Regulation of Haemopoiesis in Long-term Bone Marrow Cultures. IV. Glycosaminoglycan Synthesis and the Stimulation of Haemopoiesis by β-D-Xylosides

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ABSTRACT Sulfated glycosaminoglycans (GAGs) are distributed in consistent and distinctive patterns between the cell surface and the growth medium of haemopoietically active long-term bone marrow cultures. Heparan sulfate is the main cell surface component and chondroitin sulfate is the major sulfated species in the medium. When the cultures are supplemented with β-D-xylosides a significant increase in chondroitin sulfate synthesis is observed but no stimulation of heparan sulfate synthesis occurs. The chondroitin sulfate accumulates in the culture medium in β-D-xyloside-treated cultures but the composition of sulfated GAGs in cell-surface derived material is unaffected. β-D-xylosides also stimulate the production of haemopoietic cells without any apparent alteration in the adherent stromal cells of the marrow cultures. Equivalent increases are obtained in cells at all stages of development so that a fivefold increase in pluripotent stem cells (CFU-S) is matched by fivefold increase in the granulocyte-macrophage progenitors (GM-CFC) and in mature granulocytes. The stimulation persists for many weeks in β-D-xyloside-treated cultures. These results indicate that the sulfated GAGs may play an important role in the regulation of haemopoiesis.

The prolonged maintenance of haemopoiesis only occurs in association with the appropriate cellular environment in vivo (1, 2). The long-term production of haemopoietic cells in culture also depends on the formation of an adherent layer of bone marrow-derived stromal cells (3, 4) similar in structure and organization to the corresponding haemopoietic environment of normal bone marrow (5, 6). Duplication of the haemopoietic defects of genetically anaemic mice strains S1/S1d and W/Wv in long-term cultures (7) supports the proposition that adherent stromal cells are required for haemopoiesis in vitro. Within the adherent layers of long-term cultures the self-renewal of pluripotent stem cells (CFU-S) occurs for up to 20 wk and haemopoietic cells in all stages of development can be detected (8, 9, 10). These include the whole range of haemopoietic progenitor cells, e.g., the granulocyte/macrophage progenitor cell (GM-CFC) and certain mature cell types (11). Furthermore, in all aspects so far studied, the haemopoietic cells produced are identical to their in vivo counterparts (12).

Although the cellular environment is evidently a prerequisite for haemopoietic activity, the question remains whether it fulfills a directive or permissive role. Our approach to this problem has been to use the stromal cell-dependent long-term bone marrow cultures to investigate various aspects of cell-to-cell interactions and the importance of diffusible regulatory molecules in haemopoietic cell development (12). As part of these studies we have been investigating a class of polysaccharide molecules which are components of the extracellular matrix and the external cell surface membrane. These are the sulfated glycosaminoglycans (GAGs) which normally exist in association with proteins in the form of proteoglycan (13). The GAGs are present in largest quantities in connective tissues, in which they are essential for the physical properties of elasticity and compressibility (14). However, their functions extend beyond those of structural elements, and changes in GAG production and distribution during discrete phases of morphogenesis and organ regeneration are closely correlated with events such as cellular migration and differentiation (15). Sulfated GAGs, particularly heparan sulfate, are widely distributed in cell surface membranes where they may play an important role in cellular recognition (16). Thus, these complex molecules are candidates for influencing haemopoietic cell development.

Sulfated GAGs have been identified in haemopoietic tissue
but their functions remain obscure (17, 18, 19). There are no data available on GAGs in bone marrow cultures. In the present study we have determined the structure and distribution of GAGs in the adherent layer and in the spent medium of haemopoietically active marrow cultures and, in an attempt to assess the physiological role of GAGs, we have evaluated the effects on haemopoiesis of β-D-xylosides, agents known to stimulate sulfated GAG synthesis in a variety of other cell types (20–22).

