ANTICANCER RESEARCH *24*: 2713-2716 (2004)

Expression of CD40 Ligand in CD40-positive Murine Tumors Activates Transcription of the Interleukin-23 Subunit Genes and Produces Antitumor Responses

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Abstract. *CD40-positive dendritic cells (DCs) are stimulated with CD40 ligand (CD40L) and subsequently secrete a number of cytokines including interleukin (IL)-23, which is involved in cell-mediated immune responses. Expression of CD40 ligand (CD40L) on tumors can activate host immune systems and produce antitumor effects against the tumors. We examined a possible mechanism of the antitumor responses: tumor cells expressing CD40 can transcribe DCs-derived cytokine genes by the expressed CD40L. For the purpose, CD40-positive A11 and -negative P29 murine lung tumors cells, both of the same origin, were transfected with the CD40L gene (A11/CD40L and P29/CD40L). The growth rate in vitro of A11/CD40L and P29/CD40L cells was not different from that of the respective parent tumors; however, the growth in vivo of A11/CD40L tumors in syngeneic mice was significantly retarded and the growth retardation of P29/CD40L tumors was marginal. Transcription of the p40 and p19 genes, IL-23 subunit genes, was up-regulated in A11/CD40L cells compared with parent A11 cells, whereas this up-regulation was not observed in P29/CD40L cells. Since expression of IL-23 in tumors can produce antitumor effects, the present data suggest that the CD40/CD40L interaction can activate cytokine transcripts in certain tumors and consequently contribute to antitumor responses.*

Forced expression of foreign genes in a tumor can produce antitumor effects against the tumor by augmenting host immune responses (1). In particular, cytokines that promote differentiation of naive T cell into T helper type 1 (Th1), are effective in activating cell-mediated immunity (2). Dendritic cells (DCs) play a crucial role in an antigen presentation process: capture of putative tumor antigens followed by expression of the antigens on their cell surface (3). Immature DCs, after the acquisition of tumor antigens, must be activated to become mature to present the antigens to T cells. This activation is mediated by a number of stimulatory signals such as tumor necrosis factor-α and lipopolysaccharide. CD40 is expressed on DCs and stimulation of CD40-mediated signals, by anti-CD40 antibody and soluble CD40 ligand (CD40L), is a pathway to activate DCs (3). Mature DCs then produce a number of cytokines including heterodimeric cytokines such as interleukin (IL)-12, IL-23 and IL-27 (4, 5): IL-12 is composed of the p35 and the p40 subunits, IL-23 consists of the p35-related p19 and the same p40 molecules and IL-27 is comprised of the p28 and the EBI3 molecules. Although the respective receptors are differentially expressed on T cells (5), the cytokines coordinately promote Th1 development (4, 5). The CD40/CD40L interaction on DCs is thereby one of the early processes to initiate cell-mediated immunity.

Previous studies showed that expression of CD40L in tumors achieved antitumor effects (6). The mechanism includes cell growth inhibition that is partly due to enhanced apoptosis, which is evidenced by transduction of CD40L in malignant hematopoietic cells (6). Human solid tumors also ectopically expressed CD40 and ligation of CD40 inhibited proliferation of tumor cells, although the CD40/CD40L interactions in a certain tumor supported cell survival (7). Another mechanism of the CD40-mediated antitumor effects is activation of immune responses (6). CD40 ligation enabled hematological malignant cells to secrete a number of cytokines and chemokines, which increased immune responses against the malignant cells. Although the CD40L-mediated immune responses are primarily restricted in hematopoietic cells, expression of CD40L in solid tumors also enhanced cell-mediated immunity against the tumors. Presumably, DCs that migrated into CD40L-

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Key Words: CD40, CD40 ligand, IL-23.

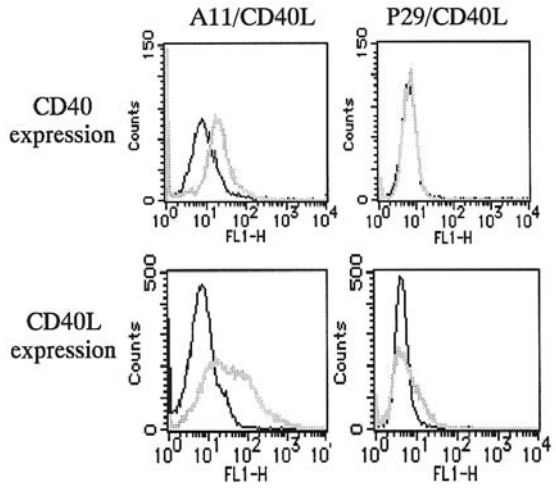

Figure 1. *Expression of CD40 and CD40L on A11/CD40L and P29/CD40L cells (thin lines). Staining profiles with control antibody (thick line) are also shown.*

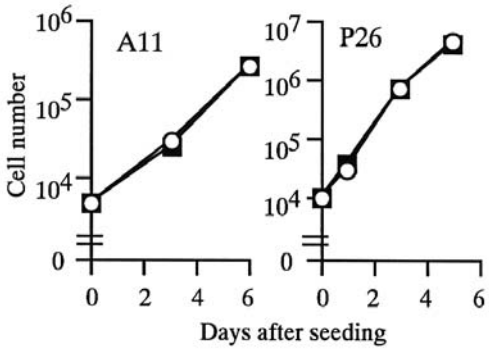

Figure 2. *In vitro proliferation of parent (open circle) and CD40L-expressed A11 and P29 cells (closed square). Standard error bars are too small to be described.*

expressed tumors were activated to present tumor-associated antigens and consequently initiated T cell-mediated immunity.

