A Role for the Interleukin 1 Receptor in the Synergistic Antitumor Effects of Human Interleukin 1α and Etoposide against Human Melanoma Cells

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ABSTRACT

To investigate the possibility that anticancer drugs combined with cytokines may show increased activity, human tumor cells were treated with combinations of human recombinant interleukin 1α (rIL-1α) and etoposide (VP-16). The cytotoxicity of these combinations was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay using rIL-1α-sensitive A375-C6 melanoma cells and A375-CS cells, a clonal variant line that is resistant to IL-1α. Data were analyzed for synergism by the median effect principle of T.-C. Chou and P. Talalay (J. Biol. Chem., 252: 6438-6442, 1977). At a dose ratio of VP-16 to rIL-1α of 12:1 unit/ml in either simultaneous or sequential exposure (VP-16 first), the calculated combination index values indicated synergistic cytotoxicity toward both A375-C6 cells and A375-CS cells. IL-1α treatment 24 h prior to VP-16 exposure had no advantage over simultaneous treatment. Surface IL-1α receptors on both A375-C6 and A375-CS cells were measured using 125I-radiolabeled rIL-1α binding; A375-CS cells had 701 ± 128 (SD) receptor molecules/cell and A375-CS cells only had 58 ± 33 receptor molecules/cell. The dissociation constants for IL-1α were similar in both cell types (19 ± 6 pm for A375-C6 and 17 ± 2 pm for A375-CS). The specific binding of rIL-1α to the surface IL-1α receptors of both sensitive and resistant cells was significantly increased in a dose-dependent fashion by the prior treatment with VP-16 (1.75-fold on A375-C6 cells and 3.5-fold on A375-CS cells). VP-16 also enhanced the internalization of receptor-bound rIL-1α, suggesting that a possible mechanism of the synergistic cytotoxicity of rIL-1α and VP-16 might be related to the modulation of rIL-1α receptors by VP-16, resulting in increased internalization of rIL-1α.

INTRODUCTION

IL-1α, a cytokine produced predominantly by monocytes and macrophages, has many diverse activities in vivo and in vitro including antitumor effects (1). Direct antitumor activity of IL-1 has been reported against malignant melanoma in vitro, breast cancer cells in vitro, and murine pancreatic cancer in vivo (2-8). However, the efficacy of IL-1 alone against various cancers is limited because of its severe host toxicity in the clinic. Although the mechanism(s) by which IL-1 induces cell killing is not clear yet, the binding of IL-1 to its membrane receptors and subsequent internalization are essential for cytotoxicity.

VP-16, a synthetic derivative of podophyllotoxin, is clinically active against several human cancers (9). The mechanism of the antitumor effects of VP-16 has been suggested to result from its binding and stabilization of the cleavable DNA-topoisomerase II complex, resulting in both DNA single- and double-strand breaks. In addition, metabolic activation of VP-16 by cytochrome P-450 and prostaglandin synthase to reactive species has also been indicated to contribute significantly in tumor cell kill (10-13).

Recently, combinations of cytotoxic agents and biological response modifiers have evolved as an important modality for treating cancers, because it is assumed that these agents are different from chemotherapeutic agents (14, 15). IL-1 showed synergistic antitumor effects in combination with another cytokine, tumor necrosis factor-α, toward malignant melanoma (16) and chondrosarcoma (17). Recently, studies from our laboratory have shown that synergistic antitumor effects of rIL-1α combined with VP-16 for A375-C6 melanoma cells, which are sensitive to IL-1, and A375-CS cells, a variant line 10-fold resistant to IL-1 (18). In this study, we have investigated the interactions between rIL-1α and VP-16 by comparing several drug administration schedules and dose ratios of the 2 agents. Additionally, we examined the effects of each agent on the cellular accumulation of the other. A possible mechanism of the synergism between rIL-1α and VP-16 may be related to the increased membrane receptor binding and internalization of rIL-1α following VP-16 treatment.

