ABSTRACT

It has long been known that complex interactions occur between tumors and normal host immune cells. The human melanoma cell line A375 has been used previously as an indicator cell for tumor cell cytotoxicity mediated by monocytes. During other studies on this tumor cell line, we noted that the conditioned media harvested from A375 cultures induced the human monocytic cell line U937 and human blood monocytes to release the cytokine tumor necrosis factor (TNF). We characterized this tumor factor which induced TNF release by monocytic cells. Purification was performed using ammonium sulfate precipitation, ion exchange (DEAE) chromatography, gel filtration, and reversed-phase high performance liquid chromatography. The factor copurified with granulocyte-macrophage colony-stimulating factor (GM-CSF). The purified material caused the release of TNF by U937 cells and stimulated formation of granulocyte-macrophage colonies in methyl cellulose. TNF release by U937 cells in response to A375-conditioned medium was inhibited by neutralizing antibodies to GM-CSF. The TNF-inducing activity in A375-conditioned medium was completely removed by an anti-GM-CSF affinity column. Western blotting using antibodies to GM-CSF confirmed a single M, 27,000 band in A375-conditioned medium. We found that recombinant human GM-CSF stimulated TNF production by the same cells as the tumor-conditioned medium. These data show that A375 human melanoma cells produce GM-CSF, which in turn causes TNF production by cells in the monocytic lineage. The combination of GM-CSF production by the tumor and TNF production by immune cells may influence not only tumor growth but also some of the paraneoplastic syndromes associated with malignancy such as hypercalcemia, cachexia and leukocytosis.

INTRODUCTION

It has long been appreciated that the immune system is activated by the presence of a tumor and that tumor growth may depend not just on the characteristics of the tumor itself but also on the defense mechanisms which host cells mount (1, 2). Although all of the signals which are responsible for tumor cell-host cell interactions are not known, recently it has become apparent that several of the cytokines which may be involved include GM-CSF and TNF. The human melanoma cell line A375 has often been used as an indicator target cell line for cytotoxic effects of immune cells. Grabstein et al. (3) found that recombinant human GM-CSF induced normal peripheral blood monocytes to become tumoricidal for A375 cells. Carinistra et al. showed that human GM-CSF induced expression of TNF by peripheral blood monocytes and by the human monocytic cell line U937 (4) and that antibodies to TNF completely inhibited the cytokotic activity released by monocytes in response to GM-CSF (5).

During the course of other studies with the human melanoma cell line A375, the target cell line used to show that GM-CSF induced monocyte-mediated cytotoxicity (3), we noted that conditioned medium harvested from A375 tumor cell cultures induced TNF production in human blood monocytes and the human monocytoid cell line U937 by the secretion of a soluble factor. By multiple criteria, we have identified this soluble factor which causes TNF production as GM-CSF. These results suggest that in this human tumor, production of GM-CSF by the tumor may retard tumor growth by causing release of cytotoxic cytokines of host cell origin.

MATERIALS AND METHODS

Culture Media and Reagents. RPMI 1640, Eagle's minimal essential medium, and PBS were from Hazleton (Lenexa, KS), trypsin-EDTA was from Gibco (Grand Island, NY), and FCS was from HyClone (Logan, UT). BSA, PMA, HEPES, and Tris buffer were from Sigma (St. Louis, MO).

The reagents and apparatus for electrophoresis and Western blotting were from Bio-Rad (Richmond, CA) unless specified. All the reagents for purification of GM-CSF from A375-conditioned medium were from Sigma, unless specified.

Cytokines and Antibodies. rhTNF, rhTGF-α, and rh-β interferon were kindly provided by Dr. H. Michael Shepard (Genentech, Inc., South San Francisco, CA), and rhGM-CSF by Dr. Steven C. Clark (Genetics Institute, Boston, MA). Monoclonal mouse anti-rhGM-CSF antibody was purchased from Genzyme (Boston, MA), rhEFGF was from Collaborative Research (Lexington, MA), and polyclonal goat anti-rhTNF was from R & D Systems (Minneapolis, MN).

