Growth Stimulation of Human Bone Marrow Cells in Agar Culture by Vascular Cells

By Søren Knudtzon and Børge Thing Mortensen

Human vascular cells are capable of stimulating granulopoiesis in agar culture of human bone marrow cells. This effect was obtained by including vein fragments in the culture or by using endothelial cells separated from the vein of human umbilical cords as feeder cells. Furthermore, the stimulatory capacity of conditioned medium obtained from cord veins was found to be highly active in comparison to that obtained from peripheral leukocytes. Endothelial cells within the bone marrow cavity are suggested as a local source of factors regulating granulopoiesis in humans in addition to the monocyte.

To obtain colony growth of human bone marrow cells human leukocytes have been found to be a major source of CSF, and the cell responsible has been identified as the monocyte by several investigators. Recent observations indicate that the monocyte is of importance for the regulation of granulopoiesis in humans.

In this report, human vascular cells are shown to release CSF using human bone marrow cells as target cells, and endothelial cells in the bone marrow cavity are suggested as a possible local source of CSF in vivo in addition to the monocyte.

MATERIALS AND METHODS

Human bone marrow cells were cultured and stimulated either by feeder cells or by conditioned medium prepared from the same cells. Human leukocytes and vascular cells were compared as a source of CSF.

Leukocyte Feeder Layers

Leukocyte feeder layers were prepared as described previously but containing between $10^5$ and $10^6$ human peripheral leukocytes per petri dish (35 mm, Falcon). These cells were mixed with McCoy’s 5A medium, 15% human serum, and 0.5% agar, and 1 ml of this mixture was added to each plate.
Vascular Cell Feeder Layers

Vascular cell feeder layers were prepared from the saphenous vein, a splenic vein, or the umbilical cord vein. The saphenous vein was removed by the stripping procedure, placed in sterile test tubes containing saline and used within 1-2 hr after the operation. The veins in a total length of 10-20 cm were opened longitudinally with a scissors, and the remaining blood was washed out thoroughly using 500 ml of saline. The vessel wall was cut into 1-2-mm pieces and each incorporated in an underlayer of 1 ml of medium, 15% human serum, and 0.5% agar.

In some experiments, splenic vein fragments, obtained during splenectomy, and umbilical cord fragments prepared after cesarean sections were used.

Endothelial cells from umbilical veins were obtained from umbilical cords 1-2 hr after normal deliveries, using a method described recently with a few modifications. The umbilical vein in a total length of 40-60 cm was perfused with 200 ml of sterile saline to remove the remaining blood, and the vein lumen was filled (20-30 ml) with a collagenase solution (Sigma, type I, 1 mg/ml NaCl). Each end of the cord was ligated and the cord kept at 37°C for 15 min in a waterbath. The endothelial cells were flushed out with 50 ml of Hanks’ balanced salt solution (HBSS, calcium and magnesium free) and washed once prior to making a cell count. The ratio between endothelial cells and remaining leukocytes was calculated in a smear after staining with May-Grunwald-Giemsa. Nigrosin was used to estimate cell viability. Feeder layers were prepared containing 5-7 x 10^5 nucleated cells per dish. Endothelial cells from three to four umbilical cords were pooled to provide a sufficiently high number of cells for the feeder layer preparation.

The cells obtained from the umbilical cord were examined by electron microscopy (electron microscope studies were performed by Dr. Bo Hainau, Department of Pathology, Finsen Institute). The cell pellet was fixed in Karnovsky’s fixative and 0.5% and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 301 electron microscope.

Underlayers containing medium, agar, and human serum but no cells were always included in the experiments as controls. The feeder layers containing either leukocytes or vascular cells were placed in a humidified incubator at 37°C constantly flushed with 7.5% CO_2 in air, and used within a week.

