RAPID COMMUNICATION

Inhibition of Human B-Cell Lymphoma Growth by CD40 Stimulation

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CD40 is a molecule present on B lymphocyte lineage cells that is important in B-cell differentiation and activation. Signaling through CD40 has been shown to exert costimulatory signals on normal B cells resulting in proliferative and differentiation responses. Examination of several B-cell lymphomas showed cell-surface expression of the CD40 molecule. Incubation of these lymphomas with anti-CD40 antibodies resulted in significant growth inhibition in vitro. Cross-linking of the CD40 antibodies resulted in even greater inhibition of proliferation. A recombinant soluble human CD40 ligand was also shown to inhibit lymphoma proliferation. When various human B-cell lymphomas were transferred into mice with severe combined immune deficiency, the treatment of the mice with anti-CD40 antibodies resulted in significant increases in survival showing that anti-CD40 is efficacious after in vivo administration. Thus, CD40 stimulation by either the antibody or soluble ligand directly inhibits human B-cell lymphoma growth and therefore, may be of significant clinical use in their treatment. This is a US government work. There are no restrictions on its use.

MATERIALS AND METHODS

Mice. C.B-17 severe combined immune deficiency (SCID) mice were obtained from the Animal Production Facility (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD) and were not used until 6 to 8 weeks of age. SCID mice were kept under specific-pathogen-free conditions at all times. The mice were housed in microisolator cages and all food, water, and bedding were autoclaved before use. SCID mice received trimethoprim/sulfamethoxazole (40 mg trimethoprim and 200 mg sulfamethoxazole per 320 mL drinking water) in suspension in their drinking water.

Antibodies. Anti-human CD40 (m2 and m3 hybridomas, mouse IgG1 antibody) was provided by Immunex (Seattle, WA). These antibodies have been shown to be specific for CD40 and exerted costimulatory responses on normal human B cells. We wanted to determine the effects of anti-CD40 on the growth of various human B-cell lymphomas both in vitro and in vivo. We report here that CD40 stimulation is capable of inhibiting the growth of several B-cell lymphomas and inducing regression of established tumors in vivo.

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human B-cell lymphomas was determined by flow cytometry and immunofluorescence. The protocol for flow cytometric analysis has been described previously. Briefly, cells were washed and counted using a Coulter counter (Coulter Electronics, Hialeah, FL). The cells were then adjusted to the appropriate cell concentration and were blocked with 2% human AB serum to prevent nonspecific binding of Ig. The cells were then incubated with the appropriate primary antibody of either anti-CD40 or isotype-matched mouse IgG1 myeloma protein (Cappel, West Chester, PA). The cells were then washed and incubated with a fluoresceinated (FITC) secondary antibody, a goat-antimouse IgG (kindly provided by Dr Kristin Komischke, PRD DynCorp, Frederick, MD). After incubation the cells were washed and fixed in 1% paraformaldehyde. The cells were then analyzed using an EPICS flow cytometer (Coulter). For the immunofluorescence studies, one million cells were spun using a cytopsin and fixed for 10 minutes with Biomek immunostaining-kit blocking reagent (Biomek Corp, Foster City, CA). The primary antibodies (anti-CD40 or msIgG) were then incubated at 1 μg/mL for 2 hours at room temperature. The secondary antibody (donkey-antimouse IgG directly coupled to FITC; Jackson ImmunoResearch laboratories, Bar Harbor, ME) was then incubated for 1 hour at room temperature. After extensive washing the cells were coverslipped with Gel Mount (Biomenta) and were examined with a Zeiss LSM-310 confocal laser-scanning microscope (Zeiss, Oberkochen, Germany) having the following configuration: 25 mW Argon and HeNe lasers (488, 514, and 543 maximum lines) with image acquisition X-Y scan, Z-series scan, and three-dimensional visualization. Photomicrographs were prepared with a Condonics NP600 printer (Condonics, Middleburg Heights, OH). For comparison of fluorescent intensity, we selected identical parameters for each image (scanning time, laser light source, contrast, and brightness).