MATERIALS AND METHODS

Drugs and Reagents. rIL-1α (specific activity, 2.26 X 10⁶ units/mg protein; Mn, 18,063) was generously provided by Dainippon Pharmaceutical Co. (Osaka, Japan). VP-16 was a gift from Bristol Myers Co. (Syracuse, NY). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, glycine, and dimethyl sulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640, trypsin-EDTA, fetal bovine serum, antibiotic mixture, and phosphate buffered saline were purchased from Gibco Laboratories (Grand Island, NY). 125I-labeled rIL-1α (specific activity, 2000 Ci/mmol; Mn, 18,200) was purchased from Amersham (Arlington Heights, IL).

Cells. Human melanoma A375-C6 (IL-1α-sensitive clone) cells and A375-CS (IL-1α-resistant clone) cells, kindly provided by Dr. Kouji Matsushima, National Cancer Institute, were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotic mixture under standard culture conditions at 37°C in a humidified 5% CO₂ atmosphere.

Cytotoxicity Assay. The antitumor activity of drugs was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide assay (19). For the sequential administration, the first drug was removed and the cells were washed once by drug-free medium before adding the second drug. The evaluation for the synergy of the combinations of rIL-1α and VP-16 was carried out by the analysis developed by Chou and Talalay (20-22).

125I-rIL-1α Receptor Binding Assay. The cells (1.2 X 10⁶/well in 12-well tissue culture plates) were incubated with a range of concentrations of 125I-labeled rIL-1α (specific activity, 2000 Ci/mmol) in 0.5 ml of cold RPMI 1640 supplemented with 10% fetal bovine serum in the absence or presence of a 1000-fold excess of unlabeled rIL-1α for 2 h at 4°C. After the cells were washed with cold complete medium, 0.5 ml of 1 N NaOH was added to dissolve the cells and release the rIL-1α. Subsequently, the contents of the wells were transferred into scintillation vials, the wells were rinsed with 0.5 ml of 1 N HCl, and 10 ml of Hydrofluor (Manville, NJ) were added to each vial for liquid scintillation counting of the bound 125I-labeled rIL-1α.

To investigate the effects of VP-16 on the IL-1α receptor, the cells...
were maintained in culture in the presence or absence of several concentrations of VP-16 for 24 h, and then evaluated for $^{125}$I-labeled IL-1α receptor binding as described above.

Internalization of rIL-1α. The kinetic analysis of intracellular rIL-1α was carried out as reported previously (23, 24) with minor modifications. Briefly, the cells were treated with 5 ng/ml of $^{125}$I-labeled rIL-1α for 0–48 h at 37°C, then exposed to an ice-cold solution of 50 mM glycine-150 mM sodium chloride (pH 3.0) for 8 min. The acid-labile fraction, which corresponded to cell surface IL-1α labeling, was removed and cells were then lysed by adding 1 N NaOH. The intracellular, acid-resistant $^{125}$I-labeled IL-1α was then quantitated by liquid scintillation counting. To analyze the effects of VP-16 on the intracellular rIL-1α, the cells were treated with sublethal doses of VP-16 for 24 h before adding $^{125}$I-labeled IL-1α to the cells.

Effects of rIL-1α on Cellular Uptake of VP-16. After treatment with 84 units/ml of rIL-1α for 24 h, cells were exposed to a drug mixture containing 0.5 μCi/ml $^{3}H$-labeled VP-16 plus unlabeled VP-16 dissolved in fresh complete culture medium (final concentration, 10 μM). Cells were incubated at 37°C under 5% CO₂ atmosphere for various times, and the uptake of VP-16 was carried out as described by Politi and Sinha (25).

Statistical Analysis. Data were analyzed by the Student's t test and values of $P < 0.05$ were considered statistically significant.