Conditioned Media

Conditioned media were prepared from umbilical cord cells and from leukocytes. The umbilical vein was flushed with saline, filled with medium and 10% human serum (20-30 ml), and both ends were ligated. To avoid bacterial contamination the cord was then immersed in Rodalon for 3 min and rinsed with saline. After 1-2 days of incubation in a waterbath at 37°C, the vein was emptied, the medium sterilized by filtration through 0.2-μm Millipore membranes, and stored at -20°C until use. The endothelial cell number per vein was calculated to be approximately 1-2 x 10^8 cells assuming an area of 100 sq μm per cell. This figure corresponds to 5-10 x 10^6 cells per ml of medium. To investigate the possibility that other cells in the cord could participate in the production of the stimulating factor, the vein was filled with medium after removal of the endothelial cells by collagenase treatment. However, it was found impossible to harvest any medium from these cords since they were partly dissolved by the collagenase treatment after only a few hours. As an alternative method, feeder layers were prepared containing sterile umbilical cord fragments (2 x 2 mm) obtained from cesarean sections. Each fragment contained either part of the vein or perivascular tissue only.

Conditioned medium from normal leukocytes was prepared by suspending these cells in medium with 10% human serum, 5 x 10^6 cells per ml, and incubating for 3 days at 37°C before harvest. After centrifugation the medium was removed and stored at -20°C until use.

The conditioned medium was only used if tests for bacterial contamination were negative.

Bone Marrow Cells

The bone marrow cells for the overlayers were obtained by a posterior iliac crest aspirate from normal volunteers or from patients with untreated malignant lymphomas in stages I and II (no marrow involvement). The aspirate, 1-2 ml, was mixed with heparin (100 U), and after 2 hr at room temperature the buffy coat was removed and the cells washed once in McCoy’s 5A medium.
GROWTH OF BONE MARROW CELLS

Table 1. Cluster Formation by Human Bone Marrow Cells (5 x 10⁴ Cells Per Plate) Stimulated by Vascular Cells or Peripheral Leukocytes

<table>
<thead>
<tr>
<th>Source of CSF in the Cultures</th>
<th>&gt; 5 Cells</th>
<th>&gt; 20 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenic vein fragment</td>
<td>80 ± 10</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>10⁶ leukocytes</td>
<td>180 ± 20</td>
<td>41 ± 10</td>
</tr>
<tr>
<td>Control†</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mean ± 1 SD, counted at day 7.
†Containing 10% human serum only.

The number of cells was counted in a hemocytometer and a smear made for differential count. The bone marrow cells were plated in each petri dish mixed with McCoy's SA medium, 10% human serum, and 0.3% agar in a total volume of 1 ml on top of the feeder layers or in a single layer mixed with 100 μl of conditioned medium. In some experiments the bone marrow cells were cultured with a vein wall fragment included in the same layer without any feeder layer. After gelling, the plates were replaced in the incubator. Triplicate cultures were made in all experiments.

After incubation for 7 days the number of clusters (greater than five cells) was counted using a dissecting microscope at x 40 magnification. In some experiments clusters exceeding 20 cells or colonies (> 50 cells) were counted separately. For morphologic studies 20-30 clusters or colonies were removed from the plate using a Pasteur pipette, and the single cluster or colony was smeared and stained with May-Grünwald-Giemsa. The human serum used in the experiments was obtained from normal volunteers and patients with polycythemia vera who underwent therapeutic phlebotomy. The serum was stored at -20°C until use.

RESULTS

When human bone marrow cells are cultured with serum in a single layer, without a feeder layer, the number and size of the clusters formed has been shown to be dependent upon the number of cluster-stimulating bone marrow cells in the culture. Thus, no clusters are formed when a low number of bone marrow cells are cultured. A low number of bone marrow cells (5 x 10⁴) was therefore used in our first experiments in order to make the assay for CSF activity as sensitive as possible. In Table 1 it is seen that no clusters were formed in the control cultures (without an exogenous source of CSF), but when a splenic vein fragment was included in the single layer culture, cluster formation was clearly stimulated. The number of clusters (greater than five cells) obtained per plate in this experiment was about half that obtained with addition of a feeder layer containing 10⁶ leukocytes.

A stimulatory effect is also demonstrated when a vein fragment is included in a feeder layer. In Fig. 1 the effect of feeder layers containing vein fragments is seen to be comparable to the effect of feeder layers containing leukocytes (10⁵–10⁶). No stimulation was seen when underlayers contained vein fragments which had been frozen and thawed three times in dry ice–ethanol.