**Proliferation assay.** The effect of anti-CD40 on B-cell lymphoma growth in vitro was determined by [3H]-thymidine incorporation. The cell lines were split 24 hours before assays were performed. Cells were resuspended in culture medium to a concentration of 1 x 10^6/mL, and 100 μL was plated in 96-well flat-bottom microtiter plates (Corning Glass Works, Corning, NY) already containing 100 μL of appropriately diluted reagents of anti-CD40, M2, and M3 clones (Immunex) or mouse IgG myeloma protein (Cappel). Seventy-two hours later, 1 μCi of [3H]-thymidine/well (specific activity, 6.7 Ci/mmol; [New England Nuclear Research Products, Boston, MA]) was added for the final 8 to 18 hours to culture. Cultures were harvested onto glass fiber filters with a PhD Cell Harvesting System (Cambridge Technology, Inc, Cambridge, MA), and [3H]-thymidine uptake was assayed by liquid scintillation on an LKB System (Cambridge Technology, Inc, Cambridge, MA). For the proliferation assay was then performed as described earlier. For determination of the effects of soluble human CD40 ligand, RL or TU2C cell lines were incubated with either titrations of COS-7 control supernatants or COS-7 supernatants containing soluble human CD40 ligand (supplied by Immunex). The recombinant soluble human CD40 ligand has been shown to induce the proliferation of human B cells in vitro. Values are reflected as percent inhibition of proliferation in comparison to control supernatants.

**RESULTS**

**Surface expression of CD40 on various human B-cell lymphomas.** We first examined the cell surface expression of CD40 in RL and TU2C cell lines by immunofluorescence and flow cytometric analysis. Both lymphomas showed surface expression of CD40 molecules, although to varying degrees (Fig 1). The RL tumor line that was isolated from a patient with diffuse large cell lymphoma was relatively homogeneous for CD40 expression as indicated by immunofluorescence and the narrow peak of positive cells in the flow cytometric analysis (Fig 1A and inset). The EBV-induced lymphoma line TU2C, obtained from a huPBL-SCID chimeric mouse, had a broader range of CD40 expression with very bright and dim cells present (Fig 1B and inset). EBV-induced B-cell lymphomas derived from SCID mice have previously been shown to be heterogeneous and oligoclonal and this may account for the differential expression of CD40 in these tumors. Similar results were obtained through the examination of other EBV-induced B-cell lymphoma lines with respect to CD40 expression (data not shown). We then ascertained the effects of soluble anti-CD40 antibodies on the proliferative potential of these various B-cell lymphomas in vitro.

**Effects of anti-CD40 on human B-cell lymphoma proliferation in vitro.** Incubation with anti-CD40 significantly inhibited the proliferation of RL, DB, TU2C, and CHIM62 cell lines tested with an optimal inhibition of thymidine incorporation (40% to 60%) occurring at 1 to 10 μg/mL of soluble antibody depending on the lymphoma when compared with isotype-matched control antibody (Fig 2, A-D). No inhibitory effects of soluble anti-CD40 were detected on the Raji cell line (Fig 2E). We have found that 10 to 100 μg of anti-CD40 are saturating amounts in this assay. The effects of soluble anti-CD40 antibody were then compared with those of immobilized anti-CD40 on lymphoma growth to determine if cross-linking the antibody would result in greater inhibition. The data show that cross-linking the antibody results in significantly (P < .001) greater growth inhibition of the RL lymphoma cell line (Fig 3A). Thus, anti-CD40 appears to directly inhibit human B-cell lymphoma proliferation in vitro and cross-linking appears to augment the inhibitory signals. We found that soluble anti-CD40 had no effect on the proliferation of Burkitt's lymphoma (Raji) cells (Fig 2E). This is in agreement with previous reports that detected no effect of CD40 stimulation on Burkitt's lymphoma cell lines. However, if anti-CD40 was cross-linked, significant (P < .001) inhibition could now be detected (Fig 3B) indicating that CD40 stimulation is also capable of inhibiting the proliferation of Burkitt's lymphoma cell lines. These results show that the in vitro assay conditions are critical in evaluating the effects of anti-CD40 on B-cell lymphoma growth and that anti-CD40 appears capable of providing inhibitory signals to a variety of B-cell lymphomas.

