Production of Growth-inhibitory Activity in Serum-free Medium by Human Monocytic Leukemia Cells

Edwin V. Gaffney, Shiow-Chuan Tsai, Marie L. Dell'Aquila, and Susan E. Lingenfelter

The Microbiology Program, The Pennsylvania State University, University Park, Pennsylvania 16802

ABSTRACT

Serum-free medium conditioned by activated cells of the acute monocytic leukemia line, THP-1, was examined for growth-inhibitory activity with several established human cell lines. Free-floating clusters of THP-1 cells were activated into adherent nonproliferating cells by a 24-hr exposure to 10^{-7} M mezerein in Roswell Park Memorial Institute 1640 medium containing 1% fetal bovine serum. Adherent cells were incubated for an additional 24 hr in serum-free medium containing insulin (5 μg/ml). Dose-response studies revealed that a cervical carcinoma (HeLa), a melanoma (A375Ag5), and several mammary carcinoma cell lines (MCF-7, BT474, MDA-MB415, and T47D) were growth inhibited by this conditioned medium. We concluded, from the results of thymidine release assays and from experiments on reversibility, that inhibition was a cytostatic and not a cytolytic response. In contrast, THP-1 conditioned medium stimulated the growth of two mammary lines (ZR75-1 and HBL-100), a lung type II carcinoma (549), and a colon adenocarcinoma (SW48). Preliminary characterization showed that the inhibitory activity was stable to acid and urea treatment but was destroyed by trypsin and sodium dodecyl sulfate. Molecular sieve chromatography of acetic acid-extracted material separated the inhibitory and stimulatory components.

INTRODUCTION

The numerous cytostatic and cytolytic activities associated with sera- and cell culture-conditioned fluids (16) may reflect a class of negative growth regulators which play an important role in the control of tumor development and normal cell differentiation. It is well known that the macrophage-monocyte cell series secretes a wide spectrum of negative growth mediators including interferon (23), cytotoxin (4-6), and tumor necrosis factor (1). Following exposure to interferon-inducing agents, macrophages in mixed cell populations have been shown to inhibit the growth of malignant but not normal cells. In addition, Schultz et al. (22) showed that medium conditioned by macrophage cultures contained both growth-inhibiting and -stimulating activities. The purification, biochemical characterization, and clinical application of many of these mediators have been difficult because either very small amounts are produced in vitro or they, like tumor necrosis factor (7), are one component in a complex biological fluid.

Recently, Rovera et al. (19, 20) and Cassileth et al. (2) reported that phorbol esters, including mezerein, induced cells of a promyelocytic leukemia line, HL-60, to function as monocytes. Tsuchiya et al. (26) showed that TPA\textsuperscript{3} induced maturation of the acute monocytic leukemia cell line, THP-1 (25). Phorbol ester-treated cells acquired the morphological and functional characteristics of macrophages, such as increases in phagocytosis, Fc receptors for IgG, and CO\textsubscript{2} production from glucose. When we exposed free-floating clusters of THP-1 cells to the plant diterpene ester, mezerein, they rapidly attached to culture flasks and demonstrated the morphological features described for activated macrophages.

The current study examines the effects of serum-free medium conditioned by mezerein-activated THP-1 cells on the growth of several human cell lines.

MATERIALS AND METHODS

Induction and Preparation of Conditioned Medium. Cells of the human monocytic leukemia line, THP-1 (25), were grown in 150-sq cm flasks in RPMI-1640, supplemented with 5% FBS and 2 mm glutamine, in a 5% CO\textsubscript{2}-humidified atmosphere at 37°. Cells were pooled to a concentration of 2.5 × 10^6/ml medium containing 1% FBS and were inoculated to 530-sq cm tissue culture plates. Mezerein (CCR, Inc., Eden Prairie, Minn.) was added to a concentration of 10^{-7} M, and cultures were incubated for 24 hr. Cell layers were then washed 3 times with serum-free medium and incubated for an additional 24 hr in 100 ml medium containing insulin (5 μg/ml; Sigma Chemical Co.). Conditioned medium was centrifuged at 10,000 × g and dialyzed against 3 changes of distilled water for 3 days.

THP-1 conditioned medium was concentrated 20-fold by ultrafiltration through a 150-mm YM-10 membrane in an Amicon-stirred cell. The concentrated medium was then centrifuged at 10,000 × g, filter sterilized, and stored frozen. Lowry (15) protein assays showed a 30% loss of protein in the culture fluids during dialysis and concentration. The protein content of the concentrated medium was approximately 300 μg/ml.