RESULTS

Combination Cytotoxic Effects of IL-1α and VP-16. The median dose values of VP-16 and rIL-1α are shown in Table 1. The reduction of the median dose of VP-16 for A375-C5 IL-1-resistant cells from the single administration compared with the median dose of the combination administration was greater than that of A375-C6 IL-1-sensitive cells. Based on the values of median doses obtained from our previous experiments on the cytotoxicity of rIL-1α and VP-16, we fixed the ratio of doses as VP-16:rIL-1α = 12 (nm)/1 (unit/ml) to analyze for synergism by the combination effect method of Chou and Talalay (20–22). This ratio of VP-16 and rIL-1α was equal to 4800:1 on molar basis based upon the $M_r$, 18,000 for rIL-1α.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Single</th>
<th>Combination</th>
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<tbody>
<tr>
<td></td>
<td>VP-16 (nm)</td>
<td>rIL-1α (units/ml)</td>
</tr>
<tr>
<td>Simultaneous exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A375-C6</td>
<td>225</td>
<td>6.3</td>
</tr>
<tr>
<td>A375-C5</td>
<td>382</td>
<td>51.9</td>
</tr>
<tr>
<td>Sequential exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP-16 6 h prior to rIL-1α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A375-C6</td>
<td>4769</td>
<td>6.2</td>
</tr>
<tr>
<td>A375-C5</td>
<td>4727</td>
<td>51.7</td>
</tr>
<tr>
<td>rIL-1α 24 h prior to VP-16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A375-C6</td>
<td>612</td>
<td>24.9</td>
</tr>
<tr>
<td>A375-C5</td>
<td>691</td>
<td>88.3</td>
</tr>
</tbody>
</table>

A Cytotoxic effects of VP-16 and rIL-1α alone and in combination were determined by median dose values. The values represent the average determined by at least 3 independent experiments.

B Dose of VP-16 was calculated from the value in the column labeled “Both” x 11/12 and rIL-1α was calculated from the value in the column labeled “Both” x 1/12. Values show the calculated relative contributions of each agent to the median dose cytotoxic effect.

For both the IL-1-sensitive A375-C6 cells and the IL-1-resistant cloned A375-C5 cells, the combination treatment with IL-1α and VP-16 was much more effective than single-agent treatments. The comparison between the simultaneous and the sequential administration of these agents to A375-C6 cells, in which rIL-1α was given 24 h prior to VP-16, indicates a strong synergism for both the sequential and the simultaneous administrations (Fig. 1A). Likewise, the comparison between effects of the simultaneous and the sequential administration, in which VP-16 was given to A375-C6 cells 6 h prior to rIL-1α, revealed strong synergism of the sequential drug administration (Fig. 1B). Similar results were also obtained for A375-C5 cells (Fig. 2). The plots of the combination index showed that the best synergism was observed for simultaneous drug administration for A375-C6 cells, and for A375-C5 cells, sequential drug administration with VP-16 given prior to rIL-1α (Fig. 3).
data also indicate better synergism for A375-C5 cells (Fig. 3B) than for A375-C6 cells (Fig. 3A). The best ratio of the 2 drugs for the maximal synergism was found to be 12 to 48 (VP-16):1 (rIL-1α), when the ratios were varied between 3:1 and 192:1 (data not shown).

Receptor Binding of rIL-1α. The specific binding of the rIL-1α to its membrane receptor for both cells is shown in Fig. 4A; the IL-1-sensitive cells (A375-C6) have 12-fold more receptors than IL-1-resistant cells (A375-C5). Scatchard plots (Fig. 4B) were used to calculate dissociation constants and receptor numbers of the IL-1-sensitive A375-C6 cells and the IL-1-resistant A375-C5 cells (Table 2).

Effects of VP-16 on rIL-1α Receptor. As depicted in Fig. 5, when A375-C6 cells were incubated with sublethal doses of VP-16 for 24 h, the rIL-1α receptor level was increased 1.75-fold by 2.4 μM VP-16 (P < 0.05). The receptor content of A375-C5 cells was increased 3.5-fold by the pretreatment with 2.4 μM VP-16 (P < 0.01). Since the exposure of VP-16 to the cells was 24 h, not 120 h as in the cytotoxicity experiments, VP-16 alone (0.24 and 2.4 μM) showed very little toxicity to these cells (6.5 and 19% and 5.5 and 8.6% to A375-C6 and A375-C5 cells, respectively).

