Enhancement of Fas-mediated Apoptosis in Renal Cell Carcinoma Cells by Adriamycin

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ABSTRACT

Anti-Fas monoclonal antibody (mAb) kills Fas-expressing cells by apoptosis. Several anticancer agents also mediate apoptosis and may share common intracellular pathways leading to apoptosis with Fas. Thus, we reasoned that combination treatment of drug-resistant cells with anti-Fas mAb and drugs might overcome their resistance. We investigated whether anticancer agents enhance Fas-mediated apoptosis and cytotoxicity against renal cell carcinoma (RCC) cells. Treatment of ACHN RCC cells with anti-Fas mAb in combination with 5-fluorouracil, vinblastine, IFN-α, or IFN-γ did not overcome resistance to these agents. However, combination treatment with anti-Fas mAb and Adriamycin (ADR) resulted in a synergistic cytotoxic effect. Furthermore, synergy was also obtained even when the exposure time was shortened from 24 h to 0 or 2 h. Synergy was also achieved in four other RCC cell lines and five freshly derived human RCC cells. Treatment with anti-Fas mAb in combination with epirubicin or pirarubicin also resulted in a synergistic cytotoxic effect on ACHN cells. Similar results were achieved with a combination of humanized anti-Fas mAb and ADR. Incubation of ACHN cells with ADR augmented the expression of Fas and p53, but not Bcl-2, Bax, or caspase-3. However, the activity of caspase-3 itself was apparently enhanced after treatment with ADR alone or combined treatment with anti-Fas mAb. The synergy obtained in cytotoxicity with anti-Fas mAb and ADR was also achieved in apoptosis. Exposure of ACHN cells and freshly derived RCC cells to ADR enhanced their susceptibility to lysis by peripheral blood lymphocytes and tumor-infiltrating lymphocytes. This study demonstrates that combination treatment of RCC cells with anti-Fas mAb and ADR might overcome their resistance. The sensitization required a low concentration of ADR and a short exposure time, thus supporting the potential in vivo application of a combination of ADR and anti-Fas mAb or immunotherapy in the treatment of ADR- and/or immunotherapy-resistant RCC.

INTRODUCTION

The intrinsic or acquired resistance of cancer cells to chemotherapyapeutic drugs remains a major obstacle to the successful treatment of human cancer. No chemotherapeutic drug available at present is effective against RCC. New therapeutic modalities, such as combination treatment with biological response modifiers and anticancer agents, have been considered as a possible means to reverse drug resistance. Our previous study (1) has shown that treatment with anti-IL-6 mAb or anti-IL-6 receptor mAb in combination with anticancer drugs resulted in a significant improvement of cytotoxicity and synergy against a variety of drug-sensitive and -resistant RCC cells.

Fas (Apo-1/CD95), a receptor for Fas ligand, belongs to the tumor necrosis factor family and mediates apoptosis in Fas-expressing cells (2–4). Fas-mediated apoptosis plays an important role in cytotoxic T cell-mediated and natural killer cell-mediated cytotoxicity and apoptosis against cancer cells (5, 6). Activated cytotoxic T cells and natural killer cells up-regulate Fas ligand on their surfaces and induce apoptosis in cancer cells expressing Fas. Cross-linking of Fas with anti-Fas mAb causes apoptosis in Fas-bearing cells (7, 8). For years it has been known that several anticancer agents also induce apoptosis and may share common intracellular pathways leading to cell killing with Fas (9–11). We reasoned that combination treatment of drug-resistant cells with anti-Fas mAb and drugs might overcome their resistance.

In this study, we investigated whether the resistance of RCC cells to anticancer agents or biological response modifiers could be overcome by combination treatment with anti-Fas mAb and anticancer agents or biological response modifiers such as ADR, 5-FU, VLB, IFN-α, or IFN-γ. Furthermore, this study explored possible underlying mechanisms that may be involved in the reversal of drug resistance.

