ABSTRACT

The human ovarian carcinoma cell line, NIH:OVCAR-3, possesses high affinity receptors for interleukin-1 (IL-1). Binding experiments with \(^{125}\text{I}-\text{IL-1}\) indicate a dissociation constant of approximately 55 pM and the presence of approximately 7800 receptors/cell. These receptors bind both IL-1\(\alpha\) and IL-1\(\beta\) and internalize IL-1. Proliferation of NIH:OVCAR-3 cells is inhibited by IL-1. Half-maximal inhibition is observed with 2–3 units/ml of IL-1\(\alpha\) or IL-1\(\beta\). A maximal effect (80% inhibition of cell proliferation) is achieved by treatment of cells with \(10^8\) units/ml of IL-1 for 3 days. The antiproliferative effect of IL-1 is blocked by IL-1 receptor antagonist. Light and electron microscopy studies show that IL-1 treatment causes cytostatic changes and a reduction in the number of mitotic figures in NIH:OVCAR-3. IL-1 stimulates prostaglandin E\(_2\) release by NIH:OVCAR-3 cells, but this response is unrelated to the antiproliferative effect of IL-1. Interferon-\(\alpha\) (IFN-\(\alpha\)) also inhibits growth of NIH:OVCAR-3 cells in a concentration-dependent manner. Combination of IFN-\(\alpha\) and IL-1 gives synergistic inhibition of NIH:OVCAR-3 cell proliferation. IL-1 alone or in combination with IFN-\(\alpha\) or other agents may be useful for treatment of human ovarian cancer.

INTRODUCTION

The IL-1\(^2\) proteins, IL-1\(\alpha\) and IL-1\(\beta\), are produced by activated monocytes and other cell types and possess a diverse range of biological activities. The activities include the capacity to modulate T- and B-cell function, stimulate proliferation of fibroblasts and mouse thymocytes, induce PGE\(_2\) release, stimulate synthesis of other cytokines such as IL-6 and tumor necrosis factor, and evoke systemic changes such as fever and the acute phase response (see Refs. 1–4 for specific references). These and other studies suggest that IL-1 has important effects on neurological, hematological, metabolic, and endocrine systems and is a key mediator in immune, inflammatory, and host defense mechanisms (1). Comparison of the amino acid sequence for IL-1\(\alpha\) and IL-1\(\beta\) shows that there is <30% identity in amino acid sequence between the two proteins (5–7). Despite this difference, IL-1\(\alpha\) and IL-1\(\beta\) elicit similar biological responses and bind to the same receptor (8, 9). Two types of IL-1 receptors, both capable of binding IL-1\(\alpha\) and IL-1\(\beta\), have been identified (10, 11). The first type is present on T-cells and fibroblasts and the complementary DNA encoding for this receptor has been molecularly cloned (12). The second type is present on B-cells and macrophages and has not been entirely characterized at the molecular level.

Several studies suggest that IL-1 may act directly to inhibit proliferation of certain tumor cells or to exert antitumor activity indirectly, e.g., by augmentation of cellular immune responses. IL-1 has been found to be cytotoxic or cytostatic for the human melanoma cell line A375 in vitro (13, 14). IL-1 has also been shown to inhibit growth of certain mammary carcinoma cell lines in vitro but either to have no effect or to stimulate growth of other tumor cells (15). IL-1 was found to be identical with tumor inhibitory factor-2 and to inhibit the growth of the human rhabdomyosarcoma cell line A673, human mammary carcinomas, and human adenocarcinoma of the lung (16). Finally, IL-1 inhibited growth of certain human and mouse myeloid leukemic cell lines (17). Other studies have also suggested that IL-1 may have antitumor activity in vivo through mechanisms other than direct inhibition of tumor growth, such as by augmentation of immune responses or other mechanisms (18–20).

In the present study, we examined the effect of IL-1 on the proliferation of the human ovarian carcinoma cell line, NIH:OVCAR-3. The NIH:OVCAR-3 cell line appears to be a suitable system for studies of the biology of ovarian carcinoma (21–23). Here we report that IL-1 inhibits proliferation of the NIH:OVCAR-3 cell line and that this inhibition is increased in a synergistic fashion by IFN-\(\alpha\).

