Fibroblasts Induce Heparin Synthesis in Chondroitin Sulfate E Containing Human Bone Marrow-Derived Mast Cells

By Leon Gilead, Osnat Bibi, and Ehud Razin

Human bone marrow-derived mast cells (hBMMCs), differentiated in vitro in suspension culture and under the influence of human peripheral blood mononuclear cells conditioned medium (hCM), were tested for their response to recombinant human interleukin-3 (rhIL-3) and for their behavior in different microenvironments. The hBMMCs were incubated in the presence of rhIL-3 and the changes in their proliferation rate were determined. Recombinant rhIL-3 induced a more than sixfold increase in 3H-thymidine uptake into the hBMMC DNA in a dose-dependent manner. Human CM used as a control for proliferation response induced a more than eightfold maximal proliferation rate increase. Rabbit anti-rhIL-3 completely inhibited hBMMC 3H-thymidine uptake induced by rhIL-3 and decreased the hCM-induced proliferation by approximately 50%. These hBMMCs were cocultured with four different myotomic C-treated cell monolayers and assayed for phenotypic changes. After only 2 days in coculture with either embryonic mouse skin-derived fibroblasts (MESFs) or human skin-derived fibroblasts (HSFs), a marked increase in granule number and density was noted on staining with toluidine blue. Mast cells that initially stained alcin blue / safranin at day 0 of coculture became alcin blue / safranin during the coculture period. Human BMMC proteoglycan synthesis shifted from approximately 85% chondroitin sulfate E to approximately 60% heparin within 14 to 19 days of coculture with the MESF monolayer and to approximately 50% heparin within 19 days of coculture with the HSF monolayer. None of the above-mentioned changes were noted in cocultures of hBMMCs with 3T3 cell line fibroblast monolayers or in cocultures with bovine vascular endothelium (BVE) cell monolayers. These results demonstrate microenvironmental effects exerted by the MESF and HSF monolayers on IL-3–dependent hBMMCs similar to those reported in the conversion of murine mast cell phenotype.

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cultured analogue of human MCs. Several attempts have been made to generate human MCs in culture. These cultured cells were identified as MC-like, \(^{11}\) basophilic/basophil-like cells, \(^{12,13}\) or basophils. \(^{14}\) Most recently, it was reported that nucleated cells from human umbilical cord blood differentiated into human skin-like MCs in vitro after 11 to 14 weeks in coculture with 3T3 fibroblasts. \(^{15}\) We have developed an in vitro culture technique for growing human bone marrow-derived basophilic cells. These cells show a striking similarity to hBMMCs by the following criteria: the expression of surface IgE Fc receptors, their granular ChS-E proteoglycan, their main arachidonic acid product (LTC, \(_{3}\) \(_{4}\)), generated on immunologic activation, their low histamine content, their morphology on transmission electron microscopy, their cell surface determinants, and their growth dependence on factors derived from activated human T cells. \(^{26}\) Therefore, we have designated these MCs as human BMMCs (hBMMCs) rather than basophils.

The availability of recombinant human IL-3 (rhIL-3) \(^ {17}\) has triggered a series of attempts to generate hBMMCs from bone marrow precursors. After 2 weeks' culture in the presence of rhIL-3, human bone marrow or cord blood gave rise to colonies in which approximately one third of the cells were basophils. \(^{38}\) We have reported the preliminary finding that hIL-3 is an hBMMC growth factor \(^ {39}\) and, more recently, it was reported that 1% to 5% of "mast-like cells" appeared in colonies after 3 weeks in agar interface cultures in the presence of rhIL-3. \(^ {40}\)

We present data supporting our preliminary report concerning the response of hBMMCs to rhIL-3 \(^ {39}\) and examine the influence of different microenvironments on the hBMMC phenotype.