Cell Lines. The A375 human melanoma, the U937 human histiocytic lymphoma, the SaOS-2 human osteosarcoma, and the NCTC-L929 murine fibrosarcoma were obtained from the American Type Culture Collection (Rockville, MD). The HOSO human maxillary carcinoma was kindly provided by Dr. Toshiyuki Yoneda, of our group. The UMR-106 rat osteosarcoma was kindly provided by Dr. T. John Martin (University of Melbourne, Australia). The A375, U937, SaOS-2, and UMR-106 cell lines were cultured in 10% FCS-RPMI 1640 medium. The NCTC-L929 cells were cultured in 10% FCS-Eagle's minimal essential medium. For preparation of CM, tumor cells were grown until confluent; then the medium was discarded, the flasks washed three times with PBS, and either 0.1% BSA-RPMI or 50 mMV HEPES-RPMI medium was added. After 2 days the conditioned medium was collected, centrifuged to remove debris, and stored at −20°C until use.

Human Blood Monocytes. Peripheral blood was drawn from healthy volunteers and the mononuclear cell fraction was separated by gradient centrifugation on Ficoll-Paque (Pharmacia, Piscataway, NJ). Aliquots of 1.2 × 10⁶ cells in 1 ml of 5% FCS-RPMI were seeded in 24-well plates and, after 1 h of incubation at 37°C in 5% CO₂, washed three times with PBS to remove the nonadherent cells. The adherent cells (monocytes) were then cultured in 0.5 ml of 5% FCS-RPMI with the indicated treatments for 24 h, at the end of which the media were collected for TNF assay.

Treatment of U937 Cells. U937 cells, 1 × 10⁶ cells/ml, were incubated for 24 h, unless indicated, with various dilutions of cytokines or tumor-conditioned media in 5% FCS-RPMI in the absence or presence of 10 ng/ml of PMA.

TNF Cytotoxicity Assay. TNF was assayed biologically as cytotoxic activity against NCTC-L929 cells. Each sample was assayed in duplicate in serial dilutions in 96-well plates as described (6). TNF activity was quantitated as the reciprocal of the dilution that results in 50% survival. One cytotoxic unit is the amount of TNF that results in 50%
survival of the indicator cells. TNF specificity was shown by using anti-human TNF antibody.

**TGFα Assay.** TGFα was measured as EGF equivalents by radioreceptor assay on U-106 as described (7).

**Immunofinity Column.** Monoclonal anti-rhGM-CSF antibody, 500 μg, was covalently bound to 2 ml of Amino Link, cross-linked, 6% beaded agarose gel, using Immuno Pure Antibody/Antigen Immunobilization Kit 1 (Pierce, Rockford, IL). The antibody-linked gel, loaded in a 1 x 0.5-cm column, had the capacity to retain 500 ng of rhGM-CSF. A375 CM, 500 ml in 50 mM HEPES-RPMI, were applied to the column at 4°C by a peristaltic pump at the rate of 0.5 ml/min. The column was washed of the unbound protein with 16 ml of RPMI, and the retained material was eluted using 12 ml of 0.1 M glycine, pH 2.8. Fractions of 1 ml were collected and immediately neutralized with 50 μl of 1 M Tris-HCl, pH 9.5.

**Electrophoresis and Western Blotting Procedure.** Fractions containing TNF-inducing activity as determined by their capacity to stimulate TNF production by U937 cells were pooled, dialyzed extensively against 50 mM NH4HCO3, pH 8.3, at 4°C in a M,3500 molecular weight cutoff membrane (Sephadex G-200 gel filtration column (100 x 2.5 cm). The bound protein was eluted by a stepwise gradient of increasing concentrations of sodium phosphate, pH 8.3, and then applied to a DEAE-cellulose anion exchange column (8 x 2.5 cm). The protein elution profile was monitored by a silver nitrate staining kit from Bio-Rad.