MATERIALS AND METHODS

Reagents. Escherichia coli-derived recombinant human IL-1\(\alpha\) and IL-1\(\beta\) (specific activities of 3 \(\times\) 10\(^5\) units/mg) were provided by Dr. S. Roy (Hoffmann-La Roche Inc.). E. coli-derived recombinant human IFN-\(\alpha\) (specific activity of 2–4 \(\times\) 10\(^5\) units/mg) and monoclonal antibody to IFN-\(\alpha\) A, L18 (24), were provided by Dr. F. Khan, R. Bartell, and Dr. M. Brunda (Hoffmann-La Roche). E. coli-derived recombinant human IL-1 receptor antagonist was obtained from Synergen (Boulder, CO). Insulin, transferrin, and sodium selenite supplement and indomethacin were obtained from Sigma Chemical Company (St. Louis, MO). \([^{3}H\text{]Thymidine (2 Ci/mmol)}\) was purchased from DuPont-NEN (Boston, MA).

Cell Culture. NIH:OVCAR-3 cells (ATCC HTB 161; American Type Culture Collection, Rockville, MD) were maintained in tissue culture medium containing RPMI 1640 without phenol red ( Gibco Laboratories, Grand Island, NY), insulin (5 \(\mu\)g/ml), transferrin (5 \(\mu\)g/ml), sodium selenite (5 ng/ml), sodium bicarbonate (0.15%) and 10% FBS (HyClone Laboratories, Logan, UT). The FBS was charcoal stripped as described previously (25).

Preparation of \(^{125}\text{I}-\text{labeled IL-1}. \) \(^{125}\text{I}-\text{IL-1}\) was prepared (8) with modification. Briefly, 27 \(\mu\)g of recombinant human IL-1\(\alpha\) was reacted with 2 mCi of Na\(^{25}\text{I}\) (IMS-30; Amersham, Arlington Heights, IL) in the presence of Enzymobeads (Bio-Rad Laboratories, Richmond, CA) for 15 min at room temperature. The radioactive mixture was placed on a 10-ml Bio-Gel P10 column which had been equilibrated with elution buffer [Dulbecco’s phosphate-buffered saline without calcium chloride and magnesium chloride (GIBCO Laboratories), 1 mg/ml bovine serum albumin (Sigma), 1 mM EDTA]. The peak fraction containing \(^{125}\text{I}-\text{IL-1}\) was reserved. The radioactivity in the peak fraction was precipitated \(\approx\)98% by trichloroacetic acid (8). The \(^{125}\text{I}-\text{labeled IL-1}\) retained essentially all bioactivity as determined in the D10.G4.1 cell proliferation assay (26) and had a specific activity of 50–70 \(\mu\)Ci/\(\mu\)g.
Determination of Receptor-binding Characteristics. Culture medium from confluent NIH:OVCAR-3 cells grown in 12-well tissue culture cluster plates (Costar, Cambridge, MA) was removed by aspiration and replaced with 0.40 ml of binding buffer [RPMI 1640-5% FBS-25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2]. Radiolabeled IL-1 was added at the indicated concentrations in the presence (nonspecific binding) or absence (total binding) of 50 nM IL-1 (final volume, 0.50 ml). The cells were incubated for 3 h at 4°C with gentle shaking (Mini-Orbital Shaker; Belco Glass, Inc., Vineland, NJ). In order to terminate the assay, the binding medium was removed by aspiration and cells were washed three times with 0.50 ml of ice cold binding buffer. The washed cells were detached by addition of 1.0 ml of 0.5% (w/v) sodium dodecyl sulfate (Sigma). A 0.80-ml aliquot was removed and bound radioactivity determined by use of a Packard 5780 Auto-Gamma Counter.

