An oral CD40 ligand gene therapy against lymphoma using attenuated Salmonella typhimurium

Mitsuyoshi Urashima, Hideaki Suzuki, Youki Yuza, Masaharu Akiyama, Noriko Ohno, and Yoshikatsu Eto

CD40 ligand (CD40L) has a great potential as a novel treatment for B-cell lymphoma (BCL). It has previously been demonstrated that a nonvirulent strain of Salmonella typhimurium mutant (ST) can be used not only as a vehicle in oral genetic immunization via the intestinal mucosa, but also as an enhancer of interferon γ- and tumor necrosis factor α-mediated immunity. After confirming that human CD40L can up-regulate expression of Fas, B7-1, and B7-2 molecules on murine BCL cells in vitro, we transfected the human CD40L gene into S typhimurium mutant (ST40L), which was administrated orally to determine whether it was able to prevent the growth of BCL in mice. Expression of human CD40L was confirmed immunohistochemically with protein being detected in the Peyer’s patches of mice immunized with ST40L. Moreover, human soluble CD40L had been detectable until 7 to 8 weeks after oral administration of ST40L. Although ST alone exhibited some protective effects, ST40L demonstrated a significantly greater protection against the development of CD40 positive BCL compared with the control. In the surviving mice that had been treated with ST40L, a small and hard nodule was formed at the injection site, which was found to be composed of infiltrating lymphocytes expressing Fas ligand. These results have the potential to be a simple, effective, and above all, safe immune-gene therapy against BCL.

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Materials and methods

Cells and mice

A20 is a BCL cell line derived from BALB/c mice and expressing CD40 as well as the major histocompatibility complex (MHC) class I and class II h-2d, IgG, and Fc receptor (American Type Culture Collection [ATCC], Rockville, MD). The level of B7-2 on the surface of A20 cells was observed to be low with B7-1 being undetectable. The wehi3 leukemia

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A20 cells were cultured with NIH3T3/vt (control) and NIH3T3/CD40LT (CD40L) cells (100 A20 cells/1 NIH3T3 cell) for 48 hours in 10% FBS + RPMI1640. Cells were stained with FITC conjugated antibodies against mouse Fas, B7-1, and B7-2 molecules. Antibody-coated cells were enumerated by flow cytometric analysis. Isotype control antibody staining were adjusted to be less than 5%, and percentage of positive cells were calculated. Intensity was judged according to previous report.23

### Table 1. Effect of human CD40L on expressions of Fas, B7-1 and B7-2

<table>
<thead>
<tr>
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<th>Fas</th>
<th>B7-1</th>
<th>B7-2</th>
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<tbody>
<tr>
<td>NIH3T3/vt</td>
<td>45% (+)</td>
<td>5% (-)</td>
<td>23% (+)</td>
</tr>
<tr>
<td>NIH3T3/CD40LT</td>
<td>94% (+)</td>
<td>39% (+)</td>
<td>74 (++)</td>
</tr>
</tbody>
</table>

A20 cells were cultured with NIH3T3/vt (control) and NIH3T3/CD40LT (CD40L) cells (100 A20 cells/1 NIH3T3 cell) for 48 hours in 10% FBS + RPMI1640. Cells were stained with FITC conjugated antibodies against mouse Fas, B7-1, and B7-2 molecules. Antibody-coated cells were enumerated by flow cytometric analysis. Isotype control antibody staining were adjusted to be less than 5%, and percentage of positive cells were calculated. Intensity was judged according to previous report.23

### Salmonella strain and CD40L

The auxotrophic ST aroA- strain SL5000 was kindly donated by Dr Bruce A. D. Stocker (Stanford University School of Medicine, Stanford, CA) and used as a gene carrier.20 The full-length human CD40L gene was cloned into pCDL-SR using a CMV promoter (ATCC).21 The auxotrophic ST aroA- strain SL5000 was grown in 100 mL L broth (Sigma Diagnostics, St Louis, MO) to A600 of 0.6, chilled on ice, and harvested by centrifugation (15 minutes, 1000g at 4°C). The pellet was suspended in a final volume of 200 µL in 10% glycerol. Aliquots (40 µL) were mixed with 1 to 2 µL DNA in a chilled microcentrifuge tube and transfected to chilled cuvettes (0.2 cm electrode gap). A single pulse of 12.5 kV/cm (2.5 kV, 200 O, 25 µF) was applied and 1 mL of prewarmed SOC was immediately added as previously reported.24 The bacteria were transferred to 17 x 100 mm polypropylene-tubes and shaken for 1 hour at 37°C before being plated onto LB agar with ABPC (50 ng/mL). Groups of 6 to 8 female BALB/c mice were fed with 0.5 mL phosphate-buffered saline (PBS) containing 10^9 colony-forming units in all injected mice within 25 days. This is in concordance with previous reports.22 Animal care was in accordance with institutional guidelines.

### Figure 1. Presence of transduced human CD40L protein in mice treated with ST40L.

(A) HE staining of intestine (×100) of BALB/c mice killed 1 week after oral administration of ST40L. (B) Immunostaining of Peyer’s patches using antihuman CD40L Ab (×400) of BALB/c mice killed 1 week after oral administration of ST40L. (C) Immunostaining of Peyer’s patches using antihuman CD40L Ab (×400) of BALB/c mice killed 1 week after oral administration of ST.
by growth in 400 µg/mL G418 and then subcloned. The cells were harvested at 70% confluence, washed 3 times in PBS (Sigma) to remove G418, and fixed in 1% formalin (Sigma) for 10 minutes at room temperature. After further washing with PBS, the cells were cultured with A20 cells (100 A20 cells/1 NIH3T3 cell) for 48 hours in 10% FBS plus RPMI1640.

