Synergistic Cytotoxicity and Apoptosis by Apo-2 Ligand and Adriamycin against Bladder Cancer Cells

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ABSTRACT

Resistance to conventional anticancer chemotherapeutic agents remains one of the major problems in the treatment of bladder cancer. Hence, new therapeutic modalities are necessary to treat the drug-resistant cancers. Apo-2 ligand (Apo-2L) is member of the tumor necrosis factor ligand family, and it induces apoptosis in cancer cells. Several cytotoxic anticancer drugs, including Adriamycin (ADR), also mediate apoptosis and may share the common intracellular pathways leading to cell death. We reasoned that combination treatment of the drug-resistant cancer cells with Apo-2L and drugs might overcome their resistance. Here, we examined whether bladder cancer cells are sensitive to Apo-2L-mediated cytotoxicity and whether Apo-2L can synergize with ADR in cytotoxicity and apoptosis against bladder cancer cells.

Recombinant human soluble Apo-2L (sApo-2L), which carries the extracellular domain of Apo-2L, was used as a ligand. Cytotoxicity was determined by a 1-day microculture tetrazolium dye assay. Synergy was assessed by isobolographic analysis.

Human T24 bladder cancer line was relatively resistant to sApo-2L. Treatment of T24 line with combination of sApo-2L and ADR resulted in a synergistic cytotoxic effect. Synergy was also achieved in the ADR-resistant T24 line (T24/ADR), two other bladder cancer lines, and three freshly derived human bladder cancer cell samples. In addition, T24 cells were sensitive to treatment with sApo-2L combined with epirubicin or pirarubicin. The synergy achieved in cytotoxicity with sApo-2L and ADR was also achieved in apoptosis. Intracellular accumulation of ADR was not affected by sApo-2L. Incubation of T24 cells with sApo-2L down-regulated the expression of glutathione S-transferase-π mRNA.

This study demonstrates that combination treatment of bladder cancer cells with sApo-2L and ADR overcomes their resistance. The sensitization obtained with established ADR-resistant bladder cancer cells and freshly isolated bladder cancer cells required low subtoxic concentrations of ADR, thus supporting the in vivo potential application of combination of sApo-2L and ADR in the treatment of ADR-resistant bladder cancer.

INTRODUCTION

Previous studies have demonstrated that a variety of cancer cells have different degrees of sensitivity and resistance to various kinds of anticancer cytotoxic agents (1). When anticancer chemotherapeutic agents are administered, only the drug-sensitive cancer cells are deleted, and cancer cells with acquired resistance develop (2). Consequently, drug resistance (inherent or acquired) is the major cause of failure in cancer chemotherapy. Therefore, new effective therapeutic modalities are necessary to treat the drug-resistant cancers.

Apo-2L (also known as TRAIL) is a member of the TNF ligand family, and it induces apoptosis in various transformed cell lines, such as Fas ligand (3, 4). Unlike Fas ligand, the transcripts of which are predominantly restricted to stimulated T cells and sites of immune privilege, expression of Apo-2L is detected in a lot of normal human tissues, most predominantly in spleen, lung, and prostate (3). The receptors for Apo-2L, DR4, and DR5 are also expressed in multiple human normal tissues (5, 6). Interestingly, Apo-2L is not cytotoxic to most normal tissues in vivo; however, it has marked apoptotic potential for cancer cells (3, 4). In contrast to injection of Fas ligand and anti-Fas mAb, which is lethal to mice, injection of Apo-2L is well tolerated in mice (7, 8). Thus, Apo-2L may have potential utility as an anticancer agent.

Fas ligand and TNF-α as well as Apo-2L induce apoptosis (9, 10). Several anticancer drugs, including ADR, also mediate apoptosis and may share common intracellular signaling pathways leading to cell death. Indeed, we have reported that treatment with ADR in combination with anti-Fas mAb or TNF-α resulted in significant potentiation of cytotoxicity and synergy against a variety of sensitive and resistant human cancer cells (11, 12). This study investigated whether the resistance of
bladder cancer cells to ADR, one of anticancer agents used clinically against bladder cancer, could be overcome by combination treatment with Apo-2L and ADR.