**MATERIALS AND METHODS**

**Cell culture.** Mast cells were obtained as previously described. \(^ {46}\) Briefly, human bone marrow aspirates-enriched mononuclear cells following Ficoll-Hypaque density sedimentation (1.072 g/mL) were resuspended at a concentration of 2.5 \times 10^6 cells/mL in RPMI-1640 medium containing 10% fetal calf serum (heat inactivated 45 minutes at 56°C), 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 100 \( \mu \)g/mL penicillin, 100 \( \mu \)g/mL streptomycin, 50 \( \mu \)mol/L \( \beta \)-2-mercaptoethanol (enriched medium), and 50% human conditioned medium (hCM). Human conditioned medium was prepared as follows: Peripheral blood mononuclear cells (PBMCs) were isolated from a pool of two buffy coats by centrifugation over Ficoll-Hypaque (1.072 g/mL) for 10 minutes at 800g. Human culture medium was prepared by culturing 5 \times 10^6 PBMCs/mL in fetal calf serum-devoid enriched medium containing 10 \( \mu \)g/mL concanavalin A for 48 hours at 37°C in 5% CO\(_2\) humidified atmosphere. The culture supernatant was then inactivated by incubation for 45 minutes at 56°C. Nonadherent cells were collected twice a week, centrifuged for 5 minutes at 400g, and resuspended in fresh culture medium. After 12 to 15 days in culture, the percentage of hBMMCs was determined by toluidine blue staining, and only cultures containing more than 90% hBMMCs were used in the following experiments. At that time, no adherent cells were found in the cultures. Seven percent of the cells were identified as lymphocytes, whereas the other 3% of cells could not be identified by Wright's stain.

**3H-thymidine incorporation.** Human BMMCs (more than 90% pure) plated in 96-well microtiter plates (Nunc, Denmark) at a density of 2 \times 10^5 cells/well in 0.1 mL enriched medium. Various doses of rhIL-3, rabbit anti-rhIL-3 serum together with rhIL-3 (kindly provided by Dr S.C. Clark, Genetics Institute, Cambridge, MA), mouse IL-3 (kindly provided by Dr J. Ihle, Memphis, TN), or hCM with or without rabbit anti-rhIL-3 serum were added to triplicate wells, and the cells were incubated for 48 hours at 37°C in 5% CO\(_2\). Enriched medium alone was used as control. A total of 0.5 \( \mu \)Ci 3H-thymidine (2 \( \mu \)Ci/nmol) (Neyeqy Nuclear Research Center, Beer Sheva, Israel) was added to each well 12 hours before harvesting onto glass-fiber filter paper (Titertek, UK) in an automated cell-harvester unit (Brandel, Rockville, MD) and 3H-thymidine incorporation was quantified by \( \beta \)-scintillation counting.

**Preparation of monolayers.** Fibroblasts were prepared from the skin of 19 to 20-day-old BALB/c mouse embryos as described previously. \(^ {23,41}\) The fibroblasts were plated in Waymouth medium at a concentration of 1 \times 10^5 cells/plate (35 mm petri dishes, Nunc). Monolayers of Swiss albino mouse skin-derived 3T3 fibroblast cell line (American Type Culture Collection, Rockville MD) were prepared similarly. \(^ {14,15}\) Human skin-derived fibroblasts (HSFs) were prepared as previously described. \(^ {42}\) Briefly, human skin dermis was separated from sterile skin biopsy specimens 2 to 3 mm in diameter. Explants were plated in minimum essential medium (BioLab, Jerusalem, Israel) containing 10% newborn calf serum (BioLab) at a concentration of five to 10 pieces per 35-mm plate. Monolayers were used after the fibroblasts were replated at least 10 times. Bovine vascular endothelium cells (kindly provided by Dr I. Vlodavsky, Hadassah Hospital, Jerusalem, Israel) were obtained from aortic arches of fetal and adult cattle as previously described. \(^ {43}\)

Before the addition of hBMMCs to the cultures, the cells were treated with a cytostatic dose of 0.4 \( \mu \)mol/L mitomycin C (Sigma Co, St Louis, MO) \(^ {44}\) for 30 minutes at 37°C followed by three washes in phosphate-buffered saline. This treatment was shown to be cytostatic, but not cytocidal, in all of the monolayers.

**Coculture and separation of hBMMCs from the monolayers.** Three million hBMMCs were cultured for various periods of time in 3 mL medium on mitomycin C-treated MESFs, 3T3, HSFs, or on bovine vascular endothelium monolayers. The coculture medium contained 50% enriched medium and 50% hCM. Medium was changed every 6 to 7 days, and nonadherent cells were counted, sedimented, and resuspended in new coculture medium. Cell samples were stained with toluidine blue and alcian blue/safranin counterdye (AB/S counterdye) each time the medium was changed.