MATERIALS AND METHODS

Cell Lines and Tumor Explants. ACHN, Caki-1, Caki-2, A704, and NC65 RCC cell lines (12, 13) were cultured in RPMI 1640 (Life Technologies, Inc., Glasgow, United Kingdom) supplemented with 25 mm HEPES (Life Technologies, Inc.), 2 mm l-glutamine (Life Technologies, Inc.), 1% nonessential amino acid (Life Technologies, Inc.), 100 units/ml penicillin (Life Technologies, Inc.), 100 μg/ml streptomycin (Life Technologies, Inc.), and 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.), referred to hereafter as complete medium.

Fresh RCC cells derived from five patients were separated from surgical specimens as described previously (14, 15). The histological diagnosis revealed that all patients had RCC of the alveolar type and clear cell subtype. Their histological classification and staging according to the TNM classification was as follows: (a) Patient 1, T2N0M0, grade 2; (b) patient 2, T3N0M0, grade 2; (c) patient 3, T4aN0M0, grade 2; (d) patient 4, T2N0M0, grade 2; and (e) patient No 5, T4aN0M0, 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 three washes in complete medium, the cell suspensions were laid on discontinuous gradients consisting of 4 ml of 100% and 4 ml of 75% Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) in 15-ml plastic tubes and centrifuged at 400 × g for 30 min. Cancer cells were collected from the 75% interface, and lymphocyte-rich mononuclear cells were collected from the 100% interface. Cell suspensions enriched with cancer cells were sometimes contaminated by macrophages, mesothelial cells, or lymphocytes. To eliminate further contamination of host cells, we layered the cell suspension on discontinuous gradients containing 25%, 15%, and 10% Ficoll-Paque and centrifuged them at 25 × g for 7 min. Cancer cells depleted of lymphocytes were collected from the bottom, washed, and suspended in complete medium. Cells having less than 5% contamination with nonmalignant cells were used as RCC cells.

Effector Cells. PBLs were obtained from heparinized peripheral blood before anticancer therapy as described previously (16). The blood was immediately centrifuged for 20 min at 400 × g. Mononuclear cells and polymorphonuclear cells were collected from the interface of erythrocytes. The cells were suspended in complete medium. The cell suspension was layered on 100% Ficoll-Paque and centrifuged at 400 × g for 30 min. The lymphocyte-rich mononuclear cells were collected from the 100% interface. The cells were washed three times in RPMI 1640 and suspended in complete medium.

The mononuclear cells were incubated at 37°C for 1 h on plastic dishes that had been precoated with autologous serum at 37°C for 15 min in a humidified 5% CO2 atmosphere. After incubation, the nonadherent cells were collected,

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2 The abbreviations used are: RCC, renal cell carcinoma; mAb, monoclonal antibody; IL, interleukin; ADR, Adriamycin; 5-FU, 5-fluouracil; VLB, vinblastine; PBL, peripheral blood lymphocyte; TIL, tumor-infiltrating lymphocyte; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pNA, p-nitroanilide; EPI, epirubicin; THP, pirarubicin; wt, wild-type; TNM, tumor-node-metastasis.
ENHANCED FAS-MEDIATED APOPTOSIS BY ADR

Synergistic Cytotoxicity of Anti-Fas mAb and ADR. We examined whether treatment of the ACHN RCC cell line with anti-Fas mAb in combination with ADR, 5-FU, VLB, IFN-α, or IFN-γ resulted in synergistic cytotoxic activity. There was no synergistic effect of anti-Fas mAb in combination with 5-FU, VLB, IFN-α, or IFN-γ (data not shown). However, when ACHN cells were treated with a combination of anti-Fas mAb and ADR, a significant potentiation of cytotoxicity and synergy was achieved (Fig. 1). Furthermore, the synergistic effect was achieved when the treatment with anti-Fas mAb and ADR was shortened from 24 h to 8 or 2 h (Fig. 2) and when the treatment was extended to 48 h (data not shown).