It has thus been demonstrated that cluster and colony growth is stimulated by vascular cells. Because of the close spatial relationship in vivo between granulopoietic cells and endothelial cells in the marrow, the possibility that endothelial cells produce CSF was further investigated. Feeder layers containing endothelial cells and leukocytes were prepared, and human bone marrow cells were included in the upper layer. After 7 days of incubation, colonies of 50–100 cells were seen using both kinds of feeder layers. The results of different experiments are summarized in Table 2.
Using light microscopy, the vascular cells obtained from the umbilical vein were rather uniform in size, slightly elongated with a round nucleus and a granular cytoplasm, measuring 30–50 μm in length. A few muscle cells were seen occasionally, and cord blood leukocytes were also present in a small number (less than 5% of the total cell number). With electron microscopy, Weibel-Palade bodies, cytoplasmic structures only seen in endothelial cells, were demonstrated. More than 75% of the endothelial cells were viable by nigrosin.

**Table 2. Colony Growth of Human Bone Marrow Cells Using Leukocytes or Endothelial Cells as Feeder Cells**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>No. of Colonies*</th>
<th>Leukocytes No. of Cells</th>
<th>No. of Cells</th>
<th>Endothelial Cells No. of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 ± 6</td>
<td>10^6</td>
<td>56 ± 5</td>
<td>7 x 10^5</td>
</tr>
<tr>
<td>2</td>
<td>62 ± 19</td>
<td>10^6</td>
<td>90 ± 5</td>
<td>7 x 10^5</td>
</tr>
<tr>
<td>3</td>
<td>50 ± 6</td>
<td>5 x 10^5</td>
<td>20 ± 3</td>
<td>5 x 10^5</td>
</tr>
<tr>
<td>4</td>
<td>150 ± 15</td>
<td>5 x 10^5</td>
<td>25 ± 5</td>
<td>5 x 10^5</td>
</tr>
<tr>
<td>5</td>
<td>115 ± 25</td>
<td>5 x 10^5</td>
<td>150 ± 5</td>
<td>5 x 10^5</td>
</tr>
<tr>
<td>6</td>
<td>40 ± 5</td>
<td>5 x 10^5</td>
<td>135 ± 10</td>
<td>5 x 10^5</td>
</tr>
<tr>
<td>7</td>
<td>50 ± 6</td>
<td>5 x 10^5</td>
<td>100 ± 2</td>
<td>5 x 10^5</td>
</tr>
</tbody>
</table>

*Number of colonies >50 cells ± standard deviation. 2 x 10^6 bone marrow cells per plate, incubation time 7–10 days.

†Patient No. 6, normal volunteer; other patients, localized neoplastic diseases not involving bone marrow.
Table 3. Cluster Formation by Human Bone Marrow Cells (10^6 Cells Per Plate) Stimulated by Conditioned Medium (CM) or Leukocytes

<table>
<thead>
<tr>
<th>Source of CSF in the Cultures</th>
<th>Number of Clusters * ± 1 SD</th>
<th>Cluster Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM (cord vein), 0.1 ml per plate</td>
<td>75 ± 5</td>
<td>5–50</td>
</tr>
<tr>
<td>CM (leukocytes), 0.1 ml per plate</td>
<td>10 ± 2</td>
<td>5–10</td>
</tr>
<tr>
<td>10^6 leukocytes in feeder layer</td>
<td>100 ± 6</td>
<td>5–50</td>
</tr>
<tr>
<td>Control†</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

*Counted at day 7.
†Containing 10% human serum only.

dye exclusion. Because of the small number of endothelial cells obtained from a cord vein (1–2 x 10^6), feeder layers of more than 5–7 x 10^5 cells per plate were not prepared.