To separate the hBMMCs from the different fibroblasts, the cells from each plate were dispersed with 0.1% trypsin, washed twice, and resuspended in 30 mL of Dulbecco modified Eagles medium containing 15% horse serum. The cell suspension was then incubated in three tissue culture dishes (100 x 20 mm, Nunc) for 1 hour at 37°C in a CO\(_2\) incubator. Within 1 hour, most of the fibroblasts adhered to the plates, whereas the hBMMCs remained in suspension and were enriched up to a purity of 90% ± 5% (mean ± SE, n = 4) as determined by toluidine blue staining. Cell viability was determined by trypan blue dye exclusion. \(^ {14}\) Human BMMCs could not be separated from the vascular endothelial cells of the coculture and therefore were analyzed only by AB/S counterdye, in which hBMMCs could be distinguished from the endothelial cells by their size and histologic characteristics.

**Histochemical staining.** Primary cultured hBMMCs and nonadherent hBMMCs from the cocultures were air-dried on glass slides, incubated for 5 minutes with a solution of 0.5% alcian blue/0.3% acetic acid, \(^ {1,14}\) washed with distilled water, incubated for 5 minutes with a solution of 0.1% safranin/0.1 acetic acid, washed, air-dried, and examined microscopically (AB/S counterdye). Toluidine blue staining was used to determine the percentage of hBMMCs in the cultures.
Quantitative analysis was performed on hBMMCs separated from three different hBMMC-MESF monolayer cocultures. For this analysis, cells were sampled at different time points in the coculture period, enriched as described, counted, and resuspended at a concentration of 5 × 10⁶ cells/mL. Slides were prepared at a density of 20 to 40 cells/high-power field (× 1,000). The slides were AB/S stained as described, and three different randomly selected high-power fields of each slide were examined microscopically. Cells showing MC morphology were counted and assigned into three categories: AB⁺/S⁻, AB⁻/S⁺, and AB⁻/S⁻. The results were obtained by calculating the percentages of cells assigned to each category from the three different cocultures after each coculture period and mean percents ± SE were calculated.

Proteoglycan analysis. The presence of cell-associated, newly synthesized proteoglycan was performed on hBMMCs before culturing on the fibroblast monolayer (more than 90% hBMMCs) and at indicated times during the coculture. Cells were incubated for 24 hours at 37°C in fresh culture medium containing 100 μCi/mL of 35S-sulfuric acid (4,000 Ci/mmol) (New England Nuclear). The fibroblast monolayers were 35S-labeled as controls in conditions similar to coculture labeling. The radiolabeled coculture hBMMCs were dispersed and separated from fibroblasts as described above. Separated hBMMCs and fibroblasts labeled both before and after the coculture were sedimented at 400g, resuspended, and incubated for 2 minutes at 4°C in 0.2 mL 0.05 M sodium acetate containing 1% (wt/vol) Zwittergent 3-12 detergent (Calbiochem-Behring Corp, La Jolla, CA), 0.1 mol/L EDTA, 5 mmol/L benzamidine, and 1 mmol/L sodium iodoacetamide, followed by the addition of 0.6 mL 0.05 M sodium acetate and 4 mol/L guanidine-HCl (GnHCl) containing 50 μg heparin carrier (Sigma Co). 35S-labeled proteoglycans were partially purified by CsCl density gradient ultracentrifugation under dissociative conditions. The bottom third of the centrifuge tubes, which contained 80% of the 35S-labeled proteoglycans, was dialyzed for 1 day against water, lyophilized, and applied to PD-10 columns. The presence of 35S-labeled heparin and chondroitin glycosaminoglycans was assessed by determining the susceptibility of the proteoglycans to nitrous acid degradation and chondroitinase ABC and AC (Sigma Co) digestion. Heparitinase and heparanase (Seikagaku Kogyo Co, Tokyo, Japan) were used to distinguish 35S-labeled heparan sulfate from heparin. Digests and hydrolysates were chromatographed on the PD-10 columns, with degradation being assessed by determining the shift in 35S radioactivity from the void volume to the included volume of the column.