**Purification of TNF-inducing Activity from A375 CM.** One liter of A375 CM in 0.1% BSA-RPMI was subjected to precipitation in 80% (NH4)2SO4 while stirring at 4°C for 4 h. After centrifugation at 15,000 rpm for 45 min at 4°C, the pellet was resuspended in H2O, dialyzed at a molecular weight cutoff of 3500 against 50 volumes of 0.01 M sodium phosphate, pH 8.3, and then applied to a DEAE-cellulose anion exchange column exchange solution (8 x 2.5 cm). The bound protein was eluted by a stepwise gradient of increasing concentrations of sodium phosphate, pH 8.3, from 0.01 to 0.1 M P04. The protein elution profile was monitored spectrophotometrically at 280 nm. The fractions collected at 0.04 and 0.06 M sodium phosphate, containing most of the TNF-inducing activity, were pooled, dialyzed against 50 mM NH4HCO3, pH 8.3, lyophillized, resuspended in 50 mM NH4HCO3, pH 8.3, and applied to a Sephadex G-200 gel filtration column (100 x 2.5 cm). The fractions corresponding to the peak of TNF-inducing activity were pooled, dialyzed against 50 mM NH4HCO3, pH 8.3, and resuspended in pyridine acetate buffer before application to a Nova Pak C18 reverse phase HPLC column, 1 x 0.39 cm (Waters, Milford, MA). The buffer system consisted of: A, 0.2 M pyridine-0.9 M acetic acid, pH 4.0; B, 50% A and 50% 1-propanol (all from Burdick & Jackson, Muskegon, MI). The gradient was linear from 100% A to 30% A and 70% B in 60 min.

**Thymidine Incorporation.** The effect of TNF or U937 tumor-conditioned medium on the growth of A375 cells was determined as follows. A375 cells (1000/well/80 μl) were seeded in 96-well plates. After 24 h, 80 μl of control or treatment medium were added; after 72 h, the cells were labeled with 0.25 μCi/well of [3H]thymidine (ICN, Irvine, CA). After 6 h, the medium was aspirated from the plate and 100 μl/well of trypsin-EDTA were added. After 10 min the cells were collected by a PHD cell harvester (Cambridge Technology, Watertown, MA) onto filter paper discs. The radioactivity retained by the filters was measured by a liquid scintillation counter (Beckman, Fullerton, CA) and the effects of each treatment were calculated as the percentage of change compared with the control.

**Colonies Formation Assay in Methylocellulose Using My-10° Cells.** A375-CM-conditioned medium and partially purified TNF-inducing activity from A375-conditioned medium were tested for colony-stimulating activity using panned My-10° bone marrow cells as described previously (11). Mononuclear cells were isolated using Ficoll-Paque (Pharmacia) and the nonadherent fraction was separated by adherence overnight to plastic. The nonadherent fraction was enriched for colony-forming cells by positive indirect immunoadherence using human progenitor cell antibody (Becton Dickinson, Mountain View, CA), which is the commercial preparation of monoclonal anti-My-10 antibody (12), and the "panning" technique of Engleman et al. (13). The cells were cultured as described previously (11) in duplicate, and the total number of colonies per dish was counted on day 14.

## RESULTS

During the course of studies on the mechanism of hypercalcemia which occurs in the A375 tumor, we found that conditioned medium harvested from cultured A375 cells consistently stimulated the human monocytes cell line U937 and human monocytes to release TNF activity (Tables 1 and 2). This activity could clearly be ascribed to TNF, inasmuch as it was abolished by anti-rhTNF antibody (data not shown). A time course study of TNF production by U937 cells in response to A375 tumor cell-conditioned medium is shown in Fig. 1. In all these experiments, the U937 cells were costimulated with the phorbol ester PMA, known to cause differentiation of U937 cells to a mature phenotype capable of differentiated cell function (14). No TNF activity could be detected in the media of U937 cells cultured in absence of PMA (data not shown). Normal human macrophages were also tested for TNF production in response to A375-conditioned medium. We saw that the A375-conditioned medium specifically stimulated TNF production by monocytes. This stimulation was more evident in presence of a secondary stimulus such as γ-interferon or PMA (Table 1).

