Production of Colony-stimulating Factor by Sarcoid Granulomas *in vitro*

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To examine whether sarcoid epithelioid cells of granulomatous lesions produce colony-stimulating factor (CSF), a growth factor of monocyte-macrophage series, the primary culture of sarcoid epithelioid cells was carried out from 16 biopsied lymph nodes which contained sarcoid granulomas. CSF activity was demonstrated at high titer in the conditioned media from all the epithelioid cell cultures. The conditioned medium of the epithelioid cell culture was harvested, concentrated by Amicon YM-10 membrane, and loaded on a Sephadex G-75 column. Two distinct peaks of CSF activity were noted just before and after bovine serum albumin region. The molecular weights were estimated at about 75,000 and 36,000, respectively. Both CSF fractions stimulated colony formation by murine bone marrow cells. The lower molecular weight CSF was active on human bone marrow cells, but the higher molecular weight CSF did not stimulate colony formation by human bone marrow cells.

Key Words: Colony-stimulating factor, Macrophage growth factor, Sarcoidosis, Epithelioid cells, Granuloma, Culture.

Sarcoidosis is a chronic granulomatous disease of unknown cause. A major constituent cell of the granulomas is the epithelioid cells which is believed to originate from blood monocytes and tissue macrophages. In studying the pathogenesis of sarcoidosis, it is critical to understand the mechanisms underlying the granuloma formation. It has been known that peripheral blood monocytosis is often seen in patients with sarcoidosis. In addition, alveolar macrophages from sarcoidosis patients have shown an increased mitotic activity. These observations suggest that the increased number of monocyte-macrophage-epithelioid cell series in sarcoidosis patients probably involves processes that stimulate proliferation of precursor for these cells. Enhanced production of monocyte-macrophages, the precursor for sarcoid epithelioid cells, may be related to the disease activity. Recently, we have succeeded in the cultivation of sarcoid epithelioid cells *in vitro* which produce large amounts of angiotensin-converting enzyme and lysozyme. This study is an attempt to see whether the sarcoid epithelioid cells produce colony-stimulating factor (CSF), a growth factor for monocyte-macrophage series *in vitro*.

**MATERIALS AND METHODS**

**Source of cells:**

The materials used were biopsied lymph nodes from 16 patients with active sarcoidosis. The diagnosis of sarcoidosis was made on the basis of clinical and chest roentogenograms and pathologic examination of biopsied specimens from scalene lymph nodes. Each lymph node contained non-necrotizing granulomas. Acid-fast bacilli,
fangi, or other microbial agents were not demonstrated in any of the specimens by microscopic and culture examinations.

**Culture medium:**

The growth medium used for tissue culture was F-10 synthetic medium supplemented with 10% fetal bovine serum (FBS) (Grand Island Biological Co., Grand Island, N.Y.). Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to the medium just before use.

**Tissue culture:**

Lymph nodes were rinsed twice with F-10 medium, cut free from the encapsulating connective tissue, and cut into small pieces with surgical scissors as described previously. The pieces were washed again and incubated in F-10 medium with 10% FBS at 37°C in a humidified atmosphere of 5% CO₂ in air. In a few days, epithelioid cells began to migrate from the attached pieces to form a monolayer. Immediately after the epithelioid cells attached to grow, cultures were washed with F-10 medium to remove floating or loosely-attached cells such as lymphocytes. A few days later, the explant tissues themselves were removed from the culture vessels, leaving cells grown in the monolayer.

**Morphologic examinations:**

The cells grown on glass coverslips were fixed in isotonic-buffered formalin (10% vol/vol) and stained with Giemsa solution. Cultured cells were also photographed without stain using a phase-contrast microscope. For electron microscopic examination, the cells grown on plastic coverslips were fixed with 2.5% glutaraldehyde and 2% formaldehyde in 0.1M sodium-cacodylate buffer (pH 7.4), postfixed in 1% OsO₄ in the same buffer, and embedded in Epon 812 after dehydration. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined in a Hitachi HU-12 electron microscope. For identification of the epithelioid cells, the cells in monolayer were stained with peroxidase.

**Angiotensin-converting enzyme:**

Angiotensin-converting enzyme (ACE) activity was determined spectrophotometrically in the culture medium by using a modification of the method originally described by Cushman and Cheung.