RESULTS

Effect of rhIL-3 on hBMMCs' 3H-thymidine uptake. In three separate experiments, primary hBMMCs (more than 90% purity), differentiated in vitro after 2 weeks in the presence of hCM, were examined for a proliferative response to rhIL-3 in a dose-dependent manner with a maximum increase in 3H-thymidine uptake of more than sixfold over background at a dose of 40 U (Fig 1A). This response was completely inhibited by the addition of a 1:100 dilution of rabbit anti-rhIL-3 serum. Human conditioned medium, which was used as a reference for positive growth induction in these assays, induced a dose-dependent increase in the incorporation of 3H-thymidine into DNA, with a maximum increase of eightfold over background at a concentration of 25% hCM (Fig 1B). Rabbit anti-rhIL-3 decreased the hBMMC 3H-thymidine uptake induced by hCM by approximately 50% (two experiments) (Fig 1B), whereas rabbit serum, which was used as control, did not affect the proliferative response of the cells to either rhIL-3 or hCM. Mouse IL-3 failed to induce 3H-thymidine uptake into DNA in the hBMMCs.

Coculture. To evaluate changes in hBMMC phenotype induced by different microenvironments, the cells were cocultured with four different types of monolayers and their histology and proteoglycan synthesis determined. Two of the monolayers were fibroblasts of mouse origin (MESFs and 3T3), one was of bovine origin (aortic arch-derived bovine vascular endothelial [BVE] cells) and one was of human origin (HSFs). All the monolayers were used after they had been replated more than 10 times. Control monolayers of each type were stained and examined for the presence of mast cells originating from the monolayer itself, and the monolayer proteoglycan composition was determined (Table 1). Before the cocultures were initiated, the monolayers were used to treat mitomycin C in a cytostatic dose. Approximately 25% of the hBMMCs initially plated (3 × 10⁶ on each monolayer) adhered to the surface of the fibroblast monolayers within 48 hours. In contrast, almost no adherence of the hBMMCs plated on the BVE monolayer was noted. While the number of cells plated on the fibroblast monolayers did not change significantly throughout the coculture period, as determined by counting sampled nonadherent cells, the number of hBMMCs on the vascular endothelium monolayer decreased gradually to about 0.5 × 10⁶, and some endothelial cells detached from the monolayer. Trypan blue exclusion dye for cellular viability in the fibroblast cocultures revealed a viability of more than 90% in the nonadherent cells and of more than 80% in the trypsin

![Fig 1. Dose response curve of 3H-thymidine uptake by hBMMCs induced by different concentrations of rhIL-3 (■—■) and rhIL-3 with 1:100 diluted rabbit anti-rhIL-3 serum (■—■) (A) and by different concentrations of hCM (△—△) and hCM with 1:100 diluted rabbit anti-rhIL-3 serum (▼—▼) (B). The data represent one of three experiments, and each point is presented as the mean ± SE of triplicates. Data of the ▼—▼ line represent two experiments.](image-url)
Table 1. 35S-Proteoglycan Composition of the Different Monolayers

<table>
<thead>
<tr>
<th>Monolayer</th>
<th>ABCase Degradation by</th>
<th>ACase Degradation by</th>
<th>Heparitinase Degradation by</th>
<th>Heparinase Degradation by</th>
<th>Nitrous Degradation by</th>
</tr>
</thead>
<tbody>
<tr>
<td>MESFs</td>
<td>Control</td>
<td>80 ± 9</td>
<td>7 ± 1</td>
<td>ND</td>
<td>10 ± 1</td>
</tr>
<tr>
<td></td>
<td>Coculture</td>
<td>84 ± 8</td>
<td>10 ± 1</td>
<td>ND</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>3T3</td>
<td>Control</td>
<td>84 ± 10</td>
<td>10 ± 1</td>
<td>ND</td>
<td>11 ± 2</td>
</tr>
<tr>
<td></td>
<td>Coculture*</td>
<td>40 ± 6</td>
<td>37 ± 5</td>
<td>54 ± 7</td>
<td>56 ± 7</td>
</tr>
<tr>
<td>BVE</td>
<td>Control</td>
<td>65 ± 10</td>
<td>14 ± 2</td>
<td>30 ± 3</td>
<td>32 ± 3</td>
</tr>
<tr>
<td></td>
<td>Coculture</td>
<td>63 ± 8</td>
<td>17 ± 4</td>
<td>32 ± 7</td>
<td>38 ± 5</td>
</tr>
</tbody>
</table>

*Since it was not possible to separate the BVE cells from mast cells, the results are of proteoglycans derived from the mixed coculture population after 19 days of the coculture.