Receptor Internalization Studies. Internalization of 125I-IL-1 was performed essentially as described for a mouse fibroblast cell line (27). Confluent NIH:OVCAR-3 cells in 12-well tissue culture cluster plates were incubated with 100 pm 125I-IL-1 for 3 h at 4°C with shaking. Unbound radiotracer was then removed by washing adherent cells three times with ice cold binding buffer and 0.50 ml of cold binding buffer was added. Cells were transferred to 37°C and, at the indicated times, released, surface bound, and internalized radioligand was determined. Released 125I-IL-1 was determined by directly measuring radioactivity in cell culture supernatants. Surface-bound 125I-IL-1 was determined by washing cells for 45 s with acid buffer (100 mM NaCl-50 mM glycine HCl, pH 3.0) at 4°C and measuring the level of radioactivity in an aliquot of the acid wash. Internalized 125I-IL-1 was determined following the detachment of acid-washed cells with sodium dodecyl sulfate as described above.

[3H]Thymidine Incorporation Assay. NIH:OVCAR-3 cells were plated at 10,000 cells/well in 96-well cluster plates (Costar) in growth medium. Twenty-four h later, medium was removed and replaced with fresh growth medium containing the indicated concentrations of IL-1 or other additions. Cells were treated with IL-1 or other agent for 3 days unless indicated otherwise. The final incubation volume was 0.20 ml. On the last day of the incubation, 1 µCi of [3H]thymidine was added (20 µl/well). Cells were harvested 18 h later by use of an LKB 1295-001 cell harvester. Incorporated [3H]thymidine was determined by use of a LKB 1205 betaplate counter.

Crystal Violet Assay. NIH:OVCAR-3 cells were plated in 96-well tissue culture cluster plates at 20,000 cells/well. Twenty-four h later, medium was removed and replaced with fresh medium containing the indicated concentrations of IL-1 or other additions. The final incubation volume was 0.20 ml. The incubation was continued for 72 h at 37°C. At the end of the incubation period, medium was removed and cell monolayers were washed gently three times with Dulbecco’s calcium-, magnesium-free Hank’s balanced salt solution (GIBCO). The adherent cells were stained with 0.2% crystal violet (Sigma) in 10% ethanol (50 µl) for 5 min at 37°C. Cells were washed three times with phosphate-buffered saline and the dye was extracted with a solution made up of equal parts of 0.1 M sodium phosphate, pH 4.5, and ethanol (200 µl/well) for 30 min at room temperature. The absorbance was measured at 570 nm using a microplate reader (Molecular Devices, Menlo Park, CA).

Cell Enumeration Assay. NIH:OVCAR-3 cells, grown in 24-well tissue culture cluster plates, were incubated with the indicated concentrations of IL-1α or IL-1β. Monolayers were washed twice with 1 ml of calcium-, magnesium-free Hank’s balanced salt solution (GIBCO). Cells were detached by incubation with 0.5 ml of trypsin-EDTA solution (GIBCO) for approximately 5 min at room temperature. Cell number was determined microscopically with a hemacytometer. Cell viability was determined by trypan blue exclusion. Cell number was expressed as total number of viable cells/well.

Determination of PGE2 Levels. Confluent NIH:OVCAR-3 cells in 24-well tissue culture cluster plates were treated with IL-1 at the indicated concentrations for 24 h at 37°C. Culture supernatants were collected and the concentration of PGE2 was determined by use of a radioimmunoassay kit (NEB Research Products, Boston, MA) according to the manufacturer’s instructions.

RESULTS

IL-1 Receptors on NIH:OVCAR-3. Experiments conducted at 4°C show that NIH:OVCAR-3 cells bind 125I-IL-1α in a specific and saturable manner (Fig. 1A). Scatchard plot analysis indicates an apparent dissociation constant of approximately 55 pm and the presence of approximately 7800 receptors/cell. Binding of 125I-IL-1α is inhibited by both IL-1α and IL-1β in a concentration-dependent manner (Fig. 1B). The concentrations of competitor inhibiting binding of the radioligand by 50% are estimated to be approximately 1.25 ng/ml for both IL-1α and IL-1β. Receptors for IL-1 on NIH:OVCAR-3 cells are
Fig. 2. IL-1 is internalized by IL-1 receptors on NIH:OVCAR-3 cells. NIH:OVCAR-3 cells were incubated with $^{125}$I-IL-1$\alpha$ for 3 h at 4°C. Cells were then washed with cold binding buffer to remove unbound $^{125}$I-IL-1 and fresh binding buffer was added to the wells. Cells were transferred to 37°C (zero time point). At the indicated time points, released $^{125}$I-IL-1 (O) was determined by counting an aliquot of the supernatant in each well. The rest of the binding medium was then removed by aspiration and cells were treated for 15 s with pH 3 buffer. Surface-bound $^{125}$I-IL-1 (C) was determined by measuring radioactivity in an aliquot of the acid wash. The remainder of the acid wash was then removed and internalized $^{125}$I-IL-1 (Q) was determined as described in "Materials and Methods." Total bound (V), the sum of surface-bound and internalized $^{125}$I-IL-1.