Effects of VP-16 on Internalization of rIL-1α. Cells were treated with 2.4 μM VP-16 for 24 h before adding radiolabeled rIL-1α. For A375-C6 cells, the surface binding of rIL-1α was maximal at 120 min and then declined rapidly while intracellular rIL-1α increased with time (Fig. 6A). Similar results were also obtained for A375-C5 cells, although the levels of intracellular rIL-1α were lower than those of A375-C6 cells (Fig. 6B). VP-16 treatment increased the intracellular content of rIL-1α 1.5-fold in A375-C6 and 1.3-fold in A375-C5 cells at 48 h (significantly different, P < 0.01).
Table 2  Receptor number and affinity for interleukin-1α (Kd) of A375-C6 and A375-C5 cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of receptors (molecules/cell)</th>
<th>Kd (pM)</th>
</tr>
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<tbody>
<tr>
<td>A375-C6</td>
<td>701 ± 128*</td>
<td>19 ± 6</td>
</tr>
<tr>
<td>A375-C5</td>
<td>58 ± 33</td>
<td>17 ± 2</td>
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</table>

* Mean ± SD.

Fig. 5. Changes in the specific binding of 5 ng/ml (■) and 10 ng/ml (■) of 125I-labeled rIL-1α by VP-16 pretreatment on A375-C6 cells (A) and A375-C5 cells (B). Values are averages of 2 different experiments. Statistical analysis using the t-test showed significant differences between control and VP-16-treated values (2.4 μM) (P ≤ 0.001).

VP-16 Uptake. The cellular accumulation of VP-16 (10 μM) by A375-C6 and A375-C5 cells was similar, and the maximal drug levels of 50 ± 6 pmol/mg protein were reached by 30 min (Fig. 7). Pretreatment of tumor cells with rIL-1α at 84 units/ml for 24 h did not significantly alter the maximal intracellular levels of VP-16 in either cell line, and rIL-1α under these conditions was not cytotoxic. These observations indicate that enhanced cell killing by the combination did not result from an increase in the intracellular VP-16 concentration.

DISCUSSION

The combination treatment of A375 human melanoma cells with rIL-1α and VP-16 showed strong synergistic antitumor activity. This synergism was obtained for several different dose ratios between the 2 drugs (from 3:1 to 192:1), however, the best dose ratios for VP-16 to rIL-1α were found to be 12 to 48:1. These ratios are similar to the ratio of the individual 50% inhibitory concentrations of VP-16 and rIL-1α (12:1) and suggest that for cytotoxic synergism to occur, the concentration of each agent must be at least minimally cytotoxic. Synergism was also obtained in different administration schedules other than
simultaneous exposure. The best treatment combination was simultaneous exposure of both agents for A375-C6 cells and sequential administration for A375-C5 cells, which was VP-16 prior to rIL-1α. For both cell types, based upon the plots of the combination indices, the sequential administration of rIL-1α prior to VP-16 was less synergistic than any other schedule. These results would imply that VP-16 potentiates the cytotoxicity of rIL-1α rather than the reverse; one possible mechanism of this synergism may be that VP-16 alters the processing or induces the IL-1α receptor in these cells.

The mechanism of the direct cytotoxicity of IL-1α is not clear, but some groups have reported that the biological action of IL-1 is related to its binding to a plasma membrane receptor (26-32). Dower et al. (26-27) have reported that IL-1α binding to the IL-1R of the murine T lymphoma cell line LBRM-33-1A5, which is highly responsive to IL-1, is necessary for its cytotoxicity. Matsushima et al. (28) have shown that even though the IL-1 receptor number of human Epstein-Barr virus transfectant B-lymphocytes is low, the cells still responded to IL-1. They have also shown that the difference between A375-C6 and A375-C5 cells in rIL-1α sensitivity is related to the relative number of the IL-1R in these 2 cell lines (2).