**Colony-stimulating factor:**

Human bone marrow cells were obtained from normal volunteers (who gave informed consent) by sternal puncture with heparinized syringes. They were used after washing with culture medium and removal of erythrocytes by hypotonic lysis. Nonadherent cells were prepared by the method of Messner et al. A constant number of nucleated cells (100,000) were cultured in a single layer in 1.0 ml of the supplemented McCoy's 5A medium, containing 0.3% agar, 20% fetal bovine serum, and varying concentrations of test samples. Mouse bone marrow cells were obtained from the femur of 6–8 week-old female mice of C3H/HeN strain, and non-adherent cells were prepared as described above. Incubations was carried out at 37°C in 7.5% CO₂. On day 7 (mouse) or day 10 (human), the number of colonies (50 cells or more) was determined at 25x magnification with a dissecting microscope. Routine morphological analysis on human colonies were performed on colonies. We also used the following cytochemical methods for the identification of the cells: naphthol ASD chloroacetate esterase, naphthol ASD acetate with or without addition of sodium fluoride, Biebrich Scarlet, and acid phosphatase.

**Characterization of CSF:**

The conditioned medium of sarcoid epithelioid cell culture was harvested, centrifuged at 3,000 r.p.m. for 20 min to remove cellular debris and precipitated materials, and then stored at −20°C until use. Pooled conditioned medium (70 ml) was concentrated twenty-fold by ultrafiltration with YM-10 membrane in the presence of 0.01% polyethylenglycol. We then transferred a 1.0 ml of sample to a Sephadex G-75 column (2 x 64 cm, flow rate 10.4 ml/hr), equilibrated with 10 mM Tris-HCl buffer (pH 7.4), containing 0.15M NaCl and 0.01% polyethylenglycol. The void volume of this column was 105 ml, determined by blue dextran.

**RESULTS**

Large epithelial cells were seen to migrate and grew continuously with the tissue explants, between which small colonies of the similar epithelioid cells were scattered. Such cells grew to become confluent within a few weeks (Fig. 1). In
Fig. 1. Monolayer culture of epithelioid cells. Polygonal or round, thick cells form monolayer in culture. Phase-contrast microscopy, x 560.

contrast, few adherent cells were seen in cultures of normal lymph nodes obtained from patients with mammary cancer at surgical operation. Ultrastructure of the cultured epithelioid cells was similar to those found in granulomatous tissues (Fig. 2)\(^4\).

**Production of colony-stimulating factor by epithelioid cells:**

The confluent cultures were washed vigorously with medium to remove contaminating lymphocytes associated with the granulomatous lesions, and refed with fresh F-10 medium supplemented with 10% FBS. Then, the cells were incubated for further 7 days. At the end of the culture period, more than 94% of the cells were epithelioid cells. In the conditioned media from all the epithelioid cell cultures, significant levels of CSF activity was demonstrated (Table 1). To see whether the CSF activity in the epithelioid cell cultures is due to the contaminating lymphocytes associated with sarcoïd tissues, non-adherent cells were collected just after the epithelioid cells at-

Table 1. Granulocyte-macrophage colony-stimulating activity in sarcoïd granulomas.

<table>
<thead>
<tr>
<th>Case</th>
<th>Colony number</th>
<th>Granulocyte %</th>
<th>Colony type %</th>
<th>Macrophage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>78.5</td>
<td>2.3</td>
<td>32.6</td>
<td>65.1</td>
</tr>
<tr>
<td>#2</td>
<td>115</td>
<td>62.4</td>
<td>16.3</td>
<td>15.7</td>
</tr>
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<td>42</td>
<td>38.6</td>
<td>20.4</td>
<td>36.6</td>
</tr>
<tr>
<td>#4</td>
<td>27</td>
<td>47.4</td>
<td>11.1</td>
<td>40.5</td>
</tr>
<tr>
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<td>44</td>
<td>34.1</td>
<td>9.0</td>
<td>56.9</td>
</tr>
<tr>
<td>#6</td>
<td>96</td>
<td>76.0</td>
<td>6.7</td>
<td>17.3</td>
</tr>
<tr>
<td>#7</td>
<td>30</td>
<td>16.7</td>
<td>6.7</td>
<td>76.7</td>
</tr>
<tr>
<td>#8</td>
<td>177</td>
<td>73.4</td>
<td>9.6</td>
<td>16.9</td>
</tr>
<tr>
<td>#9</td>
<td>82</td>
<td>72.0</td>
<td>18.5</td>
<td>8.5</td>
</tr>
<tr>
<td>#10</td>
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<td>47.5</td>
<td>12.5</td>
<td>40.0</td>
</tr>
<tr>
<td>#16</td>
<td>198</td>
<td>38.0</td>
<td>7.4</td>
<td>54.6</td>
</tr>
</tbody>
</table>

Control medium (F-10\(\times\)10% FBS)

Plates of 100,000 nonadherent mouse bone marrow cells were stimulated by 0.1 ml of stimulus in triplicate cultures. Colonies of 50 cells or more were counted at day 7. The percentage of each colony type is shown.