IL-1 Inhibits Proliferation of NIH:OVCAR-3 Cells. The ability of IL-1 to inhibit growth of NIH:OVCAR-3 cells was determined. Treatment of cells with IL-1$\alpha$ for 3 days gives a concentration-dependent inhibition of cell proliferation as determined by $[^3H]$thymidine uptake (O, Fig. 3A). Half-maximal inhibition is typically observed with 2–3 units/ml of IL-1$\alpha$, while maximal inhibition is obtained with $\geq$10 units/ml. Similar results are obtained in an assay using crystal violet staining as an indicator of cell number (O, Fig. 3A). Results obtained upon enumeration of cell number by microscopic examination are similar to those obtained by $[^3H]$thymidine and crystal violet assays (Fig. 3B). Both IL-1$\alpha$ and IL-1$\beta$ have similar inhibitory effects on cell growth (Fig. 3B).

The effect of treatment of NIH:OVCAR-3 cells with 100 units/ml of IL-1$\alpha$ for various times was examined (Fig. 4). No effect is observed if cells are treated with IL-1$\alpha$ for 1 day or less. An antiproliferative effect is found within 2 days. Maximal inhibition is achieved when cells are treated with IL-1$\alpha$ for 3 days or longer.

IL-1ra Blocks Effect of IL-1. IL-1ra blocks binding of $^{125}$I-IL-1$\alpha$ to NIH:OVCAR-3 cells; the concentration of IL-1ra required to inhibit binding of the radioligand by 50% is similar to that for IL-1$\alpha$ and IL-1$\beta$ (data not shown). The ability of IL-1ra to block the antiproliferative effect of IL-1 was investigated (Table 1). While equimolar or 10-fold excess of IL-1ra to IL-1$\alpha$ is capable of internalizing IL-1 following the initial binding interaction (Fig. 2). Internalization at 37°C reaches a maximum at 60 min at which time essentially all of the receptor-bound $^{125}$I-IL-1$\alpha$ is internalized.

Table 1 Antiproliferative effects of IL-1$\alpha$ on NIH:OVCAR-3 cells is blocked by IL-1ra

<table>
<thead>
<tr>
<th>Addition (pg/ml)</th>
<th>IL-1$\alpha$</th>
<th>IL-1ra</th>
<th>$[^3H]$thymidine incorporated (cpm/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>111,000 (100)*</td>
<td></td>
</tr>
<tr>
<td>16.5*</td>
<td>None</td>
<td>38,900 (35)</td>
<td></td>
</tr>
<tr>
<td>16.5</td>
<td>16.5</td>
<td>42,400 (38)</td>
<td></td>
</tr>
<tr>
<td>16.5</td>
<td>165.00</td>
<td>44,200 (40)</td>
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<tr>
<td>16.5</td>
<td>1,650.0</td>
<td>92,200 (83)</td>
<td></td>
</tr>
<tr>
<td>16.5</td>
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<td>121,500 (109)</td>
<td></td>
</tr>
<tr>
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<td>16,500.0</td>
<td>108,400 (98)</td>
<td></td>
</tr>
<tr>
<td>330.0</td>
<td>1,650.0</td>
<td>58,900 (53)</td>
<td></td>
</tr>
</tbody>
</table>

* % of control. Numbers in parentheses, * Approximately 5 units/ml.
IL-1 IS ANTI-PROLIFERATIVE FOR OVARIAN CARCINOMA

An examination of NIH:OVCAR-3 cells present in the culture supernatants reveals a significant decrease in the numbers of mitotic figures in IL-1-treated compared with untreated (control) cells. The percentages of cells containing mitotic figures at 48, 72, and 96 h were 38.7, 32.1, and 38.4% for control cultures, respectively, and 0.2, 0.0, and 5.1% for IL-1-treated cells, respectively. IL-1-treated cells appear smaller than control cells and contain almost exclusively pyknotic nuclei.