Our study shows that following pretreatment with VP-16 the IL-1α receptor binding of A375-C6 and A375-C5 cells was significantly increased in a dose-dependent fashion. Furthermore, this prior treatment of cells by VP-16 also significantly increased the internalization of rIL-1α as well as the surface receptor binding. These results suggested that the mechanism of the synergistic antitumor effects of combination therapy may be related to the modulation of the cellular IL-1R levels by VP-16. In this regard, it should be mentioned that there are several reports of IL-1 receptor modulation by other agents. Akahoshi et al. (33) and Matsushima et al. (34) have reported that glucocorticoid hormones dramatically increased the number of IL-1Rs on human peripheral blood mononuclear cells, and that IL-1Rs in BALB/c 3T3 fibroblasts are modulated by platelet-derived growth factor (35).

We have also investigated the efficacy of IL-1α/VP-16 combination therapy in vitro against other cancer cell lines such as human breast cancer cell MCF-7, human leukemic cell HL-60, or human colon cancer cell HT-29. The combination treatment of rIL-1α and VP-16 toward these cells did not show any synergistic or even additive effects. It is interesting to note that these cells failed to show any response to rIL-1α alone, namely, the 50% inhibitory concentration value was >1000 units/ml for these cells. These results further support the hypothesis that the synergism of rIL-1α with VP-16 results from potentiation of rIL-1α cytotoxicity by VP-16, and that for synergistic antitumor effects, rIL-1α alone must be cytotoxic to cells.

The prior treatment of the melanoma cells with rIL-1α did not increase the cellular uptake of VP-16. However, it is possible that the interaction of rIL-1α and VP-16 may occur at a target of VP-16 action. It is believed that topoisomerase II is likely the intracellular target for VP-16-dependent DNA damage (36-49). It is possible that a mechanism of rIL-1α and VP-16 synergism may be related to the topoisomerase II-mediated DNA strand breaks. Utsgul et al. (41) have reported the combination of TNF, and the topoisomerase II inhibitors teniposide and amrsacrine produced dose-dependent synergistic cytotoxicity against murine L929 fibrosarcoma cells. They have further shown that L929 cells produced a rapid and transient increase in the specific activity of extractable topoisomerase I and II following TNF treatment. They concluded that this transient nature of the increase in extractable topoisomerase activity may explain the mechanism of the synergism. Since IL-1 and TNF are both produced by monocytes and have considerable overlap in biological activities, it may be that some altered interaction of VP-16 with topoisomerase II results from rIL-1α treatment.

In this regard, we have observed that the combination of rIL-1α with doxorubicin, a topoisomerase II-active drug (42), shows a strong synergistic antitumor activity against both A375-C6 and A375-C5 cells (43), lending support for this hypothesis. Our preliminary studies using a fluorometric assay for DNA unwinding have shown no increase in VP-16-induced DNA damage following treatment of these cell lines with rIL-1α. However, we are currently investigating the possibility of a topoisomerase II-directed mechanism of synergism between VP-16 and rIL-1α using more sensitive methods.

The antitumor activity of IL-1 is pleiotropic and includes manipulation of immunonetwork targeting T-lymphocyte, large granular lymphocytes and natural killer cells, and direct action toward the tumor target cells. We have focused on the direct cytotoxic effects of IL-1α using IL-1-sensitive A375-C6 tumor cells and IL-1-resistant A375-C5 cells. From our study, the mechanism of the synergistic antitumor effects of rIL-1α and VP-16 may result from the ability of VP-16 to modulate the receptor for rIL-1α and enhance the internalization of rIL-1α. This investigation of the mechanism of the interactions of conventional cytotoxic antitumor drugs like VP-16 or doxorubicin and IL-1 may be helpful in explaining the action of IL-1 itself. Furthermore, such combinations of cytotoxic agents and biomodulators might be clinically effective.

REFERENCES

SYNERGISTIC INTERACTIONS OF IL-1α WITH VP-16


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