Cell Lines. The effect of THP-1 conditioned medium was assayed with mammary cell lines (MCF-7, BT-474, MDA-MB415, T47D, HBL-100, and ZR 75-1) obtained from the cell culture bank of the Breast Cancer Task Force; a melanoma line (A375Ag5) and a lung type II carcinoma (549) obtained through the Viral Carcinogenesis Branch, National Cancer Institute; a colon carcinoma (SW48) obtained from Dr. Jorgen Fogh, Memorial Sloan-Kettering Institute; and the cervical carcinoma (HeLa S-3) from Dr. Daniel Tershak of our program. The MCF-7 and HBL-100 lines were maintained in McCoy's Medium 5A with 10% FBS. All other lines were maintained in an equal mixture of Dulbecco-Vogt modified Eagle's minimum essential medium:Ham's F-12 containing 10% FBS. Cell lines, including THP-1, were tested free of Mycoplasma. Media and serum were obtained from Grand Island Biological Co. (Grand Island, N. Y.).

Growth Assays. The effect of conditioned THP-1 medium on cell growth was assayed by several methods. Colony-forming assays were conducted by inoculating 1 × 10^3 cells in 2 ml medium to 35-mm tissue culture dishes. Cultures were maintained for 1 day at 37° in a humidified 5% CO\textsubscript{2}-95% air environment. Dilutions of conditioned THP-1 medium were then added to triplicate cultures. Cells were fixed in formal 0.9% RPMI-1640, Roswell Park Memorial Institute Medium 1640; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LPS, lipopolysaccharide.

Received December 27, 1982; accepted May 9, 1983.
Growth Inhibitor from Human Leukemia Line

The apparent molecular weight range of components for active inhibitory fractions following chromatographic separation was determined by SDS-PAGE according to the method of Weber et al. (28). Analytical slab gels comprised of a 3% stacking and a 10% separating gel were used to resolve 5 μg of protein per lane. Samples reduced with 2-mercaptoethanol were separated on a 24 x 116 x 0.75-mm gel at a constant current of 15 mA at 15°. Gels were fixed in acetic acid and stained with silver nitrate (17). Molecular weights of visible components were determined by comparison with Bio-Rad M, 10,000 to 100,000 SDS-PAGE standards.

RESULTS

THP-1 leukemia cells grew as free-floating cell clusters to a maximum density of 7 x 10^6 cells/ml in RPMI-1640 supplemented with 5% FBS. Incubation with mezerein induced 95% of the cells to attach and flatten onto plastic culture flask surfaces within 4 hr. Cells treated for 24 hr and maintained for 1 month following the removal of mezerein continued to have a flattened morphology, and mitotic figures were not observed. Between 80 and 90% of the attached cells excluded trypan blue at 4 days following activation. Flattened cells were detached from culture flasks following a 15-min incubation in trypsin:versene and transferred to new flasks with fresh medium. Ninety % of the cells reattached, but there was no increase in cell number during the subsequent week. Thus, mezerein resulted in an irreversible change in morphology and a loss of cell division potential similar to the cell maturation events described by Rovera et al. (19, 20) and Tsuchiya et al. (26).

Medium conditioned by mezerein-treated THP-1 cells was examined for inhibitory activity against the MCF-7 mammary cancer cell line. THP-1 cells were incubated for 24 hr in RPMI-1640 supplemented with 1% serum in the presence or absence of mezerein (activator phase), and attached cells were repeatedly washed with serum-free medium and maintained for 24 hr in medium alone or medium supplemented with either 1% serum or insulin (5 μg/ml; effector phase). Inhibition was measured in both a cell growth and a colony-forming assay. The results in Table 1 show that mezerein treatment coupled with the presence of insulin or serum during the effector phase markedly enhanced the level of inhibitory activity in conditioned medium. Similar quantitative results were obtained in both assay systems. Comparison of the final cell densities or number of colonies among control cultures confirmed that growth inhibition was due to a THP-1 cell product and not to the small amount of insulin present during the assays.

