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Research Article

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Extracellular Matrix Promotes the Growth and Differentiation of Murine Hematopoietic Cells In Vitro

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Abstract

We have developed a long-term culture system in which murine marrow cells are cultured on a complex extracellular matrix (ECM) that is derived from marrow and extracted with guanidine hydrochloride and dithiothreitol. Marrow cultures were established with fresh murine marrow cells and recharged at 2 wk (week 0). Phase microscopy showed a dramatically increased adherent cell layer development on ECM compared with controls within a week after recharge. By electron microscopy, this adherent layer was composed of numerous reticular cells apparently attached to the ECM which extended cytoplasmic projections to the surrounding hematopoietic cells. Adherent cellularity on ECM-coated dishes increased to 30 times the control values by week 2. Cumulative suspension cells on ECM dishes were eight times controls. ECM influenced both hematopoietic progenitor cell proliferation and differentiation. Adherent colony-forming unit-granulocyte/macrophage and colony-forming unit-megakaryocyte were >30 and 15 times the control values, respectively, by week 2 ($P \leq 0.05$). There were more mature granulocytic and megakaryocytic cells in ECM-coated dishes than in controls at all time points. This new culture system directly demonstrates that ECM is an important component of the hematopoietic microenvironment.

Introduction

The hematopoietic microenvironment is known to influence the growth and differentiation of hematopoietic cells. Although poorly defined, this microenvironment is composed of both cellular and extracellular components. In long-term bone marrow cultures, the maintenance of hematopoiesis is dependent on the establishment of an intact layer of adherent cells that are derived from the marrow stroma (1-3). Such adherent cell layers are capable of elaborating soluble factors which influence hematopoietic proliferation and/or differentiation

(4). Little is known, however, about the function of the extracellular components within the hematopoietic microenvironment. There is substantial evidence in a variety of other developmental systems that the extracellular matrix (ECM)¹ influences cell growth and differentiation (5-7). Recent studies suggest that the ECM also plays a role in the in vitro proliferation and differentiation of hematopoietic cells. Inhibitors of matrix formation are known to decrease stem cell production (8), while compounds stimulating matrix production increase stem cell proliferation (9). Finally, the stromal cell population is known to produce a variety of ECM proteins including fibronectin, laminin, and type IV collagen (10).

We hypothesize that bone marrow-derived ECM plays a crucial role in the development of the hematopoietic microenvironment. In order to test this hypothesis, we have developed a new system of murine long-term marrow cultures established on ECM extracted from bone marrow. We report that this extracellular material promotes rapid formation of an adherent stromal cell layer and, subsequently, proliferation and differentiation of hematopoietic cells. This system demonstrates the importance of ECM in the hematopoietic microenvironment.

Methods

Preparation of ECM. Femurs and tibias removed from 4- to 6-wk-old rabbits were obtained from Pel-Freez Biologicals (Rogers, AR). Rabbits were used to ensure sufficient quantities of marrow extract. The marrow was removed and immediately placed in 50 mM Tris buffer (pH 7.4) containing 3.4 M NaCl, leupeptin (1 μ g/ml), and soybean trypsin inhibitor (10 μ g/ml) (Sigma Chemical Co., St. Louis, MO) to precipitate ECM proteins, a modification of a previously described method (5). This suspension was homogenized in a Brinkman Polytron homogenizer for 30 s at high speed. The marrow homogenate was centrifuged at 1,500 g for 10 min at 4°C and the supernatant and fatty layer removed. The pellet was washed exhaustively in 3.4 M NaCl solution, digested with RNase (100 μ g/ml) and DNase (25 μ g/ml) (Sigma Chemical Co.) in Fisher's medium at