**Effects of soluble CD40 ligand on human B-cell lymphoma**
ANTITUMOR EFFECTS OF ANTI-CD40

Fig 1. Surface expression of CD40 on RL and TU2C B-cell lymphomas as determined by immunofluorescence and flow cytometric analysis. (A) Expression of CD40 on RL lymphoma cells is shown. Immunofluorescence studies and use of the confocal microscope were performed as described in Materials and Methods. Briefly, cells are incubated with anti-CD40 followed by a fluorescinated secondary antibody. Inset shows flow cytometric analysis of CD40 expression on RL tumor cells (-----) versus control antibody (-----). (A') Immunofluorescence of RL tumor cells using control antibody is shown. (B) Expression of CD40 on TU2C lymphoma cells is shown. Inset shows flow cytometric analysis of CD40 expression with anti-CD40 staining (-----) versus control antibody (-----). (B') Use of control antibody on TU2C tumor cells is shown.

growth. A soluble version of the naturally occurring ligand for CD40 was also tested for its ability to inhibit lymphoma growth in vitro. The soluble ligand used in these experiments is a recombinant molecule that contains the extracellular domain of the CD40 ligand fused to an amino-proximal 30 amino acid-modified leucine zipper motif. The ligand is secreted into the media of transfected COS-7 cells, where it exists in an oligomeric form as a result of hydrophobic interactions between the sequences comprising the leucine zipper motif. We examined the effects of soluble human CD40 ligand obtained from transfected COS-7 cell supernatants on the lymphoma cells. Because the ligand is not yet available in a purified form, each lot of supernatant was titrated to determine the concentration that yielded the optimal inhibition of lymphoma proliferation which makes a comparison with the anti-CD40 antibody difficult. The values are represented in comparison with control COS-7 supernatants. The soluble ligand was inhibitory for the various
Fig 2. Effect of anti-CD40 on the proliferation of various B-cell lymphomas is shown. The proliferation assay is described in the Materials and Methods section. Briefly, B-cell lymphomas were incubated with various concentrations of anti-CD40 (○) or control mlgG (■) antibodies and 3 days later effects on growth were evaluated by incorporation of [3H]-thymidine. Data is presented as means of CPM with standard deviation and are representative of three to four experiments. The B-cell lymphomas examined were (A) RL, a diffuse large cell lymphoma; (B) DB, another diffuse large cell lymphoma; (C) TU2C, an EBV-induced B-cell lymphoma; (D) CHIM-62, another EBV-induced B-cell lymphoma; and (E) Raji, a Burkitt’s lymphoma. Anti-CD40 significantly (P < .01) inhibited the proliferation of RL, DB, TU2C, and CHIM-62 cell lines.
ANTITUMOR EFFECTS OF ANTI-CD40

Fig 3. Effect of cross-linking on anti-CD40 mediated inhibition of (A) RL and (B) Raji B-cell lymphomas is shown. The proliferation and cross-linking experiments are described in Materials and Methods. Briefly, either soluble anti-CD40 (1 µg/mL) or anti-CD40 (1 µg/mL) that has been immobilized on goat-antimouse coated wells are placed with RL or Raji tumor cells and are assayed after 3 days. The data is presented as percent growth compared with control mlgG antibodies and is representative of three experiments. *Cross-linking significantly (P < .001) decreases proliferation of lymphoma cells compared with soluble anti-CD40.

lymphomas tested with maximal inhibition seen (50% to 80%) on RL and TU2C cell lines at a 1:5 dilution of the supernatant (Fig 4). The soluble murine CD40 ligand, which is active on both murine and human B cells, produced similar inhibitory effects on lymphoma proliferation (data not shown). Thus, in contrast with its effects on normal B cells, stimulation of CD40 by either antibody or its ligand exerts inhibitory effects on neoplastic B cells.

Antitumor effects of anti-CD40 treatment in SCID mice bearing human B-cell lymphomas. We then determined if anti-CD40 would be efficacious in the treatment of these lymphomas in vivo. SCID mice bearing either RL or TU2C lymphomas were treated at various times with anti-CD40. All mice received antisera to asialo GM1, a marker present on murine NK cells, before cell transfer to remove host resistance to the tumor.14 Significant (P < .01) increases in survival in SCID mice receiving either RL or TU2C B-cell lymphomas were noted in the recipients receiving 2-µg injections of anti-CD40 given every other day for a total of 10 injections starting on day 0 (Table 1). Recipient mice received the human lymphomas either by IP or IV injection, resulting in differential patterns of metastatic growth. Mice receiving the EBV-induced lymphomas by IP injection developed peritoneal tumors with extensive metastases in the lymph nodes and liver, whereas mice receiving the lymphomas by IV injection primarily developed renal metastases (data not shown). Treatment with anti-CD40 was capable of significantly inhibiting tumor growth and promoting survival of recipient mice regardless of the route of tumor inoculation (Table 1). No overt toxicity was detected after repeated administration of anti-CD40 in the recipients as determined by necropsy of the surviving mice. Treatment with anti-CD40 of SCID mice bearing a human melanoma that was negative for CD40 resulted in no differences in tumor growth and no effects on survival (data not shown). Treatment with anti-CD40 of SCID mice injected with RL tumor cells also resulted in significantly (P < .01) improved survival when the treatment was initiated 3 or 14 days after tumor cell transfer (Fig 5). These results indicate that anti-CD40 treatment was also efficacious when treatment was initiated with relatively large tumor burdens (>1 cm?) in the recipient mice. Addi-

