Resistance to Cytotoxic Chemotherapy Induced by CD40 Ligand in Lymphoma Cells

By Nathalie Voorzanger-Rousselot, M.-C. Favrot, and Jean-Yves Blay

The modulation of the cytotoxic effects of an anthracyclin by CD40L was investigated in five non-Hodgkin’s lymphoma (NHL) cell lines (Daudi, Raji, BJAB, BL36, BL70). Incubation with doxorubicin (DOX) increased in a dose-dependent manner the percentage of apoptosis in NHL cells. Coculture with irradiated L cells expressing CD40L (CD40L L cells), but not CDw32 (CDw32 L cells), significantly reduced (33% to 89%) the percentage of apoptosis in all five cell lines treated with 0.1 to 0.5 μg/ml of DOX, but in only three cell lines at 1 μg/ml. Interleukin-10 (IL-10), IL-6, IL-2, or tumor necrosis factor (TNF) induced no additive protective effects with CD40L L cells. In all five cell lines, DOX induced a concentration-dependent increase of the activity of the cysteine protease caspase 3. Coculture with CD40L L cells, but not with CDw32 L cells, inhibited (38% to 100%) the activation of caspase 3 induced by 0.1 to 0.5 μg/ml of DOX in all five NHL cell lines, but in only two cell lines at 1 μg/ml. Finally, the antiproliferative effect of 0.1 to 0.5 μg/ml concentrations of DOX was also partially abrogated on coculture with CD40L L cells in all five cell lines, but in only two cell lines at 1 μg/ml. Cytokines, either alone or in combination with CD40L L cells, did not affect DOX-induced inhibition of proliferation. These results indicate that CD40L inhibits the apoptosis and antiproliferative effect induced by DOX and interferes with caspase 3 activation in NHL cell lines.

© 1998 by the American Society of Hematology.

Although non-Hodgkin’s lymphoma (NHL) cells are sensitive to cytotoxic chemotherapy, primary or secondary chemoresistance frequently occurs and is the major cause of death of these patients.1,2 The biological mechanisms by which lymphoma cells acquire resistance to cytotoxic drugs are not fully understood.3 Although markers associated with the multi-drug resistance (MDR) phenotype, e.g., P-glycoprotein (P-gp), are likely to play a role in this phenomenon, in vivo, chemoresistance of NHL is not consistently associated with P-gp expression indicating the existence of additional mechanisms.4

Apoptosis, or programmed cell death, is an active phenomenon dependent on RNA and protein synthesis, which plays an essential role in many normal processes, such as embryological development and immune cell selection.4,5 Apoptosis can be induced by ionizing radiation in cancer treatment, engagement of cell surface molecules such as Fas and tumor necrosis factor (TNF)-R, or deprivation in growth factors.7,9-12 Several cytotoxic agents act by inducing the apoptosis of tumoral cells.13-17 It has recently been shown that apoptosis caused by doxorubicin (DOX) in a human T-leukemia cell line is mediated via CD95L induction and subsequent activation of the CD95 pathway.17

CD40, a member of the TNF receptor superfamily, is expressed on B lymphocytes and interacts with a ligand (CD40 L) expressed on activated T cells.18 CD40L exerts a complex modulation of B-cell apoptosis: it promotes the survival of germinal center B cells, but also induces Fas expression thereby rendering the cells sensitive to FasL or agonists.19-22 Several cytokines, in particular interleukin-10 (IL-10), IL-6, and IL-2, have also been reported to inhibit the apoptosis of normal or neoplastic B cells, in particular induced by cytotoxic agents, or Fasl.23-27 A phenomenon associated with a modulation of the expression of bcl-2, bcl-xl, and/or BAG28-30 CD40 is expressed at the surface of fresh B NHL cells and is able to trigger a proliferative signal in the presence of IL-6 and IL-10.19,31 The biological effect of a combination of cytokines and CD40L on the modulation of apoptosis of neoplastic B-cell lines induced by cytotoxic agents is not known.

