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Regulation of CD40L expression on natural killer cells by interleukin-12 and interferon γ: its role in the elicitation of an effective antitumor immune response

Abstract Effector cell functions are regulated by a number of positive signals for the mediation of antitumor immunity. The CD40 and CD40 ligand (CD40L) interaction has been implicated in the generation of effective cell-mediated and humoral immune responses, where cytokines have been shown to play a significant role in the expression of these molecules. Our earlier studies have shown that spontaneous regression of a rat histiocytoma transplanted s.c. is mediated by CD8+ CD3− NK cells. The CD40-CD40L mediation during tumor regression was of interest. Tumor-transplanted animals showed enhanced expression of CD40L on natural killer (NK) and T cells, when compared to cells from normal animals. CD40 expression on AK-5 tumor cells was also induced after s.c. transplantation. Administration of anti-(interleukin-12) (anti-IL-12) and anti-(interferon γ) (anti-IFNγ) antibodies in tumor-bearing animals showed down-regulation of the expression of CD40L on NK and T cells with simultaneous inhibition of cytotoxic activity of NK cells, cytokine release and the production of antitumor antibody. Naive NK cells, when co-cultured with fixed AK-5 cells, were induced to express CD40L. CD40L expression modulated the immune response exerted by NK cells, in part by the activation of nuclear factor kB (NF-kB). Furthermore, the signaling via CD40L through the use of anti-CD40L antibody promoted the in vitro activation of cytotoxic as well as NF-kB binding activity in NK cells from tumor-transplanted animals. These observations demonstrate that the expression of CD40L by the effector cells is regulated by IL-12 and IFNγ, and could effectively modulate the NK-cell-mediated immune response during the regression of AK-5 tumor.

Key words Cytokines · CD40-L expression · NK cells · Antitumor activity

Introduction

CD40, a 50-kDa glycoprotein is a member of the tumor necrosis factor (TNF) receptor family, which includes the TNF receptor, nerve growth factor receptor and CD95. It was initially identified on the surface of B cells [36] and is constitutively expressed on a variety of other leukocytes, such as macrophages, dendritic cells and T cells [1, 32, 33]. CD40 ligand (CD40L, CD154), a counter-receptor for CD40, also a membrane glycoprotein with a molecular mass of approximately 39 kDa, induced on T cells upon activation [10]. Eosinophils, B cells and mast cells could also be induced to express a functional CD40L [13, 14]. The CD40-CD40L interaction has been shown to be necessary for the T-cell-dependent B cell activation [10]. The efficient presentation of antigen by CD40-positive dendritic cells and the production of IL-2 stimulated the T-lymphocytes to transmit signals to B cells. Studies with monoclonal antibodies have shown that CD40 plays a critical role in the activation, proliferation and differentiation of B cells, including early gene expression [7], cell adhesion [2] and Ig isotype production [12]. In addition CD40-CD40L intercellular interaction provided the necessary second signal to drive cytokine production and clonal expansion of naive T lymphocytes activated through their T cell receptor complex [8, 16]. This interaction is similar to the well-known costimulatory pathway comprised of CD28 on the T cells and its receptor B7.1 and B7.2 on the antigen-presenting cells [17].

Cytokines like interferon γ (IFNγ) play an important role in the induction of expression of CD40 and CD40L on cells [41]. The production of IFNγ by natural killer (NK) and T cells is induced by interleukin-12 (IL-12) [40], which is generated after the interaction between the activated T cells and antigen-presenting cells via the CD40-CD40 ligand [37]. Previous studies have also shown the expression and activity of DNA-binding proteins like nuclear factor kB (NF-kB), NF-AT and AP-1 constituents [11] upon CD40 stimulation. NF-kB is involved in regulating the expression of a number of
genes, including those encoding the Ig light chain, IL-2, IL-6 and c-myc [21], and is found to be associated with an inhibitor IκB [26]. Cellular stimulation resulted in the release of NF-κB from IκB and its subsequent translocation into the nucleus [31]. Similarly, crosslinking of monoclonal antibodies to CD40 leads to enhanced NF-κB activity in B cells [3].