MATERIALS AND METHODS

Tumor Cells. The T24, J82, and HT1197 human bladder cancer cell lines were maintained in monolayers on plastic dishes in RPMI 1640 (Life Technologies, Inc., Bio-cult, Glasgow, Scotland), supplemented with 25 mM HEPES, 2 mm l-glutamine, 1% nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (all from Life Technologies, Inc.), hereafter referred to as complete medium (13, 14). The T24/ADR line is an ADR-resistant subline of the T24 cell line (11).

Fresh bladder cancer cells derived from three patients were separated from surgical specimens as described previously (15, 16). The histological diagnosis revealed that all patients had transitional cell carcinoma of the bladder. Their histological classification and staging according to the tumor-node-metastasis classification were: patient 1, T2 N0 M0, grade 3; patient 2, T1 N0 M0, grade 2; and patient 3, T1 N0 M0, grade 2. Briefly, cell suspensions were prepared by treating finely minced tumor tissues with collagenase (3 mg/ml; Sigma Chemical Co., St. Louis, MO). After washing three times in RPMI 1640, the cell suspensions were layered on discontinuous gradients consisting of 2 ml of 100% Ficoll-Hypaque, 2 ml of 80% Ficoll-Hypaque, and 2 ml of 50% Ficoll-Hypaque in 15-ml plastic tubes and were centrifuged at 400 × g for 30 min. Lymphocyte-rich mononuclear cells were collected from the 100% interface, and tumor and mesothelial cells were collected from the 80% interface. Cell suspensions that were enriched with tumor cells were sometimes contaminated by monocyte-macrophages, mesothelial cells, or lymphocytes. To eliminate further contamination of host cells, we layered the cell suspensions on a discontinuous gradient containing 2 ml each of 25, 15, and 10% Percoll in complete medium.

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**Fig. 1** The synergistic cytotoxic effect of sApo-2L and ADR used in combination against T24 cells. The cytotoxic effect of sApo-2L and ADR used in combination on T24 cells was assessed in a 1-day MTT assay (A) and estimated by isobolographic analysis (B). Columns, means of three different experiments; bars, SD. * p < 0.05 vs. ADR alone at P < 0.05.
in 15-ml plastic tubes and centrifuged them for 7 min at 25 × g at room temperature. Tumor cells that were depleted of lymphoid cells were collected from the bottom, washed, and suspended in complete medium. To remove further contamination from mesothelial cells and monocyte-macrophages, we incubated the cell suspension in plastic dishes for 30–60 min at 37°C in a humidified 5% CO₂ atmosphere. After incubation, nonadherent cells were recovered, washed, and suspended in complete medium. Usually, the nonadherent cells contained mainly tumor cells with <5% contaminating nonmalignant cells, as judged by morphological examination of Wright-Giemsa-stained smears, and were >93% viable, according to the trypan blue dye exclusion test. Cells having <5% contamination with nonmalignant cells were accepted for use as tumor cells.

**Reagents.** sApo-2L was kindly supplied by Pepro Tech (Rocky Hill, NJ). ADR (lot no. 705ACB) and EPI (lot no. 3015AG) were supplied by Kyowa Hakkou Co. Ltd. (Tokyo, Japan). THP (lot no. THPMR100) was obtained from Meiji Pharmaceutical Co. Ltd. (Tokyo, Japan). GST-π cDNA, used in making probes for Northern blot analysis, was a gift from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan).