Flowcytometric analysis

Phenotypic changes of A20 cells (1 x 10⁶/mL) treated with NIH3T3/CD40LT (1 x 10⁹/mL) or NIH3T3/vt (1 x 10⁹/mL) for 48 hours were examined using flow cytometric analysis, as previously described. Antibody-coated cells were enumerated by flow cytometric analysis using an EPICS V cell sorter (Coulter Electronics, Hialeah, FL). The following antibodies (Abs) were used: FITC-conjugated hamster antimouse CD80 (B7-1) Ab (hamster IgG) (Pharmigen, San Diego, CA), FITC-conjugated rat antimouse CD86 (B7-2) Ab (IgG2a, k) (Pharmigen), and FITC-conjugated hamster antimouse CD95 (Fas) Ab (hamster IgG) (Pharmigen).

Immunohistochemistry

Paraffin-embedded specimens were used for hematoxylin and eosin (HE) staining and Fas ligand (FasL) (Santa Cruz Biotechnology, Santa Cruz, CA) immune-staining as previously described. Moreover, anti-CD4 and CD8 Abs (Pharmigen) were used for immunostaining combined with HE staining. For immunohistochemical staining, frozen tissue sections were treated with antihuman CD40L Ab (Santa Cruz).

Measurement of soluble human CD40L in sera

Soluble human CD40L levels in the sera of BALB/c mice were quantified using the soluble CD40L ELISA kit. Data shown are mean ± SD from 3 independent experiments.

Results

We first examined the expression of Fas, B7-1, and B7-2 on A20 cells cultured with formalin fixed NIH3T3/vt or NIH3T3/CD40LT cells by immunofluorescence flow cytometry, as shown in Table 1.
Levels of Fas and B7-2 on the surface of A20 cells were found to be low, and B7-1 was undetectable when A20 cells were cultured with NEJ3T3/Ab (control). In contrast, NIH3T3/CD40LT cells upregulated the expression of Fas as well as B7-1 and B7-2 molecules.

To analyze expression of the human CD40L protein in murine tissues, samples from the small intestine, colon, liver, and spleen were analyzed by HE staining as well as immunohistochemistry. We found that in mice immunized with ST40L, the Peyer’s patches were prominent (Figure 1A), and the majority of cells in the Peyer’s patches could be seen to express the human CD40L protein (positive: brown or yellow color; negative: blue color) (Figure 1B). There were a few CD40L+ cells in spleen, but not in liver. In contrast, human CD40L was not detectable in the Peyer’s patches of mice treated with ST (Figure 1C). To further confirm the secretion of human soluble CD40L by transfected murine cells into the sera, we next examined it by ELISA (Figure 2). Human soluble CD40L protein was detectable only in BALB/c mice treated with ST40L with or without administration of BCL cells, but not detectable in mice treated with ST and/or BCL cells. The level of soluble CD40L protein in the sera peaked at 1 week after oral administration and was detectable until 7 to 8 weeks.

BALB/c mice were injected SC with 10⁵ A20 cells (Figure 3A), these mice were then orally administered with ST40L, ST, or PBS alone, and their survival monitored. Mice in the group treated with ST40L were found to have a significantly longer survival than those treated with ST or PBS (Kaplan-Meier method: Mantel-Cox, \( P < .0001 \)). The mice in the group treated with ST alone also survived for a significantly longer period than those treated with PBS alone (\( P < .0001 \)). In contrast, SC injection of an equivalent number of CD40 negative wehi3 leukemia cells showed that there was no significant difference between treatments with ST40L, ST, and PBS. When differing numbers of A20 cells (10⁵, 10⁶, or 10⁷) were injected, the survival rates of the mice were 92%, 77%, and 55%, respectively (Figure 3B). ST40L was then administered before or after tumor challenge (Figure 3C and D). When ST40L was administered 1 week before tumor cell challenge (10⁵ A20 cells, SC injection), no significant differences were observed in mice survival compared with simultaneous vaccination by SC injection of A20 cells alone. However, the efficiency of the ST40L was found to be decreased when the mice were immunized at either 3 weeks (52%, \( P < .01 \)) or 2 weeks (67%, \( P < .05 \)) before A20 cell (10⁵) SC injection. This effect was also seen when the mice were immunized at 3 weeks (42%, \( P < .01 \)), 2 weeks (69%, \( P < .001 \)), or 1 week after (70%, \( P < .02 \)) A20 cell (10⁵) SC injection.

To explore the mechanisms of the protection from BCL growth, histologic analysis was performed on tumor tissue from mice treated with ST40L, ST, or PBS alone. In the mice treated with PBS alone, no cellular infiltrate expressing FasL was observed in the surrounding tissues and inside the BCL region (Figure 4A and D). In contrast, infiltrating lymphocytes expressing FasL were observed around the vessels and also scattered in the smaller tumor tissues in the mice treated with ST (Figure 4B and E). Small hard nodules (2-5 mm in diameter) were observed at the SC injection sites of the long-term survival mice that had been treated with ST40L. On histologic analysis, these small nodules were confirmed to be the result of an accumulation of lymphocytes, and not BCL cells (Figure 4C). These lymphocytes were also found to be strongly positive for FasL expression (Figure 4F). On the other hand, lymphocytes infiltrating in the nodules were stained by either CD4+ Ab or CD8+ Ab, but the ratio of CD4+ and CD8+ cells was not 1-sided (data not shown).

Discussion

In the current study, we have demonstrated an outstanding protection from development of BCL in mice by the oral administration of an ST40L vaccine. SC injection of 10⁵ A20 cells was lethal in all injected BALB/c mice; in contrast, more than 90% of mice survived with simultaneous oral administration of ST40L. The efficiency was dependent on the dose of A20 cells administered and
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