In the present report, the capacity of CD40L to modulate the apoptosis of lymphoma cell lines induced by a cytotoxic agent was investigated. CD40L-expressing L cells were found capable of partially inhibiting the apoptosis and antiproliferative effect of DOX on lymphoma cells, as well as the activation of the cysteine protease caspase 3.2,32,33 These results uncover a new mechanism of resistance to cytotoxic agents conferred by adjacent nontumoral cells expressing CD40L.

MATERIALS AND METHODS

Cell lines and culture conditions. Lymphoma cell lines (Daudi, Raji, BJAB, BL36, BL70) were grown at 10⁵ cells/ml in RPMI 1640 (GIBCO-BRL, Gaithersburg, MD) containing 10% fetal calf serum (FCS), 100 μg/ml penicillin, 100 mg/ml streptomycin (GIBCO) and 2 mg/ml of L-glutamine. CDw32/FcRII and CD40 ligand (CD40 L) transfected Ltk (-) cell line (CDw32 L cells and CD40L L cells) were kindly provided by Dr F. Rousset and Dr J. Banchereau (Schering-Plough, Dardilly, France). The CD40L L cells were previously published and are capable of inducing normal B-cell growth and Ig secretion.22 The expression of CD40L on the cell line used in the present report was evaluated before the initiation of these experiments using anti-CD40L labeling experiments. CD40L was detectable at the surface of 95% of CD40L L cells and 40% of phytohemagglutinin (PHA)-activated T-cell line Jurkat used as control. CDw32 expressing L cells were also used in these experiments alone or in the presence of an anti-CD40 agonist antibody (monoclonal antibody [MoAb] 89) at 0.5 μg/ml to mimic the effect of CD40L. In some experiments, various concentrations of MoAb 89 were used, ranging from 0.05 μg/ml to 2 μg/ml.

DOX was added at the initiation of the culture during 24 hours (0.5 or 1 μg/ml) or 30 hours (0.1 μg/ml). A 24-hour exposure was chosen because of the long cellular half-life of this compound in vivo in NHL cells; a prolonged exposure to DOX may be more relevant to the in vivo situation, as previously reported.17,34 After 24 hours, the medium was changed payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1998 by The American Society of Hematology.

then removed and replaced with the same culture medium without DOX for 24 additional hours of culture. Irradiated (75 Gy) L-cell lines (ie, CD40L or CDw32 expressing L cells) were added at the initiation of DOX exposure at 10⁵ cells/mL. Cytokines (IL-10, IL-6, IL-2, and TNF) were added at the initiation of the culture with or without DOX. Apoptosis and caspase 3 activation were tested 48 hours after the initiation of culture.

**Drugs, cytokines, and antibodies.** DOX (Pharmacia, Paris, France) was dissolved in sterile distilled water before each experiment. Recombinant human (rh) IL-10 and MoAb 89 (anti-CD40 antibody) were provided by Dr F. Roussel and Dr J. Banchereau (Schering-Plough, Dardilly, France). The monoclonal anti-CD40L antibody was purchased from Immunotech (Marseille, France). rhIL-10 was used at 100 ng/mL; rhTNFα (Eurocetus, Amsterdam, the Netherlands) was used at 10 ng/mL. The caspase 3 (Yama/CPP-32/apopain)-specific Asp-Glu-Val-Asp (DEVD) tetrapeptide labeled with a fluorogenic substrate (7 amino-4 trifluoromethyl coumarin, ACF), DEVD-AFC, and the inhibitor DEVD-CHO (Tebu, Le Perray-en-Yvelines, France) were used at 50 µmol/L. A cell permeable inhibitor of caspase 3 (Tebu, Le Perray-en-Yvelines, France) was used at 100 µmol/L and added at the initiation of cell culture.