**Cytotoxicity Assay.** The MTT assay was used to determine tumor cell lysis, as described previously (17, 18). Briefly, 100 μl of target cell suspension (2 × 10⁴ cells) were added to each well of 96-well flat-bottomed microtiter plates (Corning Glass Works, Corning, NY), and each plate was incubated for 24 h at 37°C in a humidified 5% CO₂ atmosphere. After incubation, 100 μl of drug solution or complete medium for control were distributed in the 96-well plates, and each plate was incubated for 24 h at 37°C. Following incubation, 20 μl of MTT working solution (5 mg/ml; Sigma) were added to each culture well, and the cultures were incubated for 4 h at 37°C in a humidified 5% CO₂ atmosphere. The culture medium was removed from the wells and replaced with 100 μl of isopropanol (Sigma) supplemented with 0.05 N HCl. The absorbance of each well was measured with a microculture.

![Fig. 2](image_url) The synergistic cytotoxic effect of sApo-2L and ADR used in combination against fresh bladder cancer cells. The cytotoxic effect of sApo-2L and ADR used in combination on fresh bladder cancer cells derived from three patients (data from patient 1 are shown as a representative sample) was assessed in a 1-day MTT assay (A), and synergy was assessed by isobolographic analysis (B). Columns, means of triplicate samples; bars, SD. Similar data were found for the two other patients. *, significantly higher than those achieved by treatment with ADR alone at P < 0.05.
plate reader (Immunoreader; Japan Intermed Co. Ltd., Tokyo, Japan) at 540 nm. The percentage cytoxicity was calculated using the following formula: percentage cytoxicity = [1 – (absorbance of experimental wells/absorbance of control wells)] × 100.

**Chromatin Staining with Hoechst 33258.** Apoptosis was observed by chromatin staining with Hoechst 33258 as described previously (19). T24 cells in a chamber/slide (Miles Scientific, IL) were incubated with sApo-2L at 100 ng/ml in the absence or presence of ADR at 1 μg/ml for 12 h at 37°C in a humidified 5% CO₂ atmosphere. After incubation, the supernatant was discarded, and T24 cells were fixed with 1% glutaraldehyde in PBS for 30 min at room temperature, washed four times with PBS, and exposed to Hoechst 33258 at 10 μM for 30 min at room temperature. The cell preparations were examined under UV illumination with an Olympus fluorescence microscope. Apoptosis was defined when apoptotic bodies, chromatin condensation, or fragmented nuclei were observed.

**ADR Determination.** The ADR content in T24 cells was determined by high-performance liquid chromatography using a Hitachi model 635A (Hitachi Co. Ltd., Tokyo, Japan), as described in detail elsewhere (20).

**Northern Blotting.** Cytoplasmic RNA from tumor cells was prepared as described in detail elsewhere (21, 22). Briefly, tumor cell RNA (10 μg/lane) was electrophoresed in 1.2% agarose-2.2 M HCHO gels in 1× 4-morpholinepropanesulfonic acid buffer (200 mM 4-morpholinepropanesulfonic acid, 50 mM sodium acetate, and 10 mM sodium EDTA). The RNA was transferred to Biodyne A membranes (Poll, CA) in 20× SSC [3 mM NaCl and 0.3 mM sodium citrate (pH 7.0)]. Fifty to 100 ng of cDNA probe were labeled with [α-32P]dCTP (NEN, Boston, MA) by random oligoprimer extension. The nylon membranes were UV cross-linked and hybridized.

**Statistical Analysis.** All determinations were made in triplicate, and the results were expressed as the mean ± SD. Statistical significance was determined by Student’s t test. A P of ≤0.05 was considered significant.

**Calculations of synergistic cytotoxicity were determined by isobolographic analysis, as described by Berenbaum (23, 24). The nature of the effect of a particular dose combination was determined by isobolographic analysis, as follows: the point representing that combination would lay on, below, or above the straight line joining the doses of the two drugs that, when given alone, produce the same effect as that combination, representing additive, synergistic, or antagonistic effects, respectively.