These studies show that A375 tumor-conditioned medium...
induces cells in the monocyte lineage to produce TNF by the release of a soluble mediator. We then attempted to identify the soluble mediator responsible for TNF production by human U937 cells. Because the A375 melanoma is a producer of TGF-α (15) (we could detect in the conditioned medium a concentration of approximately 1 ng/ml), we tested TGF-α (50 ng/ml) as well as the related peptide EGF (50 ng/ml), for their effects on TNF production by U937 cells. No stimulatory effect was seen (data not shown). Conditioned medium was collected from serum-free A375 cultures, concentrated by ammonium sulfate precipitation, and fractionated by ion exchange chromatography, gel filtration, and reverse phase HPLC. A purification profile similar to that described for GM-CSF (16) was obtained, with the TNF-inducing activity eluting from a C<sub>18</sub> reverse phase HPLC column at 31% 1-propanol (Fig. 2C).

Since conditioned medium harvested from A375 tumors was behaving like GM-CSF and previous studies had shown that GM-CSF-induced TNF mediated monocyte cytotoxicity for A375 cells (3–5), we then attempted to determine if GM-CSF was the soluble factor produced by A375 cells which stimulated TNF production. Firstly, we tested A375 crude conditioned medium and purified fractions for GM-CSF activity and found it to be present (Table 3). However, in the samples treated with crude conditioned medium the colonies were smaller in size than those seen with the purified fractions or with rhGM-CSF (data not shown). The partially purified TNF-inducing activity from A375-conditioned medium was converted to GM-CSF equivalents in molarity on the basis of the TNF-inducing activity of rhGM-CSF. Recombinant human GM-CSF and purified A375-conditioned medium caused similar effects on colony size and number.

Next, we examined whether neutralizing antibodies raised against recombinant human GM-CSF abrogated the TNF-inducing activity of the A375-conditioned medium. We saw that monoclonal anti-rhGM-CSF substantially decreased the TNF-inducing activity of the A375-conditioned medium and rhGM-CSF (Table 4). The inhibition by the antibody of the activity in the A375-conditioned medium was incomplete. However, at the dilution used, the antibody completely inhibited concentrations of rhGM-CSF lower than 0.5 ng/ml and partially inhibited concentrations of GM-CSF capable of giving the same effect as the A375-conditioned medium. We then used the monoclonal antibody to construct an affinity column for GM-CSF. We found that all of the TNF-inducing activity contained in an aliquot of A375-conditioned medium was bound to the column. No activity was detected in the flowthrough. The TNF-inducing activity was eluted from the affinity column after washing with low pH glycine buffer (Fig. 3).

These data show that neutralizing antibodies to GM-CSF block the activity in A375 tumor cell conditioned medium and that all of the TNF-inducing activity in tumor cell-conditioned media can be absorbed to a GM-CSF antibody affinity column. To confirm that the activity was in fact due to GM-CSF, we examined purified fractions using Western analysis. We fractionated a purified sample obtained from immunoaffinity columns by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred the proteins to nitrocellulose. Immunoblot analysis was performed using specific monoclonal antibody to GM-CSF as primary antibody, and alkaline phosphatase-conjugated secondary antibodies in order to visualize the corresponding protein by enzymatic reaction. Recombinant human GM-CSF was used as a positive control. As shown in Fig. 4, a distinct band was present in the A375 affinity-purified conditioned medium (M<sub>r</sub>, 27,000) within the range assigned to native hGM-CSF (17).