MATERIALS AND METHODS

Establishing Long-term Bone Marrow Cultures: Long-term bone marrow cultures were established from 8- to 12-wk-old BDF1 (DBA/2 x C57Bl/6F1) donors. Femora were removed from the donors, and the marrow cells were flushed into Fisher's medium (Gibco Ltd) supplemented with horse serum (20% final concentration) (Flow Labs, U. K.), using a l fenestr per 10 ml of growth medium (3, 23). The marrow suspension was prepared in batches of 100 ml, and 10 ml aliquots were dispensed into 25 cm² base tissue culture flasks (Sterlin). The cultures were gassed with 5% CO₂ in air and incubated at 33°C. They were fed weekly by removing half the growth medium and replacing it with fresh medium. Before feeding, the cultures were gently agitated to uniformly suspend the cells, and the harvested cells were counted and suspended for CFU-S and GM-CFC content. Occasionally, the cultures were sacrificed to determine the CFU-S and GM-CFC content of the adherent layer. The growth medium was decanted and the adherent layer gently rinsed with 3–5 ml of Fischer's medium. The wash was discarded and the adherent cells were scrapped off the base of the flask with a silicone rubber policeman into 5 ml of Fischer's medium, and a single-cell suspension obtained by repeated aspiration through a pipette.

β-D-Xylosides: A stock solution of 10⁻⁵ M p-nitrophenyl-β-D-xylopyranoside (Koch-Light Lab Ltd) was prepared in Fisher's medium and filtered through a 0.22 μm filter. The growth medium was supplemented with the appropriate concentration of β-D-xyloside when the cultures were established and at every feed.

CFU-S Assay: CFU-S were assayed according to the method of Till and McCulloch (24). Groups of 8–10 recipient BDF1 mice received a dose of 800 T 12 MeV electron irradiation and on the same day were injected (i.v.) with 5-8 × 10⁴ cells/ml of the long-term bone marrow cultures. The mice were killed 8 d later, and the spleens were removed and fixed in Bouin's solution before counting the spleen colonies.

Granulocyte/Macrophage Progenitor Cell Assay: The granulocyte/macrophage colony-forming cells (GM-CFC) were assayed according to a modified procedure of the technique of Bradley and Metcalf (25). 3.5 × 10⁶ marrow cells per ml were suspended in Fischer's medium supplemented with horse serum (20% vol/vol), WEHI-3B conditioned medium (15% vol/vol) (as the source of colony stimulating factor) and 0.3% agar (final concentration) (Difco). Triplicate 1 ml aliquots of the cell suspension were plated into 35-mm petri dishes and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. After 7 d, aggregates of more than 50 cells were scored as colonies.

Initiated Thymidine Suicide Assay: The [³H]TdR suicide assay (26) was used to measure the percentage of the CFU-S population in the S-phase of the cell cycle. Cells harvested from long-term bone marrow cultures were adjusted to a concentration of 5–30 × 10⁶ cells/ml in Fischer's medium. [³H]TdR (specific activity 15 Ci/mmol, Amersham International) was added to one of duplicate 1-ml aliquots of the cell suspension to give a final concentration of 200 μCi/ml, and an equal volume of Fischer's medium was added to the other aliquots. The cells were incubated for 30 min at 37°C. After the incubation, the cells were placed on ice, diluted to the appropriate cell concentration, and an equal number of cells incubated with or without [³H]TdR were assayed for CFU-S. Detailed analysis of the statistical treatment using this technique has been reported elsewhere (27).

Radiolabeling and Harvesting of Sulfated GAG Produced by Bone Marrow Cultures: The detailed procedure for the radiolabeling and harvesting of sulfated GAGs is described by Gallagher et al. (16). Briefly, the GAGs were biosynthetically radiolabeled with 5 μCi/ml [³H]glucosamine and 10 μCi/ml Na₂[³⁵S]SO₄. The isotopes were added to the growth medium and after 48–72 h the growth medium was decanted and centrifuged at 800 g for 10 min. The supernatant (spent medium) was retained. The adherent layer was then treated with 0.05% (wt/vol) trypsin in phosphate-buffered saline, pH 7.2, for 20 min at 37°C. The trypsinized material was centrifuged at 800 g for 10 min and the supernatant and a wash of the pellet were pooled to yield the adherent layer trypsin extract. Glycosaminoglycans present in the spent medium and the adherent trypsin extract were separated by NaCl gradient elution from DEAE ion exchange chromatography. Five bone marrow cultures were used for each group.

