Human Pulmonary Macrophage Tumor Cell Cytotoxicity

By Paul Lembred, John Hoidal, Robert Vesella, and John Rinehart

In vivo animal studies support the concept that monocytes and macrophages are important in the immune surveillance of oncogenesis and in vitro activated murine macrophages are cytotoxic for tumor cells. In this study, we examined human pulmonary macrophage and blood monocyte tumor cell cytotoxicity (a measure of cytoctasis and cell kill) and cytotoxic activity (cell kill). Three human tumor cell lines and a skin fibroblast cell strain were used. Pulmonary macrophages exhibited significantly more cytotoxic and cytocidal activity than blood monocytes against all three tumor cell lines tested. In addition, blood monocytes from patients with pulmonary infection and neoplasm were significantly more cytoxic than monocytes from normal individuals ($p < 0.01$). A similar trend was observed when pulmonary macrophages were studied, but the differences were not statistically significant ($p = 0.1$). Pulmonary macrophages also exhibited limited cytotoxic activity against human fibroblasts. However, cytotoxic activity against fibroblasts but not tumor cells was abrogated by decreasing macrophage concentration in the assay system. Macrophage cytotoxic activity against fibroblasts but not tumor cells could be inhibited by catalase and enhanced by phorbol myristate acetate. These data suggest that (A) in vivo maturation of monocytes to macrophages is associated with enhanced tumorcidal activity, (B) alteration of the in vivo microenvironment by tumors or infection enhances blood monocyte and perhaps pulmonary macrophage tumor cell cytotoxicity, (C) human pulmonary macrophages exhibit specific cytocidal activity against tumors in vivo, which is not due to production of H$_2$O$_2$.

Previous investigations have suggested that macrophages and monocytes are important in the control of neoplastic proliferation. Studies in rodent systems have demonstrated that macrophages are important effector cells against tumors in vivo: (A) an inhibitor of macrophage lysosomal enzymes (trypan blue) and a specific macrophage toxin (carrageenan and silica) decrease resistance of mice and rats to transplantable tumors, (B) mice bearing sarcomas of increased numbers of macrophages containing increased numbers of macrophages have longer survivals and a lower incidence of metastasis than mice bearing less immunogenic sarcomas, and (C) mice given agents that activate macrophages (BCG) have an increased resistance to transplantable tumors. In vitro studies have further characterized the role of macrophages in resistance to neoplastic proliferation: (A) activated murine macrophages are cytotoxic and cytoctastic against tumor cells, (B) sensitized T lymphocytes in the presence of germaine tumor antigens secrete a cytophilic protein (not an immunoglobulin) that specifically “arms” macrophages. Armed macrophages on contact with specific tumor antigens become activated and tumorcidal.

In man, several clinical observations suggest that macrophages are important in the control of oncogene:

(A) macrophages have been shown to infiltrate tumors, and (B) patients who have breast carcinoma with high numbers of macrophages infiltrating the primary tumor have a lower incidence of axillary lymph node metastasis and patients with positive axillary lymph nodes, which exhibit histiocytosis, have a lower incidence of distance metastasis. In support of these in vivo observations, we have shown that blood monocytes are cytoctastic for tumor cells in vitro and that macrophages developed from monocytes in vitro are highly cytotoxic for tumor cells.

The present study was undertaken to determine: (A) if human macrophages matured from monocytes in vivo also demonstrated increased tumoricidal activity, and (B) if an altered in vivo macrophage microenvironment, possibly associated with infection or tumor, affected macrophage tumoricidal activity. Human pulmonary macrophages were used in this study because of their relative availability from patients with a variety of clinical disorders.