The synergy was specific for the anti-Fas mAb. ADR in combination with control IgM had no synergistic effect (data not shown). Furthermore, the synergy obtained with the ACHN cell line was not selective for this cell line because four other RCC cell lines, A704, Caki-1, Caki-2, and NC65, were also sensitized to anti-mAb and ADR cytotoxicity (data not shown).

To determine whether the synergy was a reflection of the properties of established cancer cell lines, we tested for synergy in five freshly derived RCC cells. In all cases, significant synergy was achieved, irrespective of the sensitivity of the cancer cells to either ADR or anti-Fas mAb when each was used alone (Fig. 3).

Altogether, these findings clearly demonstrated that treatment of RCC cell lines or freshly derived RCC cells with a combination of anti-Fas mAb and ADR resulted in a potentiation of cytotoxicity. In all cases, synergy was achieved with subtoxic concentrations of ADR.

Effect of EPI and THP on Synergy with Anti-Fas mAb. Two compounds closely related to ADR, namely, EPI and THP, were tested for their cytotoxic effect on ACHN cells when used in combination with anti-Fas mAb. Clearly, synergy was achieved with both EPI (Fig. 4) and THP in combination with anti-Fas mAb.

Synergistic Cytotoxicity of Humanized Anti-Fas mAb and ADR. With an eye toward clinical application, we examined the cytotoxicity of ADR combined with humanized anti-Fas mAb instead of anti-Fas mAb on ACHN cells. Synergy can also be achieved with ADR in combination with humanized anti-Fas mAb (Fig. 5).

Effect of the Sequence of Treatment with Anti-Fas mAb and ADR on Synergy. The findings above demonstrated that simultaneous treatment of RCC cells with anti-Fas mAb and ADR resulted in synergy. The effect of sequential treatment with these two agents was examined next. ACHN cells were treated for 6 h with one agent, and then the medium was removed, the second agent was added for 18 h,
and the cells were tested for viability. The results showed that the cytotoxicity was enhanced irrespective of the sequence of treatment (Table 1). These findings indicate that the sequence of treatment with anti-Fas mAb and ADR is not critical for obtaining synergy.

Expression of Fas, p53, Bcl-2, Bax, and Caspase-3. We have examined by immunocytochemical analysis whether ADR regulates the expression of Fas, p53, Bcl-2, Bax, and caspase-3 in RCC cells. Treatment of ACHN cells with anti-Fas mAb (0.01–1 μg/ml) and ADR (0.1–10 μg/ml) used in combination for ACHN cells was measured by a 1-day MTT assay. B, synergy was assessed by isobolographic analysis. Results are derived from three different experiments.

Induction of Apoptosis. Because both anti-Fas mAb and ADR can mediate apoptosis, we examined by acridine orange staining whether
the synergy in cytotoxicity obtained with anti-Fas mAb and ADR was also achieved in apoptosis. Each agent mediated apoptosis in some cells. When anti-Fas mAb and ADR were used in combination, almost all ACHN cells were apoptotic, and synergy in apoptosis was observed (Fig. 7). These results indicate that there was a close correlation between cytotoxicity and apoptosis after combination treatment of RCC cells with anti-Fas mAb and ADR.

**DISCUSSION**

The present study showed that anti-Fas mAb and ADR had a synergistic cytotoxic effect on RCC cells resistant to each agent when used alone. Synergy was achieved in the ACHN cell line, four other RCC cell lines, and five freshly derived RCC cells. It was achieved even when the treatment time was shortened from 24 h to 8 and 2 h and required a relatively low concentration of each agent. In addition,

![Synergistic cytotoxicity of anti-Fas mAb and EPI for ACHN cells.](image)

**Fig. 4.** Synergistic cytotoxicity of anti-Fas mAb and EPI for ACHN cells. A, the cytotoxicity of anti-Fas mAb (0.01–1 μg/ml) and EPI (0.1–10 μg/ml) used in combination for ACHN cells was measured by a 1-day MTT assay. B, synergy was assessed by isobolographic analysis. Results are derived from three different experiments.