TNF has been shown recently to be directly tumoricidal for the A375 melanoma (18). We found similar results by testing the effects of TNF on thymidine uptake by A375 cells (Fig. 5A). In addition, the TNF-containing culture media from U937 cells treated with either A375-conditioned medium or GM-CSF inhibited, as expected, the uptake of thymidine by A375 cells.
Table 3 Colony-stimulating activity of A375 conditioned media and partially purified TNF-inducing activity compared with rhGM-CSF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Colonies/dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control medium</td>
<td>3</td>
</tr>
<tr>
<td>A375 CM</td>
<td>23</td>
</tr>
<tr>
<td>1:5</td>
<td>14</td>
</tr>
<tr>
<td>1:10</td>
<td>17</td>
</tr>
<tr>
<td>Immunoaffinity-purified fraction</td>
<td>10^6 M</td>
</tr>
<tr>
<td></td>
<td>10^10 M</td>
</tr>
<tr>
<td></td>
<td>10^14 M</td>
</tr>
<tr>
<td>rhGM-CSF</td>
<td>10^10 M</td>
</tr>
<tr>
<td></td>
<td>10^11 M</td>
</tr>
</tbody>
</table>

* The immunoaffinity-purified fraction was quantified in molar equivalents of rhGM-CSF on the basis of its TNF-inducing activity compared to that of rhGM-CSF.

Table 4 Effects of monoclonal anti-rhGM-CSF on TNF production by U937 cells stimulated with either rhGM-CSF or A375 CM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TNF (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control medium</td>
<td>3</td>
</tr>
<tr>
<td>rhGM-CSF 1.5 ng/ml</td>
<td>119</td>
</tr>
<tr>
<td>rhGM-CSF 1.0 ng/ml</td>
<td>93</td>
</tr>
<tr>
<td>rhGM-CSF 0.5 ng/ml</td>
<td>62</td>
</tr>
<tr>
<td>rhGM-CSF 0.1 ng/ml</td>
<td>21</td>
</tr>
<tr>
<td>A375 CM 1/2</td>
<td>120</td>
</tr>
<tr>
<td>A375 CM 1/4</td>
<td>115</td>
</tr>
</tbody>
</table>

Antibody to hGM-CSF prevented the production of inhibitory activity by U937 cells treated with A375-conditioned medium (Fig. 5B).

**DISCUSSION**

The monocytic cell line U937 is a well-established model for the study of maturation and activation of cells of the monocyte-macrophage lineage (19, 20). GM-CSF has been shown to induce differentiation of these cells (21) and to inhibit their growth via TNF expression (4). Several investigators have used cultured A375 cells as an indicator target cell for investigating macrophage tumoricidal activity (3, 18). In our study, we found that A375-conditioned medium stimulates monocytes and U937 cells to produce TNF and that the factor produced by A375 cells which is responsible for this effect is GM-CSF.

GM-CSF is a glycoprotein originally isolated from a T-lymphoblast cell line (22) on the basis of its capacity to stimulate the formation of granulocyte-macrophage colonies in cultures of bone marrow progenitor cells. Infusion of recombinant hGM-CSF into healthy and pancytopenic monkeys led to a dramatic leukocytosis and reticulocytosis (23). An improvement of granulocyte, monocyte, and reticulocyte counts was
observed after administration of rhGM-CSF to patients with aplastic anemia and myelodysplastic syndrome (24). However, GM-CSF is not only a hematopoietic growth-regulatory factor. It controls not only the formation but also the activity of mature phagocytes. GM-CSF induces monocytes to become tumoricidal for the A375 melanoma (3); stimulates TNF production by human mononuclear cells (25), especially upon co-addition of lipopolysaccharide or γ-interferon (26); inhibits the migration of neutrophils (22); and enhances their phagocytic activity, antibody-dependent cytotoxicity, and IL-1 production (27–29). Mice transfected with the murine GM-CSF gene showed accumulation of activated macrophages in the peritoneal and pleural cavities and developed tissue damage associated with infiltration of macrophages. Most of these mice died at an early age, underweight and with muscle wasting, suggesting excessive production of the cachectic cytokines TNF and IL-1 by GM-CSF-activated macrophages (30). We show in the present study that the A375 melanoma cells cause, through the release of GM-CSF, the release of TNF by monocytes and the monocyte cell line U937. Together with earlier reported studies, these data suggest that production of GM-CSF by tumor cells may limit the growth of the tumor itself by the production of TNF by monocyte-macrophages.