MATERIALS AND METHODS

Macrophages

Following informed consent, pulmonary macrophages were obtained by sterile saline lavage during fiberoptic bronchoscopy from normal volunteers or hospitalized patients. Harvested cells were washed four times by centrifugation with Seligman’s balanced salt solution and most preparations were >95% macrophages by differential cell count of Giemsa-stained cytocentrifuge preparations. (Two preparations were 90% macrophages.) Viability determined by trypan blue exclusion was >90%. Based on the differential cell count and percent viability, 105 macrophages were plated onto 6-mm microwells (48 well plates, Linbro, New Haven, Conn.) in 0.1 ml media (Leibowitz, Waymouths, MEM or RPMI) containing 20% pooled heat-inactivated AB sera, 100 μg/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 0.3 mg/ml glucose, and 0.075% NaHCO$_3$. Macrophages were allowed to adhere for 1 hr and were used without further manipulation.
Monocytes

Monocyte monolayers were established as previously described. Briefly, blood was collected from normal donors and hospitalized patients, anticoagulated with EDTA, and separated by Ficoll-Hypaque gradient centrifugation. Interface cells were washed in SBSS 3 times at 200 g for 10 min. Differential and total cell counts were performed. Interface cells (20%-40% monocytes and 60%-80% lymphocytes) were suspended to 5 x 10^6 monocytes/ml in RPMI with 20% autologous serum; 0.1-ml aliquots were added to individual microwells (6 mm diameter, Linbro, New Haven, Conn.) and the cells were incubated 1 hr at 37°C in humidified air with 5% CO2. After 1 hr, the wells were washed with cold Hank's balance salt solution (HBSS), and 0.1 ml of final medium (see above) was added to monocyte monolayers. Monolayers were 95% monocytes and contained 0.8-1.2 x 10^6 cells, and <1 platelet/monocyte was present.

Tumor Cells

Three human tumor lines and a skin fibroblast cell strain were maintained in culture as previously described: Malme-3M, a melanoma cell line; CAK-I, a renal carcinoma cell line; SK-MES, an epidermoid lung carcinoma line; and fibroblast, HEFF. Malme-3M and CAK-I were chosen because they were the most sensitive and resistant, respectively, of eight tumor cell lines screened in the monocyte cytotoxicity assay. SK-MES was used because of its pulmonary origin. The HEFF fibroblast line was skin derived, exhibited contact inhibition, had normal chromosomes, and was used between passages 18 and 28.

Cytotoxicity Assay

Three to seven days after plating, tumor cells or fibroblasts were harvested with 0.03% trypsin (Difco: Grand Island, N.Y.), washed by centrifugation, and suspended in final medium to various concentrations in order to achieve specific effector:target cell ratios; 0.1-ml aliquots were then added to quadruplicate control wells containing 0.1 ml media or to macrophage or monocyte monolayers. Cultures were incubated for 28 hr at 37°C with 5% CO2, and at that time, 0.5 μCi tritiated thymidine, 20 Ci/mM (New England Nuclear, Boston, Mass.), was added to each well. After 48 hr, the wells were emptied, washed 5 times with cold HBSS, and the contents of each well were removed with cotton swabs as previously described. The swabs were allowed to dry for 24 hr, and the cotton tips were then counted in liquid scintillation fluid. Macrophages or monocytes did not incorporate tritiated thymidine. Percentage cytotoxicity was determined by the formula:

\[
1 - \frac{cpm \ target \ cells + effector \ cells}{cpm \ target \ cells \ alone} \times 100
\]

Cytocidal Assay

Tumor cells prelabeled with tritiated thymidine 24 hr prior to addition to microwells were used in this assay. Tumor cells were added to empty control wells or to monocyte/macrophage monolayers and were harvested at 48 hr as described above. The spontaneous release of 3H was 10%-15% at 48 hr. Percent cytocidal activity was determined by the same formula as described for cytotoxicity. We have previously shown that (A) the cytotoxicity assay measures both cytosatic and cytocidal activity and (B) that the cytocidal assay measures cell kill. By utilizing both assay systems, an estimate of these two parameters (cytostasis and cytoidal activity) of macrophage/monocyte–tumor cell interaction can be obtained.

Reagents

Phorbol myristate acetate (PMA) was obtained from Consolidated Midland Corp. (Chemical Div.), Datarol, N.Y. Catalase (bovine liver, 30,000 μ/mg) and superoxide dismutase (bovine, 3000 μ/mg) (SOD) were obtained from Sigma Chemical Company, St. Louis, Mo.

Statistical Analysis

Data were evaluated using nested analysis of variance and Fisher’s t test. When the analysis of variance yielded significant results, Scheffe’s method of computing confidence limits for variance allowed groups to be compared.