Fig 4. Effect of recombinant soluble human CD40 ligand on RL and TU2C lymphoma proliferation. The proliferation assay is described in the Materials and Methods section and the data is presented as percent growth relative to control COS-7 supernatants. (A) Effects of various concentration of the soluble ligand on RL tumor cell proliferation. (B) Effects of various concentration of the soluble ligand on TU2C proliferation. Soluble CD40 ligand significantly (P < .01) inhibited the growth of both cell lines. Data are representative of three to four experiments.
tumor-bearing mice.  Studies are currently underway to evaluate the recombinant soluble human CD40 ligand in the treatment of tumor-bearing mice.

DISCUSSION

We report here that CD40 stimulation directly inhibits the proliferation of various human B-cell lymphomas in vitro and is efficacious against a wide variety of B-cell lymphomas in vivo. This is the first report showing that CD40 stimulation can provide negative signals to a variety of transformed B lymphocytes. Previous reports concerning CD40 have focused on its ability to promote B-cell growth and prevent apoptosis. Indeed, there have been reports that CD40 stimulation does not inhibit the growth of Burkitt’s lymphomas and promotes the proliferation of follicular cell lymphomas. However, all of the previous studies used soluble CD40 antibodies with the tumor cells in vitro. We show that cross-linking markedly enhances the inhibitory signals of anti-CD40 (Fig 3) and this may account for the lack of inhibition reported by other investigators. We also observed, in agreement with the previously mentioned reports, that a Burkitt’s lymphoma cell line was not significantly inhibited by soluble anti-CD40 (Fig 3B). However, immobilized anti-CD40 does inhibit the growth of these cells in vitro and anti-CD40 significantly increases survival in mice bearing these tumors (Fig 6). Prior cross-linking of anti-IgM antibodies has also been shown to markedly enhance the inhibitory effects of surface Ig binding on B-cell lymphomas and it appears that CD40 also needs to be cross-linked for optimal inhibition. This implies that signal transduction is required to mediate the observed effects. Preliminary evidence indicates, at least with the EBV-induced lymphoma line TU2C, that anti-CD40 is capable of inducing apoptosis. However, some lymphomas undergo irreversible growth arrest without apoptosis in vitro.

Because anti-CD40 or soluble CD40 ligand can inhibit various B-cell lymphomas directly, it may not be necessary to use it as an immunotoxin or a radioimmunoconjugate, thus avoiding toxicities associated with toxins and nuclides.

Table 1. Effect of Anti-CD40 Administration on Survival in Tumor-Bearing SCID Mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Tumor (route)</th>
<th>Treatment (initiation)*</th>
<th>No. of Mice</th>
<th>Mean Day of Death</th>
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<tr>
<td>1</td>
<td>RL (IP)</td>
<td>mslgG1</td>
<td>3</td>
<td>34 ± 0</td>
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<tr>
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<td>RL (IP)</td>
<td>Anti-CD40 (day 0)</td>
<td>6</td>
<td>&gt;138 ± 26.5t</td>
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<tr>
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<td>mslgG1</td>
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<td>28 ± 0</td>
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<tr>
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<td>Anti-CD40 (day 0)</td>
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<td>TU2C (IV)</td>
<td>mslgG1</td>
<td>6</td>
<td>30 ± 1.3</td>
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<tr>
<td></td>
<td>TU2C (IV)</td>
<td>Anti-CD40 (day 3)</td>
<td>6</td>
<td>38 ± 2.6</td>
</tr>
</tbody>
</table>

* SCID mice were treated as described in Materials and Methods. All mice received anti-ASGM1 IV 1 day before tumor transfer. On the day antibody treatment was initiated, they received either mslgG control or anti-CD40 (2 µg IP administered every other day for a total of 10 injections). All experiments were performed two or three times with five to seven mice per group. Anti-CD40 treatment significantly (P < .01) improved survival of mice receiving either RL or TU2 tumor cells when treatment was initiated on day 0 or day 3.

† Three of six mice showed no evidence of tumor.

‡ One of six mice showed no evidence of tumor.