AK-5 tumor regression in s.c. transplanted animals is mediated by CD8⁺ CD3⁻ NK cells mainly through antibody-dependent cellular cytotoxicity [4]. NK cells resemble T cells in many aspects as they share the expression of several surface molecules [27]. The cytolytic activity of both NK and T cells is enhanced by IL-12, which also promotes the development of naive CD4⁺ T cells into type 1 effector cells [40, 37]. We have previously analyzed the interaction between the B7 receptor family proteins on tumor cells and their ligand CD28 on NK cells being required for an effective antitumor immunity [35]. We have also investigated the role played by the ligation between lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) during the activation of NK cell function [19]. In addition, the production of IL-12 and IFNγ by the host immune cells and the up-regulation of the T-helper 1 (Th1) type of cytokine response was found to be essential during the spontaneous regression of AK-5 tumor in syngeneic hosts [30]. Our present study demonstrates the expression of CD40L on NK and T cells after s.c. tumor transplantation, which is involved in the activation of effector function, production of cytokines, NF-κB binding activity and mediation of humoral immune response. The expression of CD40L by NK and T cells is regulated by IL-12 and IFNγ, since in vivo administration of anti-IL-12 and anti-IFNγ antibodies caused inhibition of CD40L expression on NK and T cells and suppression of cytotoxic activity of NK cells. In addition, in vitro activation of NK cells resulted in enhanced expression of CD40L by NK cells. The cytotoxic as well as NF-κB activity in NK cells was up-regulated after treatment with anti-CD40L antibody in vitro. Thus, our studies suggest a significant role for CD40 and CD40L molecules in NK cell activation and generation of an effective immune response during tumor rejection.

### Materials and methods

#### Animals and tumor

AK-5 tumor was maintained as ascites in the inbred colony of Wistar rats [22]. The solid tumors were developed by injecting 5 x 10⁵ AK-5 cells subcutaneously in rats 6–8 weeks old. The animals with s.c. transplanted tumor that had rejected the tumor were referred to as immune.

#### Antibodies and reagents

Monoclonal antibodies anti-(murine IL-2) (S4B6), anti-IFNγ (XMG1.2), anti-IL-4 (11B11), anti-IL-10 (SXC1), anti-IL-12, p70 (C17.8), anti-CD40 and anti-CD40L (Pharmingen, USA) and isotype-matched antibodies, i.e., IgG1 to IFNγ and CD40L, and IgG2a to IL-12 and CD40, were used in this study. We have previously shown the ability of mouse monoclonal antibodies to recognize rat cytokines [30]. Anti-(rat IgG), anti-(mouse IgG) or anti-(hamster Ig), conjugated to horseradish peroxidase (HRPO) and fluorescein isothiocyanate (FITC) were used as secondary antibodies. Rat recombinant IFNγ (Boehringer Mannheim, Germany), anti-(murine IL-1β) and anti-TNFα antibodies were from Genzyme, Cambridge, USA. RPMI-1640 medium (Sigma, St. Louis, Mo., USA) supplemented with 10% fetal calf serum (FCS; Sigma, USA), 100 U/ml penicillin and 50 μg/ml streptomycin (Sigma) was used for cell culture. Anti-AK-5 antibody was prepared from AK-5-tumor-bearing rats, according to the procedure described previously [35].

In vivo administration of anti-IL-12 and anti-IFNγ antibodies

Ten animals per group were injected i.p. with anti-IL-12 and anti-IFNγ mAb separately and also in combination (1 mg Ig/animal for each injection) on days 0, 1, 3, 5, 7 and 9 after tumor transplantation. The positive controls were injected with the same amount of isotype-matched antibodies. Hybridoma clones were grown in RPMI-1640 medium containing 10% FCS (RPMI-FCS). The culture supernatants were concentrated with the Minitan Millipore system (Bedford, Mass.) and affinity-purified on protein-A–Sepharose; the eluted immunoglobulin, after dialysis, was used for in vivo injections.

#### Tumor growth

Tumor growth was regularly monitored by measuring tumor diameters from day 6 after s.c. transplantation of AK-5 cells. The maximum size of tumor induced was calculated at different times and the appearance of necrosis in the tumor mass indicated the initiation of rejection.

#### Serum samples

Blood (0.5 ml) was collected from the animals through the retro-orbital plexus on alternate days and the sera were separated by centrifugation and stored at −20 °C until further use.

#### Preparation of NK and T cells

Splenocytes were prepared from normal, immune and antibody-treated animals on day 15 after tumor transplantation. Macrophages were separated by allowing them to adhere to polystyrene for 1 h. The floating cells were treated with Dynal magnetic beads coated with anti-NKR-P1 mAb 3.2.3 (Endogen Inc., USA). NK cells were separated from the beads by using Detach-a-bead (Dynal, Oslo, Norway) and the unbound fraction was passed through a nylon-wool column. Nylon-wool-non-adherent cells were used as T cells. The purity of the cell preparations was tested by flow cytometry using mAb 3.2.3 and OX-19 (Serotec, UK) for NK and T cells respectively, and the preparations with more than 95% positive cells were used in these experiments.

In vitro activation of NK cells

Formaldehyde-fixed AK-5 cells were obtained by suspending washed AK-5 cells in 3% formaldehyde for 30 min at room temperature. Fixed cells were washed thoroughly with phosphate-buffered saline (PBS) and used for the activation of NK cells. Freshly isolated normal NK cells were cocultured with fixed AK-5 cells at an E:T ratio of 50:1 for 24 h. The NK cells were then separated using mAb-3.2.3-coated Dynal beads, and used in