In this study, we examined the influence of forced expression of CD40L in CD40-positive murine lung carcinoma cells and found that the tumor cells produced antitumor effects and up-regulated the *p40* and the *p19* genes of IL-23. Since expression of IL-23 in tumors achieved antitumor effects (8, 9), the up-regulation of cytokine genes in tumors can be an alternative mechanism for CD40 ligation-mediated antitumor responses against solid tumors.

Materials and Methods

Cells and mice. A11 and P29 cells were established from Lewis lung carcinoma (10). They were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. Syngeneic C57BL/6 mice (6-week-old female) were purchased from Japan SLC (Hamamatsu, Japan).

Establishment of CD40L-expressed cells. A11 cells were transfected with the full-length mouse CD40L cDNA and then G418 (Life Technologies, Gaithersburg, MD, USA)-resistant cells were selected. They were examined for their expression of CD40L with flow cytometry. G418-resistant A11 cells transfected with vector DNA pMKITneo were used as a control.

Flow cytometry. Tumor cells were incubated with fluorescein-isothiocyanate (FITC)-conjugated anti-CD40 (PharMingen, San Diego, CA, USA) or anti-CD40L antibody (PharMingen) at room temperature for 20 min. Isotype-matched control antibody labelled with FITC was used for the background staining. The stained cells were analyzed with FACScan (Becton Dickinson, Mountain View, CA, USA) with CellQuest software (Becton Dickinson).

In vitro growth. Cells (1 x 10^4) were seeded in 6-cm dishes and counted on days 1, 4 and 6.

Animal experiments. Parent or transfected cells (2 x 10^5) were subcutaneously inoculated into mice. Tumor volumes were calculated according to the formula, tumor volume = 0.5 x A x B^2, where A and B are the larger and the smaller diameters, respectively. Statistical analysis was performed by one-way analysis of variance.

Reverse transcriptase-polymerase chain reaction (RT-PCR). First-strand cDNA was synthesized with RNA from cultured cells. Amplification of an equal amount of respective cDNA was performed for 30 cycles (19 cycles for β-actin) with the following primers and conditions: for *p35* gene expression, forward (5'-ACC TGCTGAAGACCACAGATG-3') and reverse (5'- TTTCACTC TGTAAGGGTCTGC-3') primers, and 30 sec at 92˚C for denaturation/30 sec at 57˚C for primer annealing/1 min at 75˚C for primer extension: for *p40* gene expression, forward (5'-CCAGAGA CATGGAGTCATAG-3') and reverse (5'-GGGTCTGGTTTGA TGATGTC-3') primers, and 15 sec at 94˚C/30 sec at 60˚C/1 min at 72˚C; for *p19* gene expression, forward (5'-CACAGAGCCAG CCAGATCTGAGAAGC-3') and reverse (5'-CCATGGGAACCT GGGCATCCTTAAGC-3') primers, and 15 sec at 94˚C/30 sec at 60˚C/1 min at 72˚C; for *p28* gene expression forward (5'-CCTGACATGGGCCAGGTGACAGGAGACC-3') and reverse (5'-TCACTCGAGTTAGGAATCCCAGGCTGAG-3') primers, and 30 sec at 92˚C/30 sec at 60˚C/1 min at 72˚C; for *EBI3* gene expression, forward (5'-GCCACAGAGCATGTCCAAGCTGCT CTTC-3') and reverse (5'-TCAGGATCCTCAGGGCTTATGG GGTGCAC-3') primers, and 30 sec at 92˚C/30 sec at 62˚C/1 min at 72˚C; for *β-actin* gene expression, forward (5'-ATGGATGACG ATATCGCT-3') and reverse (5'-ATGAGGTAGTCTGTCAGGT-3') primers, and 5 sec at 95˚C/10 sec at 54˚C/70 sec at 72˚C.