**RESULTS**

**Synergistic Cytotoxicity against Bladder Cancer Cells after Their Treatment with a Combination of sApo-2L and ADR**

We investigated the cytotoxic effect of a combination of sApo-2L and ADR against the T24 bladder cancer cell line. Cytotoxicity was determined by a 1-day MTT assay, and synergy was assessed by isobolographic analysis, as described in “Materials and Methods.” The T24 line was relatively resistant to sApo-2L- and ADR-mediated cytotoxicity. However, when T24 cells were treated with a combination of sApo-2L and ADR, significant potentiation of cytotoxicity and synergy was achieved (Fig. 1). Preliminary experiments demonstrated that the synergy was not observed when normal bladder cells were used as targets.

To confirm the synergy, we then examined the sensitivity of the T24 ADR-resistant variant cell line, T24/ADR, to treatment with sApo-2L in combination with ADR. Significant synergy was achieved by the combination treatment with sApo-2L and ADR (data not shown). Furthermore, synergy was also obtained in two other bladder cancer lines, J82 and HT1197 (data not shown).

On the basis of the above findings with established bladder cancer cell lines, we examined for synergy on three freshly isolated bladder cancer cells. In all three cases, significant synergy was achieved, irrespective of the baseline sensitivity of the cancer cells to either ADR or sApo-2L used alone (Fig. 2).

These findings demonstrate that treatment of established and freshly derived bladder cancer cells with a combination of sApo-2L and ADR resulted in potentiation of cytotoxicity. Furthermore, the synergy was observed with both sensitive and resistant cancer cells. In all of the above findings, synergy was achieved with subtoxic concentrations of ADR. The synergy obtained with low concentrations of the agents is of clinical relevance because high concentrations of drugs are toxic in vivo.

**Effect of the Sequence of Treatment with sApo-2L and ADR on Synergy**

The findings above demonstrate that simultaneous treatment of T24 cells with the sApo-2L and ADR resulted in synergy. The effect of sequential treatment with sApo-2L and ADR was examined and compared with treatment with both agents together. The T24 bladder cancer cells were treated for 6 h with one agent, the medium was removed, the second agent was subsequently added for another 18 h, and the cells were tested for viability. The results show that the highest percentage cytotoxicity was obtained when sApo-2L was given first or together with ADR (Table 1). Similar results were obtained

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**Table 1 Effect of sequence of treatment with sApo-2L and ADR on their cytotoxic activity against T24 cells**

<table>
<thead>
<tr>
<th>First treatment (6 h)</th>
<th>Second treatment (18 h)</th>
<th>% cytotoxicity (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sApo-2L Medium</td>
<td>ADR Medium</td>
<td>5.8 ± 0.9</td>
</tr>
<tr>
<td>ADR Medium</td>
<td>sApo-2L Medium</td>
<td>10.8 ± 1.5</td>
</tr>
<tr>
<td>Medium</td>
<td>sApo-2L</td>
<td>11.8 ± 1.0</td>
</tr>
<tr>
<td>Medium</td>
<td>ADR</td>
<td>28.7 ± 2.9</td>
</tr>
<tr>
<td>Medium</td>
<td>sApo-2L + ADR</td>
<td>74.8 ± 6.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>sApo-2L</td>
<td>ADR</td>
<td>68.9 ± 5.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADR</td>
<td>sApo-2L</td>
<td>49.4 ± 1.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> T24 cells were pretreated with medium only, sApo-2L (100 ng/ml), or ADR (10 μg/ml) for 6 h (first treatment). The medium was aspirated, and T24 cells were washed twice with RPMI 1640. The cells were then incubated with sApo-2L (100 ng/ml) and/or ADR (10 μg/ml) for 18 h (second treatment). Cytotoxicity was assessed in a 1-day MTT assay.

<sup>b</sup> Results are expressed as the mean ± SD of three separate experiments.

<sup>c</sup> Values in the combination treatment are significantly higher than those achieved by treatment with ADR alone at P < 0.05.