Feeder layers containing cord blood leukocytes, 5 x 10^3 and 5 x 10^4 cells per plate, did not stimulate colony growth. No effect of collagenase was noted when peripheral leukocytes were incubated with collagenase for 15 min at 37°C and then used as feeder cells, and the viability of the leukocytes was unchanged by this treatment (>95%). No stimulatory effect was obtained by including cell-free HBSS, used for the preparation of endothelial cells from umbilical veins, in the culture. In a few cultures, an outgrowth of fibroblast-like cells from the vein fragment was observed. These cells were adherent to the plastic surface in the bottom of the petri dish, forming a monolayer. No colony growth or monolayer formation was observed in the feeder layers containing endothelial cells.

The stimulatory effect of conditioned medium prepared from leukocytes has in our hands been found to be very low. In contrast, conditioned medium obtained from the umbilical vein was found to be highly active (Table 3). The possibility that nonvascular cells in the cord participate in this production of CSF cannot be excluded, since stimulatory activity was seen when perivascular cord tissue fragments were included in an underlayer. Yet this stimulation was small in comparison with the activity obtained when cord vein fragments were included in the underlayers.

Bone marrow cells from normal volunteers and from patients with lymphomas were equally sensitive to CSF derived from vascular cells and from leukocytes. No differences in the results were observed when either normal serum or serum from patients with polycythemia vera was included in the cultures.

Cluster and colony morphology appeared to be identical whether stimulated by leukocytes or vascular cells and consisted mainly of granulocytic cells.

DISCUSSION

The present study has shown that vascular cells, and in particular endothelial cells, can release a factor (CSF) which stimulates granulopoiesis in vitro of human bone marrow cells.

The stimulatory effect was demonstrated both when a vein fragment was included in the culture and when feeder layers containing endothelial cells were
used. Furthermore, a highly active conditioned medium could be obtained from the umbilical cord vein.

The decreasing number of clusters of more than five cells seen when bone marrow cells are stimulated by an increasing number of leukocytes (Fig. 1) needs some explanation. When cultures are followed at daily intervals, the initial rise in number of clusters is followed by a decrease, which is probably due to dispersion of mature clusters and cell death, as discussed by Moore et al.21 By increasing the number of cells in the feeder layer, this fall in cluster numbers is already seen after 2-3 days of incubation,22 and this explains the lower number of clusters of more than five cells counted at day 7 using 10⁶ leukocytes in the cultures. Thus the cluster size rather than the total number of clusters of more than five cells gives a better indication of the amount of CSF released in the culture.

The experiment using frozen vein fragments in the underlayers indicates that only viable cells can produce CSF.

Since all clusters of more than five cells were included in the study, the target cells for the stimulatory factor (or factors) are probably not only committed stem cells (colony-forming cells) but also the immediate progeny of these cells. Nevertheless, the stimulatory factor (or factors) released from vascular cells and leukocytes is referred to as CSF (colony-stimulating factor).

The CSF released from vascular cells and from leukocytes might not be identical in spite of having a similar biologic activity. From agar culture of mouse bone marrow cells it is known that CSFs extracted from different mouse tissues vary considerably in size and charge.23 The multiorgan origin of these CSFs suggests that CSF is either elaborated from different cell types or that a single cell type found in several organs is the source of CSF (e.g., macrophages, connective or vascular tissue cells).

It has recently been shown that the activity of medium conditioned by non-hematopoietic cells ("stromal cells") within the bone marrow cavity in the mouse was 8-20 times greater than the activity of medium conditioned by the hematopoietic cells in the marrow. It was furthermore demonstrated that the local capacity of the stromal cells to produce CSF increased during marrow regeneration following radiation injury, indicating that these cells might be of significant importance for the control of granulopoiesis in the mouse.9

To obtain colony growth of human bone marrow cells in vitro, only a few sources of CSF have been reported, the most effective being human peripheral leukocytes and spleen cells.10,24 Using cell separation techniques on leukocytes, the cell responsible for the production of CSF has been identified as the monocyte,11-13 and this cell is believed to be a major source of CSF in human plasma.14

The demonstration in this study that vascular cells, especially endothelial cells, can release CSF raises the possibility that these cells within the bone marrow cavity might also be of importance for the local production of CSF in humans.

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Growth stimulation of human bone marrow cells in agar culture by vascular cells

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