Abbreviation: ND, not determined.

Lanes designated "coculture" represent fibroblasts separated from mast cells after 19 days of coculture. The digested 35S-proteoglycans were chromatographed through PD-10 columns. The results are expressed as mean ± SE of three experiments.

Resuspended cells throughout the coculture. Human BMMCs from cocultures with BVE revealed a viability of approximately 60%.

Alcian blue/safranin counterdye. Primary cultured hBMMCs and adherent and nonadherent hBMMCs from the cocultures were stained using the AB/S counterdye technique to distinguish chondroitin sulfate-containing MCs (alcian blue+/safranin-) from heparin-containing MCs (alcian blue-/safranin+). All of the hBMMCs were alcian blue+/safranin- before they were cultured on the monolayers (Fig 2A and Fig 3). Human BMMCs cocultured with MESFs behaved with striking similarity to those cocultured with HSFs throughout the coculture period. Between days 5 and 7 of coculture, with either MESFs or HSFs, most of the granulated cells exhibited a mixed staining of approximately 50% alcian blue+ granules and 50% safranin+ granules (Fig 2B and Fig 3). After 19 days in coculture, only approximately 10% of the granulated cells exhibited more than 90% safranin+ granules (Fig 2C and Fig 3). No significant differences in staining pattern were noted between adherent and nonadherent hBMMCs. Human BMMC/MESF cocultures were used to determine the kinetics of the change in the hBMMC phenotype by the shift in their AB/S staining (Fig 3). Cellular nuclei stained faintly red by safranin (Fig 2A) in all the cells used in the coculture, including control monolayer cells. Toluidine blue staining revealed a marked in-
crease in the hBMMC granule size and density starting after only 48 hours in coculture. The same shifting effect has been observed in cocultures supplemented with rhIL-3 instead of hCM. In contrast to the observations in the MESF and HSF cocultures and despite the fact that hBMMCs did adhere to the surface of the 3T3 fibroblasts cell line monolayer, no evidence of a change in the staining properties of these cells was found, either when stained with toluidine blue or when stained with AB/S counterdye at any point during 2 weeks of coculture. Human BMMCs cocultured with BVE maintained their AB2/S1 staining throughout the 3 weeks of coculture and no changes were found in their toluidine blue staining. No metachromatically stained cells were found in the control monolayers of any cell type. Staining of these monolayers with AB/S counterdye resulted only in a faint red nuclear staining. A significant decrease in the number of hBMMCs has been observed after 3 weeks of culture without feeder layers but with the presence of either rhIL-3 or hCM. No phenotypic changes have been observed in these cells along the culture period.

Changes in hBMMC proteoglycan synthesis induced by MESFs and HSFs. Mast cell oversulfated proteoglycans were used as phenotypic markers to determine the shift in MC phenotype. The proteoglycans were extracted both from nonadherent and dispersed adherent MCs separated from the coculture fibroblasts by adhesion. The proteoglycans were characterized by their susceptibility to enzymatic and chemical degradation. A total of 86% ± 5% (mean ± SE, n = 3) of the primary cultured hBMMC 35S-proteoglycans were susceptible to chondroitinase ABC or AC digestion into disaccharides. These 35S-proteoglycans were resistant to nitrous acid degradation (Fig 4), as assessed by PD-10 chromatography, indicating that primary cultured hBMMCs synthesize ChS as their major proteoglycan. These findings are in accordance with our previously published data. After 19 days in coculture with MESFs, only 24% of hBMMC-derived 35S-proteoglycans were susceptible to chondroitinase AC digestion, while 60% were degraded by nitrous acid treatment (Fig 4). The latter results indicate that hBMMCs shift to synthesize heparin as their major oversulfated proteoglycan following a coculture period of 19 days on an MESF monolayer (Table 2). The MESF monolayer proteoglycans were analyzed to verify that the heparin proteoglycan was not derived from the less than 10% contaminating fibroblasts found in the MC suspension following their separation from the coculture. It was found that the MESF monolayer did not synthesize heparin before or after the coculture (Table 1); instead, it synthesized predominantly dermatan sulfate. 35S-proteoglycans derived from hBMMCs cocultured with HSFs for 19 days were found to comprise approximately 50% heparin and 30% chondroitin sulfate proteoglycans (Table 2). The proteoglycans of the HSF monolayer characterized both before and after the coculture consisted of approximately 30% heparan sulfate, 50% dermatan sulfate, and 15% chondroitin sulfate (Table 1). The characterization of proteoglycans derived from hBMMCs cocultured with BVE cells was hampered by the appearance of numerous endothelial cells from the monolayer in the suspension. Therefore, we tried to compare the composition of proteoglycans derived from the monolayer alone with that of proteoglycans derived from the coculture. No significant differences were identified between the two (Table 1), and no significant quantities of heparin were found in the monolayer or in the coculture (Table 1).

