P815 cells.

These results suggest a scenario that host APC was stimulated by CD40L to up-regulate B7 and activated CD4+ T cells, which in turn helped the development of CD8+ T cells. Requirement of CD4+ T cells for the generation of cytotoxic T cells against class II negative tumor was also recently reported by Ossendorp et al. (45). This T cell-mediated effect was systemic and could eradicate wild-type tumor at a distant site. Therefore, vaccination with the CD40L-transduced tumor cells would have a potential to eradicate
multiple metastases systemically. A similar elicitation of T cell-mediated protective immunity by CD40L-transfected tumor cells has recently been reported by Dilloo et al. (40) and Grossmann et al (41). In the former case, up-regulation of B7 and MHC molecules was noted on the CD40" tumor cells themselves and, in the latter case, in splenic APC, although they did not directly address the contribution of B7.

It may be worth noting that some reports have suggested a direct costimulatory effect of CD40L transfectants on human T cell activation (46). However, in our preliminary experiments, CD40L-P815 cells did not significantly costimulate the proliferation of anti-CD3-stimulated CD4+ T cells as compared with P815 cells (data not shown), arguing against the direct costimulatory effect of CD40L. It has been shown that CD40L stimulated macrophages and DC to up-regulate the expression of B7. Therefore, it seems likely that CD40L expressed on P815 cells acted on dermal macrophages and/or epidermal Langerhans cells to up-regulate B7. Then, these cells that took up the putative tumor Ag from the debris of CD40L-P815 cells migrated into the regional lymph node and activated the reactive T cells. Alternatively, CD40L-P815 cells might directly migrate into the regional lymph node where they activated host APC, since such a migration of P815 cells has been shown in a recent report (47). While we used poorly immunogenic P815 cells that did not spontaneously regress in syngenic hosts, it has been reported that the spontaneous rejection of highly immunogenic P815 variants was also dependent of B7 on host APC (48).

Our present results indicated a potent immunopotentiating effect of CD40L that appeared to be predominantly mediated by activation of host APC. It has been suggested by some reports that the CD40L molecules may directly transmit a costimulatory signal of CD40L that appeared to be predominantly mediated by activation of host APC. It has been suggested by some reports that the CD40L molecules may directly transmit a costimulatory signal of CD40L that appeared to be predominantly mediated by activation of host APC.

References