Radiolabeled fractions corresponding to sulfated glycosaminoglycans were pooled, dialyzed against water, and concentrated by rotary evaporation and freeze drying. Material designated as chondroitin sulfate was completely degraded to disaccharides by chondroitinase ABC and had an electrophoretic mobility identical to that of a commercial preparation of chondroitin sulfate. Heparan sulfate was identified as material sensitive to extensive depolymerization by nitrous acid (28) in which the [³⁵S]-label was found in the scission products by gel chromatography and high voltage electrophoresis as either free sulfate (derived from N-sulfated residues in the original polysaccharide chains) or O-sulfate groups associated with di- and tetrasaccharide fragments. Full details of the heparan sulfate fine structure will be published elsewhere.

RESULTS

Effect of β-Nitrophenyl-β-D-Xyloside on the Biosynthesis of GAGs in Long-Term Bone Marrow Cultures

Long-term bone marrow cultures were established and fed weekly with growth medium containing 5 × 10⁻⁴ M β-nitrophenyl-β-D-xyloside. After 5 wk the cultures were labeled for 48 h with [³H]glucosamine and Na₂[³⁵S]SO₄. The spent medium and a trypsin-extract of the adherent layer were then harvested and the glycosaminoglycans were separated by DEAE ion exchange chromatography (Fig. 1).

FIGURE 1 Influence of β-D-xyloside on sulfated-GAGs produced by mouse bone marrow cultures. Bone marrow cultures established and maintained in 5 × 10⁻⁴ M p-nitrophenyl-β-D-xyloside. After 5 wk these cultures and controls were incubated for 48 h with [³H]glucosamine and Na₂[³⁵S]SO₄. The spent medium and a trypsin-extract of the adherent layer were then harvested and the glycosaminoglycans were separated by DEAE ion exchange chromatography.
The elution profiles obtained with xyloside-treated (Fig. 1a) and control (Fig. 1b) cultures show that little difference was observed in the trypsin-extracted material. The first peak, eluting between fractions 35 and 55, was almost entirely heparan sulfate whereas the second peak (fractions 56–75) was mainly chondroitin sulfate. The radioactivity profile of the spent medium from control cultures was quite distinctive from that of the corresponding trypsin extract (Fig. 1b). Chondroitin sulfate was the main component present in the spent medium of control cultures: the second peak (fractions 56–75) was entirely chondroitin sulfate, and the first broad peak (fractions 35–55) contained mainly chondroitin sulfate (70%) and a smaller amount (30%) of heparan sulfate. In the xyloside-treated cultures, only a single broad peak of radioactivity was observed in the spent medium which was almost entirely degraded by chondroitinase ABC. The amount of chondroitin sulfate in the medium fraction from xyloside-treated cultures was 30- to 40-fold greater than that found in the medium from corresponding control cultures (note different scales for right-hand vertical axes in Fig. 1).

**Effect of p-Nitrophenyl-β-D-Xyloside on Haemopoiesis in Long-Term Bone Marrow Cultures**

Long-term bone marrow cultures were established in growth medium supplemented with a range of xyloside concentrations from $5 \times 10^{-6}$ M to $10^{-3}$ M. The cultures were fed weekly with medium containing xyloside, and the harvested cells were counted and assayed for GM-CFC and CFU-S content. The results from 10 weekly assays were averaged and are shown in Fig. 2. There is a xyloside-mediated dose-dependent increase in the production of total cells, GM-CFC and CFU-S in the treated bone marrow cultures which is at a maximum of about fivefold increase at a dose of $5 \times 10^{-4}$ M xyloside. It is also clear from Fig. 2 that there is a remarkably consistent ratio between the number of total cells, GM-CFC, and CFU-S (300:15:1) detected in the cultures, regardless of the concentration of xyloside used. The differential morphology of the suspension cells harvested from long-term cultures is not altered by the treatment with xyloside (data not shown). The duration of haemopoietic activity in bone marrow cultures is prolonged in the treated cultures. The control cultures in the experiment described in Table I ceased production of CFU-S after 12 wk and those supplemented with $5 \times 10^{-5}$ M xyloside, after 15 wk. In the bone marrow treated with $5 \times 10^{-4}$ M xyloside, haemopoiesis declined between 18 and 21 wk (Table I).