Light Microscopy Studies. Monolayer cultures of NIH:OVCAR-3 cells treated with IL-1 exhibit an abnormal detachment of cells at the edges of colonies within 24 h and throughout the 96-h incubation period. Nucleoli in IL-1-treated NIH:OVCAR-3 cells appear enlarged and less dense compared with untreated cells. Under the conditions used in this study, control cultures of NIH:OVCAR-3 cells are fully confluent by 48 h. In contrast, IL-1-treated cells achieve only approximately 20–40% confluence at 96 h.

An examination of NIH:OVCAR-3 cells present in the culture supernatants reveals a significant decrease in the numbers of mitotic figures in IL-1-treated compared with untreated (control) cells. The percentages of cells containing mitotic figures at 48, 72, and 96 h were 38.7, 32.1, and 38.4% for control cultures, respectively, and 0.2, 0.0, and 5.1% for IL-1-treated cells, respectively. IL-1-treated cells appear smaller than control cells and contain almost exclusively pyknotic nuclei.

Electron Microscopy Studies. Control (untreated) NIH:OVCAR-3 cell pellets contain typical epithelial cells, degenerating cells, and many mitotic cells. In addition, vacuolated cells and cells containing phagosomes consisting of cellular debris are observed. Cell pellets from culture supernatants derived from IL-1-treated cultures also contain "normal" appearing, degenerating, and vacuolated cells and cells containing phagosomes of cellular debris. However, mitotic cells are rare and many cells contain lipid droplets.

Synergism between IL-1 and IFN-α A. IFN-α A at concentrations of 500–50,000 units/ml inhibits NIH:OVCAR-3 cell proliferation in a concentration-dependent manner as determined by [3H]thymidine uptake (Table 2, no IL-1 added). The effect of IFN-α A is enhanced in a more than additive fashion by addition of IL-1α (Table 2). For example, while no effect of IL-1 is observed with 0.5 units/ml of IL-1 alone and 36% inhibition by 5000 units/ml of IFN-α A alone, combination of these concentrations of IL-1 and IFN-α A results in 69% inhibition in cell growth. Ninety % or greater inhibition of cell growth is observed with combinations of 2 or 62 units/ml of IL-1 and 5,000 or 50,000 units/ml of IFN-α A.

Anti-IFN α A antibody, L18, blocks the antiproliferative effect of IFN-α A but has no effect on the response to IL-1. Similarly, IL-1α blocks the antiproliferative response to IL-1 but not to IFN-α A (data not shown).

DISCUSSION

The NIH:OVCAR-3 cell line was established from the malignant ascites of a patient with progressive adenocarcinoma of the ovary (21–23). NIH:OVCAR-3 cells are resistant to clinically relevant concentrations of Adriamycin, melphalan, and cisplatin and appear to serve as a suitable system for investigations of ovarian carcinoma biology both in vivo and in vitro. Other studies have shown that IL-1 has cytoidal or cytostatic effects on certain cell types (13–17). In the present study, the effect of IL-1 on proliferation of NIH:OVCAR-3 cells was examined. NIH:OVCAR-3 cells were first shown to possess high affinity receptors for IL-1. Scatchard plot analysis from binding experiments with 35SIL-1α indicates a dissociation constant of approximately 55 pm and the presence of approximately 7800 sites/cell. These values are in close agreement with values obtained with other human cells and cell lines including keratinocytes (29), lung fibroblasts (30), synovial fibroblasts (31), endometrial epithelium (32), and neutrophils (26). Following the binding of IL-1 to its receptor on OVCAR-3 cells, 35SIL-1α is internalized, reaching a maximum in 1–2 h. It is conceivable that the IL-1/IL-1 receptor complex is targeted for the cell nucleus where it may directly modulate gene transcription as has been suggested for mouse EL-4 thymoma and 3T3 fibroblast cell lines (27).
2 days and is maximal with 3 days of treatment. At most 80% inhibition of cell growth by IL-1 is observed. The inability of IL-1 to completely inhibit cell growth suggests that there may be heterogeneity in NIH:OVCAR-3 cells and that a proportion of the cells may be IL-1 resistant. Recently, we have obtained cell cultures which lose sensitivity to the antiproliferative effect of IL-1 upon prolonged culture with IL-1.*

