Radiosensitization of Human Tumor Cells with Levamisole¹

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Abstract

Levamisole in combination with radiation and chemotherapeutic agents is being studied in clinical trials. The mechanism of interaction of levamisole with these modalities is unknown. In order to determine if there is direct interaction between radiation and levamisole, a series of colony-formation assays was performed with the use of two human tumor cell lines. Cells were exposed to 0–10 Gy of radiation, with or without the addition of 0–1000 μM levamisole. Exposure to levamisole alone had no effect on cell survival; however, the combination of continuous exposure to levamisole at concentrations approaching 1000 μM and radiation revealed a potentiation of radiation-induced cell killing.

Introduction

The drug levamisole recently gained widespread attention as a result of a large clinical trial (1) that revealed a therapeutic benefit when levamisole was combined with 5-fluorouracil in the adjuvant treatment of Stage C colon cancer. Prior to this, levamisole was known as a synthetic anthelminthic. The beneficial effect noted in the colon cancer trial prompted additional clinical trials of levamisole. These trials use levamisole in combination with chemotherapy or radiotherapy, or both. In particular, there are several ongoing trials using combinations of levamisole, radiotherapy, and 5-fluorouracil for gastrointestinal malignancies. Although interest in levamisole as an antineoplastic agent first came from knowledge of its immunomodulating effects, the combination of levamisole with chemotherapy and radiotherapy has been derived empirically, without knowledge of the actual mechanism of interaction. Determination of the basis of the beneficial role of levamisole in the treatment of malignancies will aid in the development of more effective therapeutic combinations. Toward this end, a colony-formation assay was used to investigate the interaction between levamisole and radiation in two human tumor cell lines in an effort to determine if there is any direct interaction between levamisole and radiotherapy.

Materials and Methods

Cell Lines. The continuous human tumor cell lines A549, of alveolar lung carcinoma origin, and A375, of melanoma origin, were used in this study and have been described previously (2, 3). They have been maintained by serial passage in DMEM² supplemented with 10% FBS. The cells grew in an attached monolayer and were detached from the bottom surface of growth vessels by treating the cells with 0.25% trypsin in EDTA. Cultures were maintained in polystyrene tissue culture flasks and kept in a humidified 95% air:5% CO₂ atmosphere at 37°C. The A549 cell line had an approximate population-doubling time of 16 h, whereas the A375 cell line had a doubling time of approximately 12 h.

Cell Survival. A colony-formation assay was used to determine cell survival. Exponentially growing cells were plated into triplicate 60-mm tissue culture dishes at densities of 200 to 10,000 cells in a total of 4 ml growth medium. The dishes were incubated at 37°C. Cells were irradiated or exposed to drug approximately 16 to 18 h after initial plating to allow for cell adherence and elimination of trypsin effects. Colony multiplicity, using this technique, was 1.17 ± 0.23 (SE) (4).

The dishes were incubated for 9 to 15 days to allow the surviving cells to form verifiable colonies. The dishes were removed from the incubator and stained with crystal violet, and the colonies were counted to determine the absolute and relative plating efficiencies. The absolute plating efficiencies of the A549 and A375 cell lines were approximately 0.41 and 0.23, respectively.

Irradiation. A GE Maxitron 300 X-ray machine was used for all irradiations. Cells were irradiated with 300-kV X-rays at 1.54 Gy/min for a total of 1 to 10 Gy.

Drug Exposures. Cells were exposed to levamisole (Sigma Chemical Co., St. Louis, MO) 1 to 1.5 h after irradiation by removing the initial medium, washing the plates with phosphate-buffered saline, and replacing the medium with the appropriate concentration of the drug diluted in 4 ml DMEM plus 10% dialyzed FBS. Concentrations of levamisole varied from 1 to 1000 μM. Continuous exposures were defined as those in which cells were exposed to drug for the entire period of colony formation. Brief exposures were defined as those in which the medium containing levamisole was replaced with DMEM plus 10% FBS after the cells had been exposed to drug for 1 h.

Results

Initially, radiation survival curves were constructed and analyzed to determine the radiosensitivity of each cell line. Both cell lines displayed characteristic radiation survival patterns and parameters (Table 1; Fig. 1)

To determine whether levamisole alone had any effect on cell survival, cells were exposed to medium containing 1 to 1000 μM levamisole for continuous exposures and to 1000 μM levamisole for brief exposures. At these concentrations of levamisole, there was no detrimental alteration of cell survival.