Results and Discussion

Establishment of CD40L-expressed tumors. Murine lung carcinoma A11 but not P29 cells expressed CD40 (Figure 1), although these cell lines were derived from Lewis lung

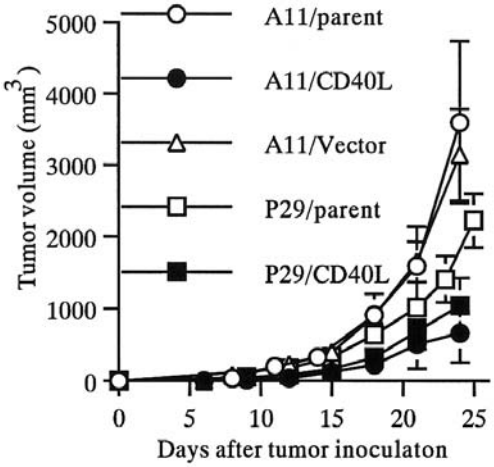

Figure 3. *In vivo growth of parent and transfected tumors in syngeneic mice (n=7). Vector DNA transfected-A11 cells (A11/Vector) were used as a control and standard error bars are also shown.*

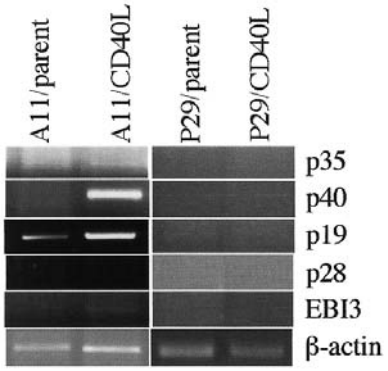

Figure 4. *Expression of cytokine genes in CD40L-expressed tumor cells. RNA extracted from parent and CD40L-expressed tumors were analyzed for cytokine gene expression with RT-PCR. The β-actin gene was used as a control.*

carcinoma. We transfected the *CD40L* gene in these cells and established G418-resistant cells (A11/CD40L and P29/CD40L). Both transfectants expressed CD40L with a different expression level (Figure 1). The proliferation *in vitro* of CD40L-expressing cells was not different from that of the respective parent cells (Figure 2). Expression of the class I antigens of major histocompatibility complexes on A11 and P29 cells was undetectable and remained unchanged after the CD40L gene transfer (data not shown).

Production of antitumor effects and cytokines. We examined whether expression of CD40L in A11 and P29 produced antitumor effects *in vivo*. The growth of A11/CD40L tumors inoculated in syngeneic mice was retarded compared with that of parent or vector DNA-transfected cells (day 24, $p<0.01$, Figure 3). In contrast, the growth retardation of P29/CD40L tumors was marginal compared with that of P29 tumors (day 23, $p<0.05$, Figure 3). Two out of seven mice inoculated with A11/CD40L cells rejected the tumors, whereas none of the mice rejected P29/CD40L cells. The antitumor effects produced by A11/CD40L cells were thereby stronger than those by P29/CD40L cells, which may partly be attributed to the expression level of CD40L.

We examined the expression of cytokine genes in these tumor cells, because CD40 ligation induced the expression in DCs. Expression of the *p40* gene, a shared subunit gene between IL-12 and IL-23, was detected in A11/CD40L but not in A11 cells (Figure 4). The *IL-12p35* gene was expressed in both A11 and A11/CD40L cells to a similar extent. In contrast, expression of the *IL-23p19* gene was greater in A11/CD40L than in A11 cells. The *p28* and the *EBI3* genes, IL-27 subunit genes and the *IFN-γ* gene (data not shown) were not expressed in A11 or A11/CD40L cells. None of the cytokine genes tested was detected in P29 or P29/CD40L cells.

CD40 ligation appeared to play a growth-inhibitory role for tumor cells *in vitro* and *in vivo* through a number of mechanisms including apoptosis induction, enhanced expression of adhesion and costimulatory molecules, and increased T cell-mediated cytotoxicity (6). Immune responses generated by CD40 ligation are primarily linked with maturation of DCs. The maturation process is accompanied by the secretion of cytokines, which are indispensable for T cell differentiation and activation (3-5). Recently, novel heterodimeric cytokines, IL-23 and IL-27 (11, 12), have been identified and they constitute a new cytokine family together with IL-12 (4, 5). Moreover, secretion of IL-23 as well as IL-12 from tumors induced systemic immunity against the tumors, which was mainly mediated by CD8-positive T cells (8, 9). In the present study, we demonstrated that the forced expression of CD40L induced expression of IL-23 genes, *p19* and *p40*, in CD40-positive A11 cells but not in CD40-negative *p*29 cells. Since expressed CD40L in A11 cells did not influence cell proliferation *in vitro*, we speculate that CD40 ligation-mediated IL-23 production from A11/CD40L tumors can contribute to the generation of immune responses against the tumors. Growth of CD40-negative P29 tumors was also retarded and thereby immunological responses against CD40L-expressed P29 tumors were independent of cytokine production but mediated by the interaction between immunocompetent cells and the tumors. The present study implies that cytokine production by a CD40-positive tumor is one of the mechanisms for the CD40L-mediated antitumor effects in addition to the immune response induced by activated DCs.

Acknowledgements

We thank Dr. Jiyang O-Wang (Laboratory for B Lymphocyte Function, Research Center for Allergy and Immunology, RIKEN Yokohama Institute, Japan) for his valuable advice. This work was supported by a Grant-in-Aid for the COE research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a Grant-in-Aid for scientific research from the Japan Society for the Promotion of Science.

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Received June 24, 2004
Accepted July 13, 2004