<sup>d</sup> Values in the combination treatment are significantly higher than those achieved when ADR was given first at P < 0.05.
when sApo-2L and ADR were used at different concentrations (data not shown). These findings demonstrate that the sequence of treatment with sApo-2L and ADR may not be critical to obtain significant synergy but may determine the extent of synergy.

**Effect of the ADR-related Cytotoxic Drugs, EPI and THP, on Synergy with sApo-2L.**

Two closely related compounds of ADR, EPI and THP, are currently used clinically. The cytotoxic effect of these two drugs used each in combination with sApo-2L was tested against T24 cells. Synergy was achieved with both EPI and THP in combination with sApo-2L (Fig. 3).

**Mechanism of Synergy Achieved by sApo-2L and ADR**

**Induction of Apoptosis.** Because both sApo-2L and ADR can mediate apoptosis, we examined by Hoechst 33258 staining whether the synergy achieved in cytotoxicity with sApo-2L and ADR was also achieved in apoptosis. Apoptosis was defined when apoptotic bodies, chromatin condensation, or fragmented nuclei were observed. No apoptosis was seen in T24 cells cultured in medium. ADR at a concentration of 1 μg/ml was cytotoxic against T24 cells and mediated modest apoptosis (Fig. 4B). Swelling of T24 cells was also observed. Likewise, sApo-2L, at a concentration of 100 ng/ml, was slightly cytotoxic against T24 cells and also mediated some apoptosis (Fig. 4C). When sApo-2L and ADR were used in combination, fragmented nuclei of T24 cells were observed. We considered almost all T24 cells apoptotic, and synergy...
in apoptosis was also observed (Fig. 4D). The apoptosis was also examined by DNA ladder assay. Treatment of T24 cells with combination of sApo-2L and ADR resulted in strong DNA ladder (data not shown). These results indicate that there was a good correlation between cytotoxicity and apoptosis after treatment of bladder cancer cells with a combination of sApo-2L and ADR.

ADR Intracellular Accumulation. A possible mechanism of augmentation of cytotoxicity may be the result of increased accumulation of ADR in the cells treated with sApo-2L. When T24 cells were treated with combination of ADR and sApo-2L, ADR accumulation inside the cells did not change significantly (Table 2).

Table 2 Effect of treatment of T24 cells with sApo-2L on the intracellular accumulation of ADR

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intracellular accumulation of ADR (ng/10^5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>35.8 ± 4.2</td>
</tr>
<tr>
<td>sApo-2L</td>
<td>33.1 ± 5.0</td>
</tr>
</tbody>
</table>

* T24 cells were treated with ADR (10 μg/ml) in combination with medium or sApo-2L (100 ng/ml) for 12 h. The medium was aspirated, and T24 cells were washed three times with RPMI 1640. The intracellular concentration of ADR was measured by high-performance liquid chromatography, as described in “Materials and Methods.” The results are expressed as the mean ± SD of three separate experiments.

Expression of GST-π mRNA. Previous findings indicated that ADR resistance may be, in part, due to up-regulation of the antioxidant enzyme GST-π expression (25, 26). T24 cells constitutively expressed mRNA for GST-π (Fig. 5). Treatment of T24 cells with sApo-2L down-regulated GST-π mRNA expression.

Altogether, these findings suggest that the synergistic cytotoxicity achieved with sApo-2L and ADR is, in part, due to up-regulation of apoptosis and down-regulation of GST-π mRNA expression.

DISCUSSION

Here, we showed that the combined treatment of sApo-2L and ADR resulted in a synergistic cytotoxicity and apoptosis
against bladder cancer cells and reversed their resistance. It has been reported that ADR augmented Apo-2-induced apoptosis in breast cell lines (27). The synergy was achieved with low concentrations of each agent, thus minimizing their toxicity in vivo and maximizing their therapeutic application in vivo. Because Apo-2L is not toxic to most of normal tissues (3, 4), a combination of sApo-2L and ADR may be a promising strategy to eliminate bladder cancer cells.