The length of the effector phase was examined to determine

| Table 1 |
|------------------|------------------|
| Incubation conditions leading to the production of growth inhibitor by THP-1 leukemia cells |
| THP-1 cells were incubated in RPMI-1640 supplemented with 1% FBS with or without mezerein. Cells were washed 3 times with serum-free medium or with medium containing either FBS or insulin. MCF-7 cells were assayed in McCoy's Medium 5A with 10% FBS and a 1:20 dilution of media samples. Average cell number and colony counts were based on triplicate cultures. |

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Inhibition of MCF-7 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-hr activator phase</td>
<td>24-hr effector phase</td>
</tr>
<tr>
<td>Cell no. x 10^6</td>
<td>Colony no.</td>
</tr>
<tr>
<td>1% FBS</td>
<td>1% FBS</td>
</tr>
<tr>
<td>1% FBS, 10^-7 mezerein</td>
<td>1% FBS</td>
</tr>
<tr>
<td>1% FBS, 10^-7 mezerein</td>
<td>1% FBS</td>
</tr>
<tr>
<td>1% FBS, 10^-7 mezerein</td>
<td>Insulin (5 μg/ml)</td>
</tr>
</tbody>
</table>

^a Numbers in parentheses, percentage of inhibition calculated from average cell number (2.58 x 10^6) and colony count (261) control cultures.

AUGUST 1983

3669
the optimal period for the collection of THP-1 medium with the greatest inhibitory activity. Mezerein-activated THP-1 cells were incubated with insulin-supplemented serum-free medium for 3 days. Aliquots of the culture fluids were collected each day to assay the amount of accumulated activity, or culture fluids were collected and replaced with fresh medium to determine the amount of inhibitory activity released each day. Inhibition was monitored by growth assay following the addition of 10% conditioned media to MCF-7 cell cultures. The results showed that medium collected during the first day of the effector phase inhibited MCF-7 cell growth by 80%. Inhibition by media conditioned during the second and third days inhibited growth by 62 and 17%, respectively. However, the level of activity present during the first day was maintained over the 3-day period of incubation. All subsequent experiments used THP-1 medium collected after a 1-day effector phase.

Macrophages can be activated by LPS for tumoricidal activity (3, 27). Phenol-extracted LPS (Escherichia coli serotype; 0127:138; Sigma) was added to THP-1 cells at a concentration of 10 μg/ml during either the mezerein activation or effector phases. Conditioned media were then tested at 5 and 10% concentrations in growth assays with MCF-7 cells. There were no differences observed in the amount of growth-inhibitory activity induced with the addition of endotoxin during either phase. In addition, LPS did not induce THP-1 activation in the absence of mezerein.

Experiments were performed to monitor the effects of conditioned medium from mezerein-activated THP-1 cells on the colony-forming ability of several human cell lines. The results illustrated in Chart 1 show that colony formation by a cervical carcinoma (HeLa), a melanoma (A375Ag5), and 4 mammary carcinoma cell lines (MCF-7, BT-474, MDA-MB-415, and T47D) was inhibited in a dose-response manner. The 50% inhibitory dose ranged from 0.9 to 1.5 μg protein per ml for HeLa, MCF-7, and BT-474 cells to 7.5 μg protein per ml for T47D cells. All cell types exhibited changes in morphology including increased vacuolization at the higher protein concentrations.

In contrast, assays with 4 cell lines showed that THP-1 conditioned medium increased the number of colonies per dish (Chart 2). A maximum 20% increase was observed in cultures of the 549 lung carcinoma line. A 47% increase was observed with the HBL-100 milk cell line and an 80% increase with ZR75-1 malignant mammary cells following exposure to 1.6 μg protein per ml. The colon carcinoma line, SW-48, was the most responsive with a 3-fold increase in the number of cell colonies. Protein concentrations above 6.4 μg/ml did not stimulate colony formation. Inhibition or stimulation of growth could not be correlated with the tissue of origin or the population doubling time for each cell line.

Experiments were conducted to determine if this growth inhibition or stimulation were the result of mezerein remaining in conditioned medium from washed THP-1 cells during the effector phase. Mezerein in insulin-supplemented RPMI-1640 was added directly to cultures of MCF-7 and HBL-100 cells preseeded for colony assay. Counts at 7 days revealed that 10⁻⁸ and 10⁻⁹ M mezerein inhibited MCF-7 cell colony formation by 62 and 19%, respectively; and HBL-100 cells, by 42 and 16%, respectively. In addition, medium originally containing mezerein at 10⁻⁷ M and dialyzed for 3 days against distilled water did not inhibit MCF-7 colony formation. The lower level of growth inhibition observed with mezerein alone, the loss of inhibitory activity following dialysis of mezerein-containing medium, the absence of growth-stimulating activity with mezerein-treated HBL-100 cells, and the data in Table 1 showing lower levels of activity in serum- and insulin-free medium collected during the effector phase led to the conclusion that the growth effects we observed with conditioned medium are the result of THP-1 cell activation.