RESULTS

Comparison of Macrophage and Monocyte Cytotoxic and Cytocidal Activity

Pulmonary macrophage cytotoxicity was reproducibly measured: one volunteer underwent 3 separate bronchoscopies and was found to have cytotoxicity levels against CAK-I of 41%, 64%, and 57%. The collective series of macrophage cytotoxic and cytocidal activities measured at 48 hr against 3 human tumor cells, lines is presented in Fig. 1. Cytotoxicity was greater than cytoidal activity in each case (p < 0.01),
because cytotoxicity includes both cytostasis (or cell growth inhibition) and cytoidal activity (or true cell kill), and the cytoidal assay measures only cell kill. Pulmonary macrophage cytotoxicity and cytoidal activity were greater than monocyte cytotoxicity and cytoidal activity ($p < 0.01$) against every tumor cell line tested (Figs. 2 and 3). Although monocytes were active in the cytotoxicity assay, they exhibited significant cytoidal activity against only SK-MES. In marked contrast, macrophages were cytoidal in each case.

The kinetics of macrophage and monocyte cytotoxicity is shown in Fig. 4. Malme-3M and SK-MES target cells were added to macrophage or monocyte monolayers and triitated thymidine was added 8 hr prior to harvesting the cells. Macrophage cytotoxicity peaked earlier and was maintained at significant levels longer than monocyte cytotoxicity. A comparison of the kinetics of macrophage cytoidal and cytotoxic activity is seen in Fig. 5. Macrophage cytotoxicity peaked within 8 hr, while cytoidal activity increased during the entire 48-hr assay.

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**Fig. 2.** Comparison of pulmonary macrophage and blood-monocyte tumor cell cytotoxicity. The number of studies is indicated in the bars.

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**Fig. 3.** Comparison of pulmonary macrophage and blood-monocyte cytoidal activity. The number of studies is indicated in the bars.

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**Fig. 4.** Comparison of the kinetics of pulmonary macrophage and blood-monocyte cytotoxicity. SK-MES or Malme-3M tumor cells were added to monocyte or pulmonary macrophage monolayers containing $10^5$ effector cells or to empty control wells. Replicate wells were pulsed 8 hr prior to termination of culture, and cytotoxicity was calculated at the indicated time intervals. Mean ± SE of three studies are shown at each time interval.
In Vivo Modification of Macrophage and Monocyte Tumor Cell Cytotoxic and Cytocidal Activity

To determine if modification of the in vivo environment altered the development of macrophage tumor cell cytotoxicity or cytocidal activity, we studied normal nonsmokers, normal smokers, and hospitalized patients with pulmonary infections and neoplasms (Table 1). All normal donors and patients were males and all but one patient smoked. Normal donors ranged in age from 22 to 40 yr, and patients were 45–71 yr of age. Patients were undergoing clinically indicated diagnostic bronchoscopy for new pulmonary masses or infiltrates. Normal donors received no drugs and only one patient was given antibiotics before bronchoscopy. There was no difference in tumor cell cytotoxicity between monocytes from normal smokers and nonsmokers and patients with infections (Table 2). In contrast, monocytes from patients with tumors demonstrated enhanced cytotoxicity. Macrophages from normal nonsmokers exhibited less cytotoxicity than macrophages from normal smokers and patients, although the differences were not statistically significant (Table 3, \( p = 0.1 \)). Suggestive differences were also observed between normals and patients in macrophage cytocidal activity against Malme-3M at 4:1 macrophage:tumor cell ratio—normals (5) 50 ± 32 versus patients (7) 65 ± 21, (mean ± SD, \( p < 0.1 \)).

Table 1. Clinical Characteristics of Patients Studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>KT</td>
<td>45*</td>
<td>Resolving pulmonary infiltrate? etiology</td>
<td>None</td>
</tr>
<tr>
<td>TD</td>
<td>57</td>
<td>Lung abscess</td>
<td>Hydrochlorothiazide; penicillin after bronchoscopy</td>
</tr>
<tr>
<td>AG</td>
<td>61</td>
<td>Pulmonary infiltrate</td>
<td>Cleared with antibiotics after bronchoscopy</td>
</tr>
<tr>
<td>LT</td>
<td>53</td>
<td>Infiltrate secondary to possible aspiration</td>
<td>Cleared with antibiotics after bronchoscopy</td>
</tr>
<tr>
<td>BT</td>
<td>67</td>
<td>Clearing infiltrate</td>
<td>Digoxin</td>
</tr>
<tr>
<td>DC</td>
<td>62</td>
<td>Diplococcal pneumonia</td>
<td>Antibiotics after bronchoscopy</td>
</tr>
<tr>
<td>GL</td>
<td>60</td>
<td>Viral pneumonia</td>
<td>None</td>
</tr>
<tr>
<td>LW</td>
<td>76</td>
<td>Fibrous pleuritis of unknown etiology, diagnosed at thoractomy</td>
<td>Triamterene, hydrochlorothiazide, chlorpropamide</td>
</tr>
<tr>
<td>GC</td>
<td>47</td>
<td>Lung abscess</td>
<td>On Clindamycin during bronchoscopy</td>
</tr>
<tr>
<td>LN</td>
<td>61</td>
<td>Probable viral pneumonia</td>
<td>Chlorthalidone</td>
</tr>
<tr>
<td>JR</td>
<td>54</td>
<td>Infiltrate</td>
<td>Antibiotics after bronchoscopy, Diuril</td>
</tr>
<tr>
<td>BM</td>
<td>71</td>
<td>Viral pneumonia</td>
<td>None</td>
</tr>
<tr>
<td>FK</td>
<td>50</td>
<td>Infiltrate</td>
<td>Propranolol, antibiotics after bronchoscopy</td>
</tr>
<tr>
<td>EP</td>
<td>60</td>
<td>Epidermoid carcinoma</td>
<td>Aminophylline</td>
</tr>
<tr>
<td>AH</td>
<td>61</td>
<td>Epidermoid carcinoma</td>
<td>Doxepin</td>
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<tr>
<td>HF</td>
<td>61</td>
<td>Small cell carcinoma</td>
<td>None</td>
</tr>
<tr>
<td>RT</td>
<td>50</td>
<td>Adenocarcinoma</td>
<td>None</td>
</tr>
<tr>
<td>RM</td>
<td>51</td>
<td>Adenocarcinoma</td>
<td>None</td>
</tr>
</tbody>
</table>

*All patients were male.

Table 2. Monocyte Cytotoxicity: Comparison of Normal Donors and Patients With Pulmonary Infection and Neoplasm

<table>
<thead>
<tr>
<th></th>
<th>Percent Cytotoxicity*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normals</strong></td>
<td></td>
</tr>
<tr>
<td>Nonsmokers (8)†</td>
<td>32 ± 17</td>
</tr>
<tr>
<td>Smokers (4)</td>
<td>36 ± 11</td>
</tr>
<tr>
<td><strong>Patients</strong></td>
<td></td>
</tr>
<tr>
<td>Pulmonary infection (6)</td>
<td>56 ± 21</td>
</tr>
<tr>
<td>Pulmonary neoplasm (3)</td>
<td>72 ± 17</td>
</tr>
</tbody>
</table>

* Cytotoxicity was measured at monocyte:Malme-3M ratio of 4:1. Numbers represent the mean ± SD. The normal group as a whole was compared to the two patient groups, since there was no difference between normal smokers and nonsmokers. †Number of patients studied in each group is shown in parenthesis.
Specificity and Mechanism of Macrophage Cytocidal Activity

Pulmonary macrophages exhibited some cytoidal activity against cultured fibroblasts (Table 4). However, macrophages were less cytoidal for fibroblasts than tumor cells. When the number of macrophages/well was reduced, macrophage activity against fibroblasts was abrogated and macrophages selectively killed tumor cells (Table 4). To determine if oxygen radicals played a role in macrophage cytocidal activity, PMA (which enhances macrophage O2 radial production), catalase, or superoxide dismutase were added to the assay system (Table 5). Macrophage-tumor cell cytocidal activity was not affected by PMA, catalase, or superoxide dismutase. However, macrophage cytocidal activity against fibroblasts was significantly inhibited by catalase and enhanced by PMA.

DISCUSSION

The data presented here suggest that (A) monocytes primarily exhibit cytostatic activity against tumor cells, and monocyte to macrophage maturation is associated with the development of enhanced cytocidal activity; (B) in vivo modification of the cellular microenvironment by pulmonary infection, tumor, or perhaps smoking is associated with enhanced monocyte and macrophage tumor cell cytotoxicity; (C) pulmonary macrophages were cytoidal for contact-inhibited fibroblasts at high macrophage concentrations. Cytoidal activity against fibroblasts but not tumor cells is eliminated at low macrophage concentrations (10 versus 2 x 104 macrophages/6-mm well); and (D) pulmonary macrophage tumor cell cytotoxicity is not mediated by H2O2, O2, or stable soluble mediators. However, macrophage cytoidal activity against fibroblasts may be due to H2O2 since it is enhanced by PMA and inhibited by catalase and not superoxide dismutase.

The observation that normal human pulmonary macrophages are cytoidal for tumor cells is at variance with data developed in animal systems: resident peritoneal and pulmonary macrophages from "normal" mice, rats, and guinea pigs are not tumoricidal and require activation before cytoidal activity can be demonstrated. The reason for the discrepancy between rodent and human studies is unclear, but the data presented here are consistent with our previous observation that macrophages cultured without activating agents from human monocytes are cytoidal for tumor cells.

Blood monocytes from patients with pulmonary neoplasms and infections exhibit greater tumor cell cytotoxicity than monocytes from normal donors. The enhanced monocyte tumor cell cytotoxicity seen in patients with infection and tumors may have been due to activation by several possible mechanisms. Bacterial, fungal, and tumor cell products have been shown to specifically and nonspecifically activate mouse macrophages to a tumoricidal state. Pulmonary macrophages from normal smokers and patients with infection and cancer exhibited somewhat greater cytotoxic activity than macrophages from normal nonsmokers (p = 0.1). In this regard, it is of interest that several independent observations support the possibility that smoking may activate macrophages and enhance cytotoxicity. Animal macrophages on exposure to tobacco smoke exhibit increased metabolic and functional activity, and in some studies, human pulmonary macrophages from smokers exhibited evidence of acti-
viation, i.e., increased lysosomal enzyme content,21 glucose metabolism,22 and membrane activity.23

Macrophages killed tumor cells with relative specificity, thus high \((10 \times 10^6 / \text{well})\) and low \((2 \times 10^4 / \text{well})\) concentrations of macrophages were tumoricidal, but macrophages killed fibroblasts only at high concentrations. Similar observations have been made in murine systems.6 8

The mechanism of macrophage tumoricidal activity has not been convincingly elucidated, but numerous studies have suggested several possibilities: (A) macrophage–tumor cell fusion with transfer of toxic lysosomal enzymes into tumor cells,24 (B) release of cytotoxic products from macrophages, including C3a,25 proteases,26 oxygen radicals,18 and undefined cytotoxins.27 To examine the possibility that \(\text{H}_2\text{O}_2\) or \(\text{O}_2\) might mediate macrophage tumoricidal activity, we added PMA, catalase, and superoxide dismutase to the tumoricidal assay. PMA has been shown to induce the release of \(\text{H}_2\text{O}_2\) from macrophages.18 Catalase and superoxide dismutase are specific scavengers of \(\text{H}_2\text{O}_2\) and \(\text{O}_2\), respectively. These agents did not affect macrophage killing of tumor cells, but catalase inhibited and PMA enhanced macrophage killing of fibroblasts. These data strongly suggest that \(\text{H}_2\text{O}_2\) may mediate macrophage killing of fibroblasts but not tumor cells. The data do not conclusively prove these points, since (A) PMA induces release of other macrophage products, such as plasminogen activator and lysozyme, and (B) the enzymes, catalase and SOD, may not be able to gain access to areas of close macrophage tumor cell contact.

These data confirm our previous findings that human monocyte-macrophages are effective and selective mediators of tumor cell cytotoxicity. This in vitro observation further supports the hypothesis that human macrophages are important in the control of oncogenesis in vivo.

REFERENCES

Human pulmonary macrophage tumor cell cytotoxicity

P Lemarbre, J Hoidal, R Vesella and J Rinehart