The cell lines were then exposed to various doses of levamisole in combination with irradiation. With continuous exposures, there was a potentiating effect on radiation-induced cell killing as concentrations of levamisole approached 1000 μM (Fig. 1). The radiation-potentiating effect is manifested as an increased slope and a reduced shoulder on the radiation survival...
Table 1  Radiation survival curve parameters

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$D_0$ (Gy)</th>
<th>$D_1$ (Gy)</th>
<th>$D_2$ (Gy)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375 cell line</td>
<td>Radiation alone</td>
<td>1.77</td>
<td>3.17</td>
<td>1.42</td>
</tr>
<tr>
<td>Radiation + 1000 µM levamisole (continuous exposure)</td>
<td>1.00</td>
<td>1.83</td>
<td>1.25</td>
<td>3.30</td>
</tr>
<tr>
<td>A549 cell line</td>
<td>Radiation alone</td>
<td>2.33</td>
<td>5.00</td>
<td>1.58</td>
</tr>
<tr>
<td>Radiation + 1000 µM levamisole (continuous exposure)</td>
<td>1.17</td>
<td>2.25</td>
<td>1.08</td>
<td>2.45</td>
</tr>
</tbody>
</table>

Fig. 1. Radiation survival curves with and without continuous-exposure levamisole. X, radiation alone; O, radiation and 1000 µM continuous-exposure levamisole. Values were calculated from the respective survival curves in Fig. 1. With the addition of 1000 µM levamisole, all values decreased, representing a steeper slope and a reduced shoulder on the curve.

Discussion

The results of studies using levamisole alone or in combination with other modalities in the treatment of cancer have been mixed (1, 5–12). Most recently, a large clinical trial revealed a therapeutic benefit when levamisole was combined with 5-fluorouracil in the adjuvant treatment of Stage C colon cancer (1). It has been suggested that the interaction of levamisole with other therapies depends on the effectiveness of the antineoplastic agent it is combined with as well as the timing, duration, and dose of levamisole (13).

Levamisole is a synthetic anthelmintic and has been used extensively in both animals and humans for this purpose. This drug has a wide variety of known activities in cells, tissues, and organisms, including inhibition of fumarate reductase in nematodes, stimulation of sympathtic and parasympathetic gan-

glia, inhibition of alkaline phosphatase, and immunomodulation (14, 15).

Levamisole is readily absorbed through the gastrointestinal tract, i.m., and s.c. It is metabolized extensively by the liver (2, 3, 16). A common treatment schedule for levamisole is 150 mg daily, divided into three p.o. administered doses per day and given for 3 consecutive days every 2 weeks. Others advocate administering levamisole on the basis of body weight, giving 2.5 to 5.0 mg/kg/day. These dosage regimens have been used for anthelmintic treatment; however, they have been derived empirically and are not necessarily optimum with regard to an antineoplastic effect and toxicity (13, 17–19).

Pharmacokinetic studies have been conducted on patients receiving a single p.o. dose of 5 mg/kg levamisole. At this dosage, levamisole has a serum half-life of 3.3 to 5.1 h, and peak plasma levels of 1.6 µg/ml (6.6 µM) are reached within 1 to 2 h (3, 16). However, the plasma, tissue, and tumor levels of levamisole are not known for an optimum therapeutic dosage regimen. This may be addressed better by the results of an ongoing Phase I clinical trial, in which pharmacokinetic and immune response parameters are being measured in patients receiving levamisole for gastrointestinal malignancies.6

The mechanism of interaction of levamisole with other antineoplastic modalities is unknown. One hypothesis is that levamisole restores the immune function in the compromised host, leading to an immune reaction against the antigenic cancer cells (13, 18). Indeed, levamisole influences virtually all functions involved in cell-mediated immunity, including enhancement of phagocytosis, chemotaxis, migration inhibition, nucleic acid and protein synthesis in resting lymphocytes, and lymphokine production. At the cellular level, the mechanism of these effects

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has been hypothesized to be related to an effect on cyclic nucleotide accumulation or calcium transfer. The immunomodulation effect appears to result in the restoration of immune responses to normal levels in compromised hosts rather than increasing immune responses in normal hosts (14, 15).

Another possibility is that levamisole directly interacts with chemotherapeutic or other cytoreductive modalities. The present study attempted to evaluate if there is a direct interaction of levamisole with irradiation. Radiation-induced cell killing was evaluated with and without levamisole in two human tumor cell lines. Continuous exposure of levamisole at doses approaching 1000 μM demonstrated significant radiosensitization. This effect was observed as an increased slope and a reduced shoulder on the radiation survival curve. This suggests that the addition of levamisole fixes some portion of cell damage which would otherwise have been sublethal. Exposures to levamisole at doses similar to these have also been noted to enhance cell killing by 5-fluorouracil in studies of colon carcinoma cell lines (20). In our study, there was no potentiation of radiation-induced cell killing from brief exposures or doses of levamisole in the range of the plasma concentrations found after a single 5-mg/kg p.o. dose in humans.

The data presented here demonstrate a direct interaction of levamisole and radiation. Further study of the pharmacokinetics of levamisole and the intriguing interactions of levamisole with radiation may lead us to a better understanding of the antitumor mechanism of action of levamisole. Knowledge of the mechanism of these interactions could lead to the development of more rational and effective treatment regimens.

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References

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