A kinetic analysis of the shift in synthesis of hBMMC proteoglycan type was carried out during a period of 19 days of hBMMC/MESF coculture (Fig 5). As can be seen, the synthesis of ChS gradually declined, whereas that of heparin began probably within the first 48 hours of coculture and gradually increased. The synthesis of heparin equaled that of ChS after approximately 6 days of coculture, and continued to increase gradually to reach a maximum of 60% of total proteoglycan synthesis at about 14 to 19 days.

DISCUSSION

Mast cells are important participants in the allergic response and may play a role in the regulation of the normal immune response. The two known MC subclasses differ in their tissue distribution and functional properties. It is therefore of considerable interest to try to understand the environmental stimuli and factors that regulate MC function and phenotype.

In the present work, we show that human BMMCs show a significant proliferative response to rhIL-3 (Fig 1A). When placed in coculture with MESF or HSF monolayers, hBMMCs shift their proteoglycan synthesis from ChS to heparin (Fig 4 and Table 2) and become more histologically "mature" with marked condensation of their granules.
change in the phenotype of the cells is also marked by a shift in staining properties from \( \text{AB}^-/\text{S}^- \) to \( \text{AB}^+/\text{S}^+ \) and to some extent to \( \text{AB}^-/\text{S}^+ \) staining (Figs 2 and 3).

Recombinant hIL-3 has been reported to stimulate the appearance of basophilic, eosinophilic, and neutrophilic myelocytes and macrophages in human bone marrow and cord blood after 2 weeks in suspension culture. However, it did not induce differentiation into MCs in such cultures.\(^{38}\) As mentioned previously, only 5% of "mast-like" cells appeared in agar interface cultures under the stimulation of rhIL-3.\(^{36}\) Our findings suggest that rhIL-3 may be one of the factors supporting the in vitro proliferation and differentiation of these cells from human bone marrow. The observation that rabbit anti-rhIL-3 inhibits by 50% hBMMC \(^3\)H-thymidine uptake induced by hCM (Fig 1B) supports this possibility and suggests that rhIL-3 is one of the factors present in hCM. It must be assumed that the hCM contains, in addition to rhIL-3, other growth and differentiation factors that participate in the induction of MC differentiation and proliferation.

Mouse MC-fibroblast monolayer interactions have been extensively investigated in the last few years. Two traditional sources of fibroblasts have been the MESF and 3T3 fibroblast cell lines. 3T3 fibroblasts have been used to induce phenotypic changes in mouse MCs.\(^{18,19,22-23}\) to support rat serosal MC survival ex vivo in a mixed coculture,\(^{17}\) and, most recently, to induce the in vitro differentiation of human cord blood mononuclear cells into human skin-like MCs after 11 to 14 weeks of coculture.\(^{36}\) Embryonic mouse skin-derived fibroblast monolayers have been used in a number of studies in coculture with murine MCs.\(^{15,16,22-23}\) Recently, it has been reported that mouse mesenteric lymph node-derived MC committed precursors or chondroitin sulfate D containing mouse lymph node-derived MCs differentiate into connective tissue-like MCs when cocultured with MESF or 3T3 cell monolayers.\(^{22-23}\) The present selection of fibroblast monolayers to attempt to induce a change in the phenotype of the hBMMC was based on these studies.