As noted above, GM-CSF induces monocyte tumoricidal activity. This effect is mediated by TNF (5), which may be either soluble or membrane-bound. In these monocyte-cytotoxicity experiments coculture was performed with target tumor cells; cell-cell contact may have been required. However, it is known that TNF inhibits the growth of several tumor cell lines (31) including, as shown in this study, the A375 melanoma suggesting that cell-cell contact is not necessary in this system. GM-CSF and TNF may play important collaborative roles in the local immune response. GM-CSF is secreted not just by activated T-cells but also by human tumor cells (as shown here), and it may be one of the major factors responsible for priming monocytes to express TNF. This relationship between GM-CSF secretion by tumor cells and TNF production by monocyte-macrophages may work as a negative feedback loop in the control of tumor cell growth.

Since we show here that a human tumor produces a factor which can indirectly mediate cytotoxic effects on this tumor, presumably a delicate balance must exist between tumor cell products and release of host cell cytokines in vivo. One important element must be the responsiveness of the tumor cells to the cytokotic cytokines produced by the stimulated normal immune cells. Moreover, in some circumstances, GM-CSF may act as a direct autocrine stimulator of tumor growth. Young and Griffin (32) have shown secretion of GM-CSF and autocrine stimulation of growth in an acute myeloblastic leukemia. However, TNF displays a wide range of biological actions and its in vivo effects may extend beyond direct tumor cell killing. TNF may influence the neoplastic process through activation of T-lymphocytes, stimulation of angiogenesis and procoagulant activity, increased prostaglandin and collagenase synthesis, induction of other cytokines, and effects on hematopoiensis (31). Furthermore, apart from its effect on the tumor, TNF may also have detrimental effects on the host such as fever, anorexia, muscular protein degradation, derangement of lipid metabolism, cachexia, stimulation of bone resorption, and subsequent hypercalcemia (31, 33).

TNF is not the only cytokine released by mononuclear cells that inhibits tumor cell replication. Other immune cell products which also inhibit tumor cell replication include TGFβ (34), the interferons, lymphotixin, natural killer cell cytotoxic factor, cytolysin and leukoregulin (35). Recently, Zarling et al. (36) identified a growth-inhibitory factor produced by activated T-cells which inhibited the growth of certain tumor cell lines including the A375 melanoma. This polypeptide growth regulator has a molecular weight of 30,000 and is released by U937 cells as well as by activated T-lymphocytes. The NH2-terminal amino acid sequence reveals that it is distinct from other growth-inhibitory factors. Receptors for this factor, called Oncostatin M, have been identified on target cells. The binding interactions are complex and probably represent several different classes of binding sites (37).

Recent papers have shown that both TNF and IL-1 stimulate GM-CSF production by fibroblasts, endothelial cells, and tumor cell lines (38–41), raising the likelihood of complex loops between tumor factors, immune cells, and other host cells. Although the overall importance of these interactions will be difficult to unravel, they may be responsible not just for regulating tumor growth but also for some paraneoplastic syndromes associated with malignancy such as leukocytosis, cachexia, and hypercalcemia. Clearly, the production of GM-CSF by A375 cells may modulate specific functions in the host with profound consequences on the balance between tumor growth and host defense.

ACKNOWLEDGMENTS

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Stimulation of Tumor Necrosis Factor Release from Monocytic Cells by the A375 Human Melanoma via Granulocyte-Macrophage Colony-stimulating Factor

Massimo Sabatini, Jeffery Chavez, Gregory R. Mundy, et al.


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