We then compared cell growth, colony formation, and cytotoxicity assays to determine their relative sensitivities in measuring the inhibitory effects of THP-1 medium. Experiments were conducted with HeLa and MCF-7 cells. The dose-response curves of Chart 3 demonstrate that the cell growth and colony-forming assays were more sensitive than was the cytotoxicity assay. The 50% inhibitory protein dose for HeLa cells was approximated at 1.0 μg/ml in the colony assay and at 1.6 μg/ml in the growth assay. A similar response with MCF-7 cells was observed in the colony assay, but the 50% inhibitory dose in the growth assay

![Chart 1. Inhibition of cell colony formation by various concentrations of protein in THP-1 conditioned medium. Cell line designations and the average colony number in control cultures of each line were: MCF-7 (△), 392; HeLa (○), 322; A375Ag5 (●), 136; BT-474 (●), 144; MDA-MB-415 (▲), 192; T47D (□), 240. Values expressed as the percentage of inhibition calculated from colony counts in triplicate control and treated cultures after 7-day incubation; bars, S.D.](image-url)

![Chart 2. Stimulation of cell colony formation by THP-1 conditioned medium. Cell line designations and the average colony number in control cultures of each line were: SW-48 (●), 100; ZR 75-1 (△), 80; HBL-100 (○), 65; 549 (□), 56. Values expressed as the percentage of stimulation calculated from colony counts in triplicate control and treated cultures after 7-day incubation; bars, S.D.](image-url)
was approximately 2.3 μg/ml. Incubation in the highest concentration of THP-1 conditioned medium resulted in the release of less than 20% of the tritium counts above the spontaneous lysis values. A preliminary experiment was conducted to determine if growth inhibition was reversible. Preseeded MCF-7 cultures were incubated in THP-1 medium at a concentration of 5% which induced approximately 80% growth inhibition by 7 days. Each day, sets of cultures were washed twice with serum-free medium and incubated in serum-supplemented medium without inhibitor. Daily cell counts of triplicate cultures revealed that growth inhibition was reversible following 6 days of exposure. Thus, the data show that growth inhibition is a cytostatic rather than a cytolytic response.

Chart 4 shows results calculated from growth studies for HeLa and MCF-7 cells treated continuously with 2 concentrations of THP-1 conditioned medium. Cells were seeded at 5 × 10^5 into 35-mm dishes. One day later, at time zero, medium samples were added, and cell numbers were counted each day thereafter. Results were expressed as percentage of inhibition of growth. THP-1 medium showed a time- and concentration-dependent effect on cell growth. Inhibition accumulated and reached a maximum in a shorter time at the lower sample concentration. Maximum inhibition at the higher dose was observed following incubation for 6 days.

MCF-7 and HBL-100 growth assays were used to examine the effects of various chemical and physical treatments on the stability of the inhibitory activity (Table 2). There was a 20% loss of activity following a 1-hr incubation of THP-1 conditioned medium at 56°, and 64% was lost with incubation at 70°. The inhibitor and growth stimulator were stable following dialysis against acetic acid, and repeated freezing and thawing had no effect. Incubation with 6 M urea for 4 days at 4° or 20° did not affect recovery of inhibitor. Only 45% of the inhibitory activity was regained when concentrated medium was incubated with 0.1% SDS for 1 hr. Trypsin destroyed the inhibitor during a 2-hr incubation.

Preliminary separation of active components in crude concentrated medium from mezerein-activated THP-1 cells was conducted by chromatography on Bio-Gel P-60 in the presence of PBS. When fractions were tested for inhibitory or stimulatory activity against MCF-7 or HBL-100 cells, all activity was eluted near the void volume. When a second sample was applied and eluted in acetic acid, inhibitory activity was retained by the column and was recovered in 4 very active fractions (Chart 5). Subsequent SDS-PAGE of these pooled acetic acid fractions resolved several components in a molecular weight range of 12,000 to 30,000. Growth-stimulating activity for HBL-100 cells was observed at an elution volume of approximately 110 to 135 ml on the P-60 column, indicating a component(s) distinct from the inhibitory factor.

| Table 2 |
|-------------------------------|----------------|-------------|
| **Physicochemical stability of inhibitory activity** | **Treatment** | **Av. cell no. x 10^5** | **% of inhibition** |
|-------------------------------|----------------|-------------|
| **Heat, 1 hr** | 0.85 | 69 |
| Control | 0.83 | 70 |
| 37° | 1.21 | 56 |
| 56° | 2.07 | 25 |
| 70° | 1.85 | 64 |
| **1 M acetic acid, 3 days** | 0.85 | 69 |
| Control | 0.87 | 68 |
| Treated | 0.82 | 70 |
| **6 M urea, 4 days at 4°** | 0.85 | 70 |
| Control | 0.83 | 69 |
| Treated | 0.89 | 64 |
| **6 M urea, 4 days at 20°** | 0.74 | 73 |
| Control | 0.74 | 73 |
| Treated | 1.85 | 33 |
| **0.1% SDS, 1 hr** | 0.65 | 80 |
| Control | 0.55 | 80 |
| Treated | 2.31 | 16 |