![Synergistic cytotoxicity of humanized anti-Fas mAb and ADR for ACHN cells.](image)

**Fig. 5.** Synergistic cytotoxicity of humanized anti-Fas mAb and ADR for ACHN cells. A, the cytotoxicity of humanized anti-Fas mAb (0.01–1 μg/ml) and ADR (0.1–10 μg/ml) used in combination on ACHN cells was measured by a 1-day MTT assay. B, synergy was assessed by isobolographic analysis. Results are derived from three different experiments.
humanized anti-Fas mAb and ADR also had a synergistic cytotoxic effect on ACHN cells. These data strongly suggest that combination treatment using anti-Fas mAb and ADR is promising from a clinical perspective.

The principal Fas-mediated apoptotic signaling pathways have been recently established. Binding of Fas ligand to Fas induces trimerization of Fas, and Fas-associated death domain binds to the trimerized Fas cytoplasmic region through the interaction of the respective death domain (21). Caspase-8 is then recruited to Fas-associated death domain through the binding of the death effector domains, which, in turn, may trigger self-activation of the cascade of caspases (22, 23). The activated caspases can cleave various substrates such as poly-(ADP-ribose) polymerase, lamin, and actin and cause morphological changes to the cells (23, 24). Because treatment of ACHN cells with ADR up-regulated the expression of Fas, the up-regulation is a possible mechanism of overcoming their resistance.

In addition to Fas, many proteins encoded by p53, bcl-2, bax, and cpp32 genes are involved in the modulation of apoptosis. The presence of functional wt p53 is closely correlated with an efficient induction of Fas-mediated apoptosis in many cell types (25–27). Overexpression of ectopic wt p53 in non-small cell lung cancer and an erythroleukemic cell line resulted in the up-regulation of Fas (28). Furthermore, it has been shown that wt p53 can activate the Fas gene in a variety of human solid cancer cell lines after DNA damage by anticancer agents (29). These findings indicate that p53 regulates

Table 1 Effect of the sequence of treatment with anti-Fas mAb and ADR on cytotoxic activity against ACHN cells

<table>
<thead>
<tr>
<th>First treatment* (6 h)</th>
<th>Second treatment* (18 h)</th>
<th>% cytotoxicity (Mean ± SD)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Anti-Fas mAb</td>
<td>6.7 ± 1.9</td>
</tr>
<tr>
<td>Medium</td>
<td>ADR</td>
<td>16.9 ± 3.1</td>
</tr>
<tr>
<td>Medium</td>
<td>Anti-Fas mAb plus ADR</td>
<td>37.8 ± 2.2$^c$</td>
</tr>
<tr>
<td>Anti-Fas mAb</td>
<td>ADR</td>
<td>37.1 ± 5.0$^c$</td>
</tr>
<tr>
<td>ADR</td>
<td>Anti-Fas mAb</td>
<td>38.0 ± 2.3$^c$</td>
</tr>
</tbody>
</table>

$^a$ ACHN cells were pretreated with medium only, anti-Fas mAb (1 μg/ml), or ADR (10 μg/ml) for 6 h (first treatment). Medium was aspirated, and ACHN cells were washed twice with RPMI 1640. The cells were then incubated with anti-Fas mAb (1 μg/ml) and/or ADR (10 μg/ml) for 18 h (second treatment). The cytotoxicity was measured by a 1-day MTT assay.

$^b$ Results are expressed as the mean ± SD of three separate experiments.

$^c$ Values for combination treatments are significantly higher than those achieved by treatment with ADR alone plus anti-Fas mAb alone ($P < 0.025$).