Adherent layers were occasionally sacrificed to assay the CFU-S content. Table II shows that, although the total cell and CFU-S content of an adherent layer is variable, in all cases assayed the number of CFU-S in the xyloside-treated cultures slightly exceeded that of the control adherent layers.

**Proliferative Status of CFU-S in Bone Marrow Cultures Treated with p-Nitrophenyl-β-D-Xyloside**

The proliferative status of CFU-S in 3- to 5-wk-old bone marrow cultures was measured by the thymidine suicide technique. It has previously been shown that in untreated bone marrow cultures the proportion of the CFU-S population in the S phase of the cell cycle is maximal in the 2-3 d after feeding, after which there is a decline to insignificant levels of proliferation. The results are shown in Table II.

*TABLE I*

<table>
<thead>
<tr>
<th>Duration of Active Haemopoiesis in Bone Marrow Cultures</th>
<th>Time after cultures were initiated</th>
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<tr>
<td></td>
<td>Total suspension cells $\times 10^6$</td>
</tr>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>wk</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.30</td>
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<tr>
<td>8</td>
<td>1.48</td>
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<tr>
<td>18</td>
<td>0.30</td>
</tr>
<tr>
<td>21</td>
<td>0.10</td>
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</table>

Syl: p-nitrophenyl-β-D-xyloside.

*TABLE II*

<table>
<thead>
<tr>
<th>CFU-S Content of Adherent Layers of Long-term Bone Marrow Cultures</th>
<th>Cells per adherent layer $\times 10^6$</th>
<th>CFU-S per adherent layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXPERIMENT</td>
<td>AGE OF CULTURES</td>
<td>CONTROL</td>
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<td>3</td>
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</table>

Syl: p-nitrophenyl-β-D-xyloside

Ex. 1: Full results not shown.

Ex. 2: Produced results shown in Fig. 2.

Ex. 3: Produced results shown in Fig. 3.

**FIGURE 2** The effect of p-nitrophenyl-β-D-xyloside on haemopoiesis in long-term bone marrow cultures. Data shown are the average of 10 weekly assays ± SEM. Figures refer to the total suspension cells, GM-CFC and CFU-S per culture. There were six cultures per group.
progressive stimulation but sustains a stimulatory effect at a constant plateau, the level of which is established in the first and the degree of amplification to mature cells remain constant.

Thus, continuous supplement of xyloside does not lead to a decrease in numbers of CFU-S merely reflects an increase in the maximum percentage of CFU-S killed by \(^{3}\text{H}-\text{TdR} \) due to the proposition that the CFU-S population increases, and the proportion of CFU-S which are diverted to differentiate and their mature progeny, i.e., the balance between self-renewal, differentiation and maturation is unaffected by xylosides. Treatment of long-term cultures with xylosides led to only slight changes in the cell-associated heparan and chondroitin sulfate but gave major differences in the GAG composition of the spent medium. In the latter case, a 30- to 40-fold increase in chondroitin sulfate occurred as a result of xyloside treatment and no heparan sulfate was detected. This effect agrees with previously published information in which xylosides were effective at stimulating chondroitin sulfate in embryonic chick cartilage cultures (20, 21). The biosynthesis of chondroitin sulfate and heparan sulfate is initiated on a \(\beta\)-xylose moiety linked to a serine residue in a protein core (22). \(\beta\)-D-xylosides (derivatives of xylose which carry a substituted aglycone group at the carbon-1 position) act as artificial initiators of chondroitin sulfate synthesis (21, 22). Despite the theoretical feasibility of xylosides to act as primers for heparan sulfate synthesis, other studies have also shown that xylosides are poor, or inactive, substrates for the assembly of heparan sulfate chains (22, 29, 30). Thus, the dramatically elevated levels of chondroitin sulfate seen in long-term culture supernatant medium after treatment with \(\beta\)-D-xyloside were perhaps not too surprising. What is intriguing, however, is the concomitant stimulation of haemopoiesis in such cultures. The enhanced haemopoiesis occurs not only after cultures are treated with \(\beta\)-nitrophenyl-\(\beta\)-D-xyloside but with other xylosides (e.g., methylumbelliferyl-\(\beta\)-D-xyloside) as well (E. Spooncer, unpublished observation).