The mechanism by which IL-1 exerts an antiproliferative effect on NIH:OVCAR-3 cells appears to be mediated by binding of IL-1 to its receptor on these cells since the effect of IL-1 is blocked by IL-1ra. IL-1ra is a specific antagonist which acts by blocking the binding of IL-1 to its receptor (33, 34). IL-1ra has been cloned and has been shown to preferentially bind to type I IL-1 receptors present on T-cells, fibroblasts, and other cell types with an affinity equal to that for the IL-1 proteins. A 100-fold or greater molar excess of IL-1ra to IL-1 is required for reversal of the IL-1 effect on NIH:OVCAR-3 cells. The requirement for excess IL-1ra suggests that there are spare receptors for IL-1 on NIH:OVCAR-3 cells and that occupation of a large proportion of these receptors by the IL-1 antagonist is essential for blocking the activity of IL-1.

Our study shows that IL-1 stimulates PGE2 synthesis by NIH:OVCAR-3 cells. While indomethacin completely blocks IL-1-stimulated PGE2 release, it has no effect on the inhibition of cell growth in response to IL-1. The mechanism by which IL-1 exerts an antiproliferative effect on NIH:OVCAR-3 cells is not known, but IL-1 may act to stimulate the production of other cytokines. Preliminary results show that IL-1 stimulates IL-6 production by NIH:OVCAR-3 cells. This ability of ovarian carcinoma cells to produce IL-6 suggests that tumor cells may be responsible for the high levels of IL-6 which have been detected in human ovarian ascitic fluid (35). We are currently evaluating whether IL-6 modulates proliferation of NIH:OVCAR-3 cells.

Light and electron microscopy studies confirm that IL-1 treatment is cytopathological for NIH:OVCAR-3 cells. IL-1 treatment leads to rapid changes in the size and density of the nucleolus, followed by a release of cells at the edge of colonies. In addition, IL-1 causes a suppression in cell division. Except for the presence of fat droplets in some of the IL-1-treated cells, it is not possible to differentiate between untreated and treated cells.

IFN-α A is well known to have antiproliferative effects on a number of cell types (36). In this study, we find that the growth of NIH:OVCAR-3 cells is inhibited by IFN-α A. Approximately 5000 units/ml of IFN-α A is required to inhibit cell growth by 50%. Maximal inhibition of 80% is achieved with 50,000 units/ml. A synergy between IL-1 and IFN-α A is observed on NIH:OVCAR-3 cells. Inhibition of NIH:OVCAR-3 cell growth is ≥99% when both agents are present at optimal concentrations. The ability of IL-1 to promote inhibition of cell growth is not due to the induction of IFN-α A synthesis by IL-1 as shown by IFN-α A antibody-blocking experiments. Similarly, IL-1ra does not block IFN-α A action, demonstrating that IFN-α A effects are not mediated through stimulation of IL-1 synthesis.

Whether a defect in IL-1 action or production may be associated with ovarian cancer is not known, but a recent report has shown that tumor-associated macrophages isolated from ascites or solid human ovarian carcinoma produce reduced levels of IL-1 (35). Our study suggests that IL-1 alone or in combination with other agents such as IFN-α A may be useful for the treatment of human ovarian cancer. Demonstration of antitumor activity by IL-1 in an animal model of human ovarian cancer may be the next step in determining whether IL-1 will be clinically useful. The ability to use the NIH:OVCAR-3 cell line as an experimental model of ovarian cancer in vivo (21–23) should facilitate such studies.

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* P. L. Kilian, K. L. Kaffka, and W. R. Benjamin, unpublished observations.


Antiproliferative Effect of Interleukin-1 on Human Ovarian Carcinoma Cell Line (NIH:OVCAR-3)

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