**Determination of apoptosis.** NHL cells were removed from 24-wells microtiter plates using gentle aspiration to avoid the removal of adherent CD40L L cells. The phenotype of the recovered cell population was consistently analyzed. The mean size of L cells was fourfold to fivefold higher than the mean size of the B NHL cell lines. In all experiments, the size and the level of CD20 expression was similar in noncocultured B NHL cell lines and in the recovered cell population, indicating that the contamination of recovered NHL cells by irradiated L cells is minimal, if it exists. The percentages of apoptotic NHL cell lines after removal from the CD40L or CD32 L cells layered ranged from 2% for the BL70 cell line to 23% for the Daudi cell line and were not found significantly different from that of cell lines cultured alone. For the quantification of apoptosis in the recovered cell population, DNA breaks characteristic of apoptotic cells were assessed by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (d-UTP) nick end labeling (TUNEL) assay (Boehringer Mannheim Corp, Indianapolis, IN). NHL cell lines were fixed with 1% paraformaldehyde, permeabilized with 0.1% Triton X100 in 0.1% sodium citrate and washed extensively. Incubation with TdT and fluorescein-labeled d-UTP provided visualization of DNA strand breaks by flow cytometry on a FACSscan instrument (Becton Dickinson, Pont de Claix, France).

In each sample, 2,000 cells were analyzed for their content in fluorescein-labeled DNA strand breaks. The intensity of fluorescence was proportional to the number of fluorescein-labeled DNA strand breaks within the cell. The threshold level of fluorescence intensity beyond which cells were considered to be apoptotic was 10³.

**Proliferation assay.** NHL cell lines (3.5 × 10⁴ cells in 200 µL) were cultured in 96-well flat-bottomed microtiter plates. DOX was added at the initiation of the culture during 24 hours (0.5 or 1 µg/mL) or 30 hours (0.1 µg/mL). After 24 hours, the medium was removed and replaced with the same culture medium without DOX for 24 additional hours of culture. Irradiated (75 Gy) L cell lines (ie, CD40L or CDw32 expressing L cells) were added at the initiation of DOX exposure at 2.5 × 10⁵ cells/mL. After 72 hours (or more) of culture, cells were pulsed with 1 µCi/well of [³H]TdR (25 Ci/mmol, Amersham, Les Ulis, France) for 1 hour. The assay group: cell lysates were directly incubated 1 hour at 30°C with DEVD-AFC substrate in the reaction buffer and dTT. Fluorescence measurements were performed after 1 (60) and 2 hours (660) of incubation. Caspase 3 activity was detected by AFC release monitored on a spectrophotofluorometer (Kontron Analytical SFM 25, Velizy, France), using an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Caspase 3 activity was calculated with the following formula: 1 unit = (dFU/min) × (calibration curve slope)⁻¹ × (1 U/1 × 10⁻⁶ mmol AFC/min), where dFU is the difference of fluorescence units: (FU of the assay group at t60 − FU of the blank group at t60) − (FU of the assay group at t0 − FU of the blank group at t0).

**Statistics.** Statistical analyses were performed using paired Student’s t-test.

**RESULTS**

Modulation of DOX-induced apoptosis by CD40L. The capacity of CD40L expressed on L cells to modulate the apoptosis induced by DOX in B-lymphoma cell lines (BJAB, Daudi, BL70, BL36, Raji) was investigated. Incubation with DOX during 24 hours at therapeutic concentrations (0.1 to 1 µg/mL) induced a dose-dependent increase of apoptosis in the five cell lines tested (Fig 1). With a shorter duration (3 hours) of incubation, DOX at 1 to 2 µg/mL also induced the apoptosis of these cell lines, while lower concentrations (0.1 to 0.5 µg/mL) of DOX were not cytotoxic (not shown).