Figures and charts are included to illustrate the data.
Chart 5. Inhibitory and stimulatory activities in THP-1 conditioned medium separated by molecular-sieve chromatography on a Bio-Gel P-60 column (1.5 x 90 cm). Medium sample was dialyzed and eluted in 1 M acetic acid. Each 4.5-ml fraction was then tested at a 10% concentration for inhibition against MCF-7 cells (C) or growth stimulation with HBL-100 (○). Values expressed as percentage of inhibition or stimulation calculated from cell counts in triplicate control and treated cultures after 7-day incubation. Average cell number in control cultures was 6 × 10⁴ for MCF-7 cells and 1.8 × 10⁵ for HBL-100 cells.

DISCUSSION

Rovera et al. (19, 20) and Cassileth et al. (2) reported that phorbol tumor promoters and the plant diterpene estrer, mezerein, rapidly and irreversibly converted cultures of a human acute promyelocytic leukemia cell line (HL-60) into adherent nonproliferating macrophage-like cells. These differentiated cells demonstrated several macrophage characteristics, including surface Fc receptors for IgG, phagocytosis, and increased acid phosphatase and nonspecific esterase activities (20). The THP-1 line used in the current study was derived from an acute monocytic leukemia and has the unique feature of retaining its monocytic properties with extended in vitro maintenance (25). The changes in morphology and division evoked by exposure of THP-1 cells to mezerein were identical to those originally described for mezerein-treated HL-60 cells (20) and TPA-activated THP-1 cells (26).

Macrophages can be activated to express cytostatic or cytotoxic activity in vitro, and most evidence suggests that macrophage cytotoxicity is selective for tumor cells (9–11, 24). Additional results showed that cytotoxic activity does not require target cell cocultivation but is induced by macrophage-conditioned culture fluids (12).

The culture fluids conditioned by activated THP-1 inhibited the growth of 6 target cell lines, but promoted the growth of 4 additional lines. The degree of susceptibility to growth inhibition was independent of the population doubling time and the tissue of origin. This latter observation agrees with previous findings that macrophage-mediated inhibition differs among cell lines (12). However, growth inhibition was not due to the classical cytotoxicity reported for other macrophage systems (21). When we compared assay techniques with the HeLa and MCF-7 cells, the thymidine release assay measuring cell destruction was much less sensitive. Colony formation and cell growth assays both resulted in sigmoidal dose-response curves with similar sensitivities for growth inhibition. Keller (13) observed that the spectrum of cell types susceptible to cytotoxicity by macrophages is much more restrictive than the range of target cells responsive to growth inhibition.

The presence of growth-stimulatory factors in THP-1 culture fluids might be expected, since growth-promoting activity by macrophages has been frequently reported (8, 14, 18, 29). Schultze et al. (22) showed that direct exposure of primary mouse macrophage cultures to the potent tumor promoter, TPA, resulted in stimulation of tumor cell growth. In contrast, cytotoxic macrophages were only recovered from mice given injections of TPA.

Our results suggest that it is possible to observe this dual role with cells activated in vitro and raises several interesting questions concerning the nature of the growth response to THP-1 conditioned medium. For example, does inhibition or stimulation of growth reflect the effects of one or more macrophage products whose expression is dictated by some target cell feature? If activities result from different growth modifiers, are they competing for the same site of action? This would, in part, explain the differences in cell line sensitivities.

A better understanding of the nature of macrophage-mediated growth modifiers has been curtailed, because most studies are conducted with primary cultures of macrophage populations which express low levels of cytostatic or cytotoxic activities for a limited period in vitro. Two features of the THP-1 cell system make it attractive for the study of effector molecules: (a) growth-inhibitory and growth-stimulatory activities are produced by a continuously replicating human leukemia cell line; and (b) activity is recoverable under serum-free conditions avoiding the problems in isolating effectors from complex protein mixtures.

REFERENCES

14. Lebowich, S. J., and Ross, R. A macrophage dependent factor that stimulates...
Production of Growth-inhibitory Activity in Serum-free Medium by Human Monocytic Leukemia Cells

Edwin V. Gaffney, Shiow-Chuan Tsai, Marie L. Dell'Aquila, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/43/8/3668

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.