Haemopoiesis is stimulated in a dose-dependent manner and is reflected by equivalent increases in the number of pluripotent stem cells (CFU-S), granulocyte progenitor cells (GM-CFC) and their mature progeny, i.e., the balance between self-renewal, differentiation and maturation is unaffected by xylosides. This increase in haemopoiesis is mainly associated with the suspension phase of long-term cultures, little, if any, change occurs in haemopoiesis associated with the adherent cell layer. Since the suspension CFU-S are derived from CFU-S in the adherent layer (9), then these observations can be accommodated by the proposition that the CFU-S population increases, yet the proportion of CFU-S which are diverted to differentiate and the degree of amplification to mature cells remain constant. Thus, continuous supplement of xyloside does not lead to a progressive stimulation but sustains a stimulatory effect at a constant plateau, the level of which is established in the first few weeks of culture. These data suggest the attractive hypothesis that xyloside treatment modifies cell:cell and cell:matrix interactions in the adherent layer (the stromal cell environment) in some way that renders it able to support higher levels of haemopoiesis than untreated adherent layers. The mechanism involved is unclear. Xylosides only altered sulfated glycosaminoglycans in the medium, not the cell-associated material. However, if these complex polysaccharides in some way regulate haemopoietic activity within the marrow stroma, then a high concentration of chondroitin sulfate in the overlying medium of bone marrow culture could modulate such control processes leading to a stimulation of CFU-S proliferation and to the establishment of a new, and more active, steady-state of haemopoiesis. Presumably, the primary effect is to modify the proliferative activity of the CFU-S. Indeed, this was found to be the case. Our data clearly show that in xyloside-treated cultures a higher proportion of the CFU-S were detected in the S phase of the cell cycle and that the duration for which a significant proportion of the CFU-S were in the DNA S phase was prolonged. Experiments are in progress to test whether this is mediated by the over-production of the factor which is known to stimulate DNA synthesis in CFU-S (32) and which can be produced by the adherent cells in long-term marrow cultures (12, 27). However, previous work has shown that addition of excess CFU-S stimulatory material to long-term cultures did not lead to an increase in the number of CFU-S, although CFU-S proliferation was stimulated (12). Presumably, the "extra" CFU-S generated either died or were recruited into differentiation. Therefore, in addition to prolonging the proliferation of CFU-S, the xylosides also enable the cultures to support both the maintenance of CFU-S and their differentiation and proliferation.

It is also of interest that the duration of haemopoiesis in \(\beta\)-D-xyloside-treated cultures is more prolonged than the corresponding control cultures. To discount the possibility that the increase in numbers of CFU-S merely reflects an increase in the spleen seeding efficiency of pluripotent cells (31), we have also measured the seeding efficiency of CFU-S from control and xyloside-supplemented cultures. No significant differences were found (data not shown).

In conclusion, although this work indicates an association between stimulation of GAG synthesis with \(\beta\)-D-xylosides and increased capacity of long-term cultures to maintain haemo-
poiesis, no “cause and effect” relationship has been formally established. It could be, for example, that the xyllosides are having a role other than the stimulation of GAG synthesis. However, the results presented here demonstrate that haemopoiesis is enhanced in xyloside-treated cultures and determine the effect directly on haemopoietic cell development.

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