All five NHL cell lines tested express CD40 at their cell surface (>95% of positive cells, not shown). Coculture of
lymphoma cell lines in the presence of irradiated (75 Gy) L cells expressing CD40L (CD40L L cells) resulted in a decrease of the percentage of lymphoma cell undergoing apoptosis (Fig 2A), as compared with lymphoma cells cultured with DOX alone or with L cells expressing CDw32, ie, the FcγRII receptor for IgG (Fig 2B). Coculture with CD40L L cells significantly (Student’s t-test, \( P < .05 \)) reduced the intensity of TdT signal as well as the percentage (33% to 89%) of apoptotic cells after treatment with DOX as compared with no CD40L L cells or with CDw32 L cells (Fig 2B, Table 1), in all five cell lines at concentrations of 0.1 to 0.5 \( \mu \)g/mL. With 1 \( \mu \)g/mL of DOX, the inhibition of apoptosis was significant only for the Daudi, Raji, and BL36 cell lines (Fig 2C). Coculture with CDw32 L cells (without anti-CD40 Ab) did not significantly affect the apoptosis of the five NHL cell lines incubated with a 0.5-\( \mu \)g/mL concentration of DOX (Table 1), or with 0.1 or 1 \( \mu \)g/mL (not shown). However, the addition of the agonist IgG anti-CD40 antibody (MoAb 89, 0.5 \( \mu \)g/mL) to the coculture of NHL cells with CDw32 expressing L cells, thus mimicking the effect of CD40L expressed at the cell surface, reduced the percentage of apoptotic NHL cells similarly to CD40L L cells (Table 1). Of note, a similar protective effect was observed with lower concentrations of anti-CD40 (0.05 \( \mu \)g/mL): for instance, in the BL70 cell line, the percentage of apoptotic cells was reduced from 57% \( \pm \) 2% to 21% \( \pm \) 1% with a 2-\( \mu \)g/mL concentration of MoAb 89 and to 19% \( \pm \) 1% with a 0.05-\( \mu \)g/mL concentration of MoAb 89 in a representative experiment. The apoptosis induced by a shorter (3 hours) exposure to DOX (2 \( \mu \)g/mL) was inhibited similarly on coculture with CD40L L cells, with a reduction of the percentage of apoptotic cells ranging from 30% \( \pm \) 2% for BL70 to 55% \( \pm \) 2.5% for Daudi (not shown).

IL-10, IL-2, IL-6, and TNF were tested for their capacity to modulate CD40L-mediated inhibition of apoptosis. The percentage of apoptotic cells induced by DOX (0.5 \( \mu \)g/mL) was significantly reduced when NHL cells were incubated with IL-10 alone in four cell lines (Table 1). IL-2 and IL-6 also marginally reduced DOX-induced apoptosis in the BJAB cell line only, while TNF alone had no effect (not shown). No additive protective effect was observed between CD40L and IL-10 in any cell line tested (Table 1) or between CD40L and the other cytokines tested.

Activation of caspase 3 by doxorubicin: Modulation by CD40L. Caspase 3 (Yama/CPP32/apopain) is a key enzyme involved in the cleavage of poly (ADP-ribose) polymerase (PARP) at the onset of apoptosis.\(^{32,33}\) DOX was found to induce a dose-dependent increase of caspase 3 activity in the five NHL cell lines (Table 2). This increase of caspase 3 activity was completely inhibited when cell lysates were preincubated with the caspase 3 inhibitor DEVD-CHO\(^{32}\) (Table 2). In all five cell lines, coculture with CD40L L cells, but not with CDw32 L cells, inhibited DOX-induced (0.1 or 0.5 \( \mu \)g/mL) caspase 3 activity by 38% to 100% (Table 2). With a 1-\( \mu \)g/mL concentration of DOX in contrast, coculture with CD40L L cells significantly reduced caspase 3 activity only in the BJAB and BL70 cell lines (Table 2).

IL-10, IL-2, IL-6, and TNF alone also significantly inhibited DOX-induced caspase 3 activity in three cell lines, respectively (Table 3). IL-10 or IL-6 exerted a significant additive inhibitory
effect on DOX-induced caspase 3 activity in the Daudi cell line only (Table 3).