No MCs were detected either by toluidine blue staining or by AB/S in control MESF cultures maintained for over 40 days. Before the coculture period, the fibroblasts were growth inhibited by mitomycin C treatment; therefore, it is most unlikely that MCs developed from progenitors in the monolayer itself. In coculture with the MESF monolayer, but not with the 3T3 monolayer, hBMMCs, initially producing ChS-E, were induced to synthesize heparin, with a gradual increase up to a 3:1 ratio in favor of heparin, during a period of up to 3 weeks (Fig 5). Control MESF and 3T3 monolayers and fibroblasts derived from the hBMMC/MESF cocultured monolayers were found to be devoid of heparin proteoglycans (Table 1). Alcian blue/safranin counterstaining properties shifted in parallel to the shift in the proteoglycan synthesis, from \( \text{AB}^-/\text{S}^- \) in the starting cells to \( \text{AB}^+/\text{S}^+ \) in most of the cells after 5 to 7 days in coculture, and even to \( \text{AB}^+/\text{S}^+ \) in approximately 10% of the cells after about 19 days of coculture (Fig 3). The failure of the 3T3 fibroblasts to induce changes in hBMMC phenotype may be attributable to a loss of function of this specific line during the numerous transfers it went through. This 3T3 fibroblast cell line has also proved to be less potent than MESFs in cocultures with mouse mesenteric lymph node-derived chondroitin sulfate D containing MCs.\(^{23}\)

Since primary murine fibroblasts have been shown to be able to induce a change in the phenotype of hBMMCs, we decided to assess the effect of fibroblasts from a human source. Primary cultured HSFs were chosen for convenience. In cultures with hBMMCs, these HSFs induced a strikingly similar response to that induced by MESFs. The hBMMCs shifted to synthesis of 50% heparin and only approximately 30% ChS (Table 2) in only 3 weeks; parallel changes in AB/S staining were also observed. It is possible that an even greater shift would have been achieved in longer term cocultures. However, in the kinetic analysis (Fig 5) of the shift in the proteoglycan synthesis in hBMMCs from the MESF cocultures, a plateau was reached after 19 days.

### Table 2. \(^3\)S-Proteoglycan Composition of hBMMCs Before and After Cocultures With MESF and HSF Monolayers

<table>
<thead>
<tr>
<th>hBMMC</th>
<th>ABCase</th>
<th>ACase</th>
<th>Heparinase</th>
<th>Heparitinase</th>
<th>Nitrous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>90 ± 7</td>
<td>86 ± 5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MESF coculture</td>
<td>30 ± 4</td>
<td>24 ± 6</td>
<td>3 ± 1</td>
<td>57 ± 5</td>
<td>60 ± 6</td>
</tr>
<tr>
<td>HSF coculture</td>
<td>32 ± 6</td>
<td>30 ± 4</td>
<td>0</td>
<td>45 ± 7</td>
<td>50 ± 8</td>
</tr>
</tbody>
</table>

All of the results are of \(^3\)S-proteoglycans derived from hBMMCs at a purity of approximately 90% (primary culture derived and coculture derived). Degradation was determined by PD-10 chromatography. The results are expressed as mean ± SE of three experiments.

![Fig 5. Kinetics of the shift in proteoglycan synthesis in hBMMCs cocultured with MESFs.](https://www.bloodjournal.org/)

The results are expressed as the percent ± SE of the total \(^3\)S-proteoglycan in each of the three separate experiments.
It is a matter of great importance to determine whether the apparent change in MC phenotype is due to a selection of a subpopulation of cells from the starting hBMMCs or whether it is due to a change within each cell in the majority of the starting population. A clue to the answer to this question is presented by the AB/S staining. Cells staining positive with both alcian blue and safranin (Fig 2B) were detected. Such mixed staining was also noted in mBMMCs, cocultured with 3T3 fibroblasts, in a previous study.18 The high correlation between the change in the histologic staining and the shift in the synthesis of proteoglycans (Figs 3 and 5) also supports the likelihood of a phenotypic change taking place within each cell, rather than a selection of cell subpopulations.

The data presented in this work support our previous findings regarding the identification of human basophilic cells developing in normal human bone marrow suspension cultures as human MCs. Moreover, this study is the first to show that hBMMCs can be induced to shift their proteoglycan production from ChS to heparin and to change their phenotype to connective tissue-like MCs in an appropriate microenvironment. The effects of the MESF and HSF monolayers are of great importance, as they may enable the identification of novel MC regulating factors, which may play a role in the physiologic "steady-state" maintenance of human MCs in vivo.

REFERENCES

Fibroblasts induce heparin synthesis in chondroitin sulfate E containing human bone marrow-derived mast cells

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