The mechanisms responsible for cellular resistance to ADR are believed to be multifactorial and include alterations in the transmembrane transport of ADR, decreased formation of DNA single- and double-strand breaks, earlier onset of DNA repair, the cytosolic quenching of ADR due to increased levels of glutathione and its related enzymes, and the decreased cellular level of DNA Topo II. Alterations in the transmembrane transport of ADR in cancer cells by P-glycoprotein or multidrug resistance-associated protein result in reduced intracellular accumulation of ADR and resistance to ADR (28, 29). The resistance to ADR has been attributed to reduced levels of DNA single- and double-stranded breaks induced by the drug in ADR-resistant P388 leukemia cells (30, 31). The ADR-resistant P388 cells appeared to display an earlier onset of DNA repair than did drug-sensitive cells (32). Some ADR-resistant cancer cells have higher levels of intracellular glutathione or its related enzymes (25, 26). Because ADR inhibits DNA Topo II, the reduced cellular level of DNA Topo II has been proposed as a possible mechanism of cancer cell resistance to ADR (32, 33).

Several possible mechanisms of resistance to the killing of cells by Apo-2L in cancer cells have been reported, such as lack of the expression of Apo-2L receptors, DR4 and DR5, and the enhanced expression of antagonistic Apo-2L receptors, Drk1 and Drk2 (5, 6, 8). The existence of multiple receptors for Apo-2L suggests an unexpected complexity in the regulation of signaling by this cytokine. Protection against apoptosis via synthesis of an intracellular protein is a well-established paradigm. The protein product of bcl-2 has been shown to inhibit DNA fragmentation induced by a variety of stimuli (34). Because Apo-2L induces apoptosis in target cells in a caspase-dependent fashion, the resistance to Apo-2L might be dependent on the level of the expression of caspases (35, 36). Further studies are required to elucidate the mechanisms responsible for the acquisition of resistance of bladder cancer cells to Apo-2L-mediated cytotoxicity.

Although the down-regulation of GST-π mRNA expression by sApo-2L is suggestive for synergistic cytotoxicity of sApo-2L and ADR, the precise mechanism of the synergy by combination treatment with sApo-2L and ADR is not fully understood. The expression of GST-π protein might have been increased by posttranslational stabilization of the protein, or the activity of GST-π itself might have been increased. The mechanisms responsible for synergistic cytotoxicity by combination treatment of sApo-2L and ADR await further investigation.

Both Apo-2L and Fas ligand are coexpressed on the cell surface of immune cells (37, 38). Apo-2L as well as Fas ligand may play an important role in cytotoxic T cell-mediated apoptosis in cancer cells (39). The cells that are resistant to Fas-mediated apoptosis are sensitive to Apo-2L-mediated apoptosis (39). In this study and in a previous report, it has been shown that treatment with ADR in combination with sApo-2L or anti-Fas mAb resulted in significant potentiation of cytotoxicity and synergy against bladder cancer cells (10). In addition, preliminary experiments showed that treatment of freshly isolated bladder cancer cells with ADR enhanced their susceptibility to lysis by autologous lymphocytes. These findings suggest that a combination of ADR chemotherapy and immunotherapy might be an alternative approach in the treatment of ADR-immunotherapy-resistant bladder cancer.

The overall response rate of patients with bladder cancer to current anticancer chemotherapeutic agents involving ADR has improved. However, drug resistance and recurrence of cancers remain major problems, and a more effective therapy is necessary for these patients. This study shows that combination treatment with sApo-2L and ADR resulted in a synergistic cytotoxicity and apoptosis against both acquired and natural ADR-resistant bladder cancer cells, and the synergistic effect is not restricted to established cell lines: it is also observed in freshly derived cancers. These findings suggest that the therapeutic use of ADR in combination with sApo-2L might be useful in patients with ADR-resistant bladder cancer.

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