CD40L increases the proliferation of doxorubicin-treated NHL cells. DOX induced a dose-dependent inhibition of tritiated thymidine uptake by the five lymphoma cell lines at 72 hours of culture (Fig 3) and also after 96, 120, 144, and 240 hours of culture (not shown). When cocultured with CD40L L cells, but not with CDw32 L cells, the proliferation of the five cell lines treated with 0.1 to 0.5 µg/mL of DOX was partially restored at 72 hours (Fig 3) and also at 96, 120, 144, and 240 hours of culture (not shown). In contrast, CD40L L cells reversed the antiproliferative effect of a 1-µg/mL concentration of DOX in only two of the five cell lines (Daudi, BL70) (Fig 3). Thymidine incorporation by NHL cells cultured with DOX alone or with CDw32 L cells was similar (not shown). Cytokines (IL-10, IL-2, IL-6, or TNF) either alone or added to the coculture of lymphoma cells with CD40L L cells did not further increase the proliferative potential of these cell lines after exposure to DOX (not shown).

DISCUSSION

The results presented here show that drug resistance to a major cytotoxic agent can be induced by a ligand expressed on adjacent nontumoral cells. Coculture of lymphoma cells with CD40L-expressing L cells (or with L cells expressing CDw32, ie, FcγRII, in the presence of an IgG anti-CD40) was found capable (1) to partially inhibit the apoptosis of lymphoma cell lines induced by therapeutic concentrations of DOX, (2) to inhibit the activation of caspase 3 induced by DOX, and (3) to increase the proliferative potential of these cell lines after the end of exposure to DOX. An external signal provided by adjacent cells, L cells in the present case, is therefore capable of inducing the resistance of lymphoma cells to the proapoptotic and antiproliferative effects of cytotoxic drugs. Of note, the addition of the same concentrations of the anti-CD40 Ab alone without CDw32 L cells did not significantly protect these cell lines against DOX-induced apoptosis (not shown). CD40 ligand mRNA has been found detectable in lymphoma tumor samples by reverse transcriptase-polymerase chain reaction (RT-PCR).31 Recently, the presence of CD40L protein in NHL tumor samples has been reported in aggressive NHL and follicular NHL tumor samples.35,36 Conceivably, CD40L expressed on nontumoral tumor cells against the cytotoxic activity of anticancer drugs. However, the protective role of CD40L in vivo still remains to be demonstrated. It must indeed be noted that the level of CD40L expression at the surface of CD40L L cells is superior to

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Caspase 3 Activity (U/2 x 10^5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Daudi</td>
</tr>
<tr>
<td>DOX (0.5 µg/mL)</td>
<td></td>
</tr>
<tr>
<td>L Cells</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>5.8</td>
</tr>
<tr>
<td>0.1</td>
<td>4.57</td>
</tr>
<tr>
<td>0.1 CD40L</td>
<td>4.49</td>
</tr>
<tr>
<td>0.5</td>
<td>7.22†</td>
</tr>
<tr>
<td>0.5 CD40L</td>
<td>4.97†</td>
</tr>
<tr>
<td>1</td>
<td>8.28*</td>
</tr>
<tr>
<td>1 CD40L</td>
<td>7.69</td>
</tr>
<tr>
<td>0.5 CDw32</td>
<td>7.26*</td>
</tr>
<tr>
<td>0.5 INH‡</td>
<td>0‡</td>
</tr>
</tbody>
</table>

* P < .05 (Student’s paired t-test) as compared with culture without DOX in 7 experiments.
† P < .05 (Student’s paired t-test) as compared with DOX in 7 experiments.
‡ Caspase 3 inhibitor (tetrapeptide DEVD-CHO, 50 µmol/L).
In conclusion, these results show a new mechanism of drug resistance of lymphoma cells triggered by CD40L expressed on adjacent nontumoral cells. The expression of CD40L by nontumoral cells in tumor microenvironment could play a role in drug resistance of lymphoma cells in vivo.
REFERENCES

19. Arman M, Delespesse G, Safarit M: IL-2 and IL-7 but not IL-12 protect normal killer cells from death by apoptosis and up-regulate bcl-2 expression. Immunology 85:331, 1995
27. Seymour JF, Tulpaz M, Cabanillas F, Wetzler M, Kurzrock R:


Resistance to Cytotoxic Chemotherapy Induced by CD40 Ligand in Lymphoma Cells

Nathalie Voorzanger-Rousselot, M.-C. Favrot and Jean-Yves Blay