Fig 5. Effects of anti-CD40 treatment on survival in RL-bearing SCID mice with varying tumor burdens. SCID mice received RL B-cell lymphomas as described in Materials and Methods. Three and 14 days after IP tumor inoculation, the mice were treated with anti-CD40 (2 µg) or mslgG control antibody (2 µg) by IP injection every other day for 20 days and a total of 10 injections. The mice were then monitored for tumor growth and effects on survival. Moribund mice were killed and they showed evidence of extensive tumor burden. Representative of two experiments with five to seven mice per group. *Anti-CD40 treatment significantly (P < .01) improved survival of tumor-bearing mice.

Fig 6. Effect of anti-CD40 administration on survival in SCID mice bearing human Burkitt’s lymphoma cells. SCID mice received Raji tumor cells as described in Materials and Methods. After IV injection of Raji tumor cells, the mice were treated 3 days later with either anti-CD40 (2 µg) or control mslgG (2 µg) administered by IP injection every other day for 20 days and a total of 10 injections. Moribund mice were killed. Each experiment contained four to six mice per group and was performed three times. *Anti-CD40 treatment significantly (P < .01) promoted the survival in tumor-bearing recipients.
and potentially negative effects on normal B cells. Use of the soluble human CD40 ligand is especially attractive because no human-antimouse antibodies would be elicited that would neutralize the antitumor effects. Thus, long-term exposure to CD40 ligand is theoretically possible. Furthermore, the selective effects of anti-CD40 on B cells makes it attractive in settings where cytotoxic therapy may be too toxic (eg, acquired immune deficiency syndrome patients, elderly patients, and posttransplant patients).

It is of interest that treatment of mice bearing the RL lymphoma with anti-CD40 resulted in mice showing no evidence of disease for at least 100 days. By contrast, statistically significant but modest inhibition of RL proliferation was noted when the lymphoma was incubated with soluble anti-CD40 in vitro (Fig 2A, Table 1). This also suggests that there may be additional yet-undefined antitumor mechanisms by which anti-CD40 can exert its effects when given in vivo. It may be that better cross-linking of the antibody is achieved in vivo as opposed to soluble antibody exposure in vitro because we have shown that greater inhibition of proliferation is obtained in vitro with immobilized anti-CD40 (Fig 3). Alternatively, it may be that antibody-dependent cell-mediated cytotoxicity may be contributing to the responses seen in vivo. In support of this, it has been reported that treatment of SCID mice bearing human EBV-induced B-cell lymphomas with nonconjugated CD21, CD23, and CD24 monoclonal antibodies could prolong their survival. It was reported that these antibodies did not inhibit the growth of these lymphomas in vitro, so the in vivo mechanism remains speculative although ADCC may be responsible. Studies are currently underway to compare the efficacy of anti-CD40 with these and other B-cell lymphoma surface markers. The data also show that anti-CD40 treatment is more efficacious if the lymphoma is also administered by IP as opposed to IV inoculation (Table 1, Fig 4). This may simply be caused by the fact that, because the antibody is administered by IP injection, this would allow for higher concentrations of the antibody to be present at the sites of tumor.

It would also be important to determine if anti-CD40 treatment selectively inhibits tumor cell growth while at the same time sparing normal B cells. We have data showing that anti-CD40 prevents human B-cell lymphomagenesis in the huPBL-SCID chimeric mouse model and at the same time promotes human B-cell engraftment in vivo (Murphy et al, manuscript submitted). This would suggest that use of anti-CD40 would be advantageous because of its selectivity for inhibiting transformed cells.

The CD40 molecule is in the tumor necrosis factor/nerve growth factor receptor family that also includes CD30 and Fas. It has been speculated that because Fas is involved in the induction of apoptosis that Fas stimulation may be applicable in the treatment of EBV-lymphomas. However, treatment of mice with anti-Fas antibodies resulted in death because of extremely toxic effects on the liver. Because CD40 expression appears restricted to B cells and monocytes, little toxicity should occur after in vivo administration. Additionally, CD40 has also been detected on some carcinomas and melanomas; it will be of considerable interest to examine the effects of anti-CD40 on the growth of these tumors.

Thus, stimulation of CD40 by either antibody or its ligand molecules may offer a biologic approach to the treatment of human B-cell lymphomas.

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REFERENCES

duced human B lymphoproliferative disease in mice with severe combined immunodeficiency. Am J Pathol 142:139, 1993


21. Stamenkovic I, Clark EA, Seed B: A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. EMBO J 8:1403, 1989


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