Table 2 Effect of treatment with anti-Fas mAb and ADR on caspase-3 activity in ACHN cells

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Caspase-3 activity$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (medium)</td>
<td>0.031 ± 0.002</td>
</tr>
<tr>
<td>Anti-Fas mAb</td>
<td>0.039 ± 0.005</td>
</tr>
<tr>
<td>ADR</td>
<td>0.057 ± 0.002$^c$</td>
</tr>
<tr>
<td>Anti-Fas mAb and ADR</td>
<td>0.193 ± 0.012$^d$</td>
</tr>
</tbody>
</table>

$^a$ ACHN cells were pretreated with medium only, anti-Fas mAb (1 μg/ml), and/or ADR (10 μg/ml) for 24 h. After treatment, caspase-3 activity was measured using a CPP32/Caspase-3 Colorimetric Protease Assay Kit.

$^b$ Results are expressed as the mean ± SD of three separate experiments.

$^c$ $P = 0.0004$.

$^d$ Values for combination treatments are significantly higher than those achieved by treatment with ADR alone plus anti-Fas mAb alone ($P = 0.0016$).
Fas-mediated apoptosis by up-regulating Fas expression and activating the Fas gene. In this study, treatment of ACHN cells with ADR caused up-regulation of p53 expression and resulted in Fas-mediated apoptosis. However, when prostate cancer DU-145 cells (mutant for p53; Ref. 30) were used as targets, no synergy was obtained. Although a more quantifiable assay such as Western blot analysis is necessary to evaluate p53 and Fas expression, these results suggest that p53 plays an important role in the synergistic cytotoxicity of anti-Fas mAb and ADR in RCC cells.

Bcl-2 protein is able to inhibit DNA fragmentation induced by a variety of stimuli (31, 32). Furthermore, overexpression of Bcl-2 inhibits Fas-mediated apoptosis in several cell lines through the inhibition of caspase-8 and caspase-3 activation (33–35). In contrast, overexpressed Bax activates apoptosis and also counters the apoptotic repressor activity of Bcl-2 (36). In present study, the treatment of ACHN cells with ADR did not affect Bcl-2 or Bax expression. Thus, Bcl-2 and Bax might not be crucial molecular mediators in the enhancement of Fas-mediated apoptosis in RCC cells by ADR.

Caspase-3 is constantly expressed in an inactive precursor form that can be activated during apoptosis (37, 38). Recently, it was shown that active caspase-3 releases a caspase-activated DNase from its inhibitory complex, which enables caspase-activated DNase to enter the nucleus and degrade genomic DNA (39). The present study showed that treatment of ACHN cells with ADR had no effect on the expression of caspase-3. Interestingly, however, the activity of caspase-3 itself was apparently enhanced after treatment with ADR alone or combination treatment with ADR and anti-Fas mAb. These findings indicate that caspase-3 activated by ADR and anti-Fas mAb is a crucial molecular mediator in the synergistic cytotoxicity.

Altogether, these results suggest that ADR enhances Fas-mediated apoptosis and cytotoxicity against RCC cells by augmenting Fas and p53 expression and activating caspase-3.

The present study showed that treatment of ACHN cells and freshly isolated RCC cells with ADR augmented their susceptibility to lysis by PBLs and TILs. The enhanced Fas-mediated cytotoxicity and apoptosis against RCC cells might be responsible, at least in part, for the augmented susceptibility of ADR-treated RCC cells to lysis by PBLs and TILs.

The chemotherapeutic drug resistance of RCC cells remains a major obstacle to successful treatment, and a more effective therapy is needed. The present study showed that anti-Fas mAb and ADR had synergistic cytotoxic activity for RCC cells and that this effect was not restricted to established cell lines but was also observed in freshly derived RCC cells. Furthermore, the sensitization required a low concentration of ADR and a short treatment time. These findings suggest that the therapeutic use of ADR in combination with Fas-mediated immunotherapy would be useful in patients with ADR- and/or immunotherapy-resistant RCC.

### REFERENCES


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