Fig. 2. Electron micrograph of a cultured epithelioid cell. The cells is characterized by finger-like or lamellar processes (\(P\)) at the cell surface and by numerous cytoplasmic vesicles with electron-dense content (arrows) in the cytoplasm. The cell also contains abundant mitochondria (\(M\)), well-developed Golgi complexes (\(G\)) and some aggregations of rough-surfaced endoplasmic reticulum. Occasional phagosomes and lysosomes are seen (\(Ly\)). x 9,400.

Fig. 3. Dose-response relationships between number of colonies and concentration of conditioned media of epithelioid cell culture, and lymphocyte culture obtained from sarcoïd lymph nodes. (\(\bullet\-\bullet\)); epithelioid cell culture, (\(\circ\-\circ\)); lymphocyte culture.

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tached to the bottom of the culture vessels. They were incubated for a week in the same conditions. More than 91% of the cells were lymphocytes and only 3–7% were macrophage-epithelioid cells in the culture. Only a trace of CSF activity was detected in these non-adherent cell cultures (Fig. 3).

These results suggest that epithelioid cells in sarcoid granulomatous lesions produce and release colony-stimulating factor.

Characterization of sarcoid CSF:

A concentrated conditioned medium of 1 ml was loaded on Sephadex G-75 column chromatography after equilibration with 10 mM Tris-HCl (pH 7.4). Two distinct peaks of CSF activity were observed. The first peak was eluted just after the bovine serum albumin region, which indicated that its molecular weight was approximately 75,000 (Fig. 4). The second peak was noted just after bovine serum albumin region, suggesting the approximate molecular weight of 36,000. While both CSF were active on murine bone marrow cells, the higher molecular weight CSF did not stimulate colony formation by human bone marrow cells (Table 2). The lower molecular weight CSF was highly active on human bone marrow cells as well as on murine marrow cells (Table 2). These results suggest that sarcoid epithelioid cells produce two different types of CSF.

Table 2. CSF activity on human bone marrow cells of Peak 1 and Peak 2.

<table>
<thead>
<tr>
<th>% of fraction added</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
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<tr>
<td>Peak 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peak 2</td>
<td>25</td>
<td>136</td>
<td>128</td>
<td>25</td>
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</tbody>
</table>

Plates of 100,000 nonadherent human bone marrow cells were stimulated by 0.1 ml of stimulus in triplicate cultures. Colonies of 50 cells or more were counted on day 10.

DISCUSSION

Sarcoidosis is characterized by a marked increase in the number of macrophage-epithelioid cells which are the main constituent cells of sarcoid granulomatous lesions. The present study demonstrated that one possible mechanism for this increase is the monocyte-macrophage proliferation that resulted from the spontaneous release of colony-stimulating factor(s), product by macrophage-epithelioid cells themselves that stimulated a clonal growth of monocyte-macrophage lineage.

CSF is considered to be a specific growth factor for granulocytes and macrophages\(^6\). This factor has been demonstrated in several tissues, including lungs\(^8\), placenta\(^9\), spleen\(^10\) and leucocytes\(^11\). Recent studies on human CSF have shown the possible existence of at least two different types of CSF obtained from human sources, one of which is only active on mouse bone marrow cells but does not stimulate colony formation by human marrow cells (Type I CSF). This type of CSF has been purified from urine, and the molecular weight was estimated as 45,000–85,000\(^{12–14}\). While this factor does not stimulate colony formation of human bone marrow cells, it stimulated the growth of human monocytes and macrophages\(^13\). The other type of CSF stimulated granulocyte-macrophage colony formation by human bone marrow cells (Type II CSF). The human active factor has an approximate molecular weight of 30,000\(^{15, 16}\). In the present study, Sephadex G-75 column chromatography revealed two distinct peaks of CSF activity on murine bone marrow cells. The higher molecular weight CSF was not active on human bone marrow.
cells. The larger size and functional feature suggested that this CSF was related to type I CSF, the macrophage growth factor\textsuperscript{12, 13}. The lower molecular weight CSF in sarcoid epithelioid cell culture was active on human as well as mouse bone marrow cells, suggesting that this factor may be type II CSF\textsuperscript{15, 16}.

It is attractive to consider that CSF produced by epithelioid cells in sarcoid granulomatous lesions are able to stimulate the proliferation of monocyte-macrophage series, which may contribute to formation of new sarcoid granulomas. This interesting possibility of positive feedback via the release of CSF from epithelioid cell granulomas in the pathogenesis and in the maintenance of the granulomatous lesions may form a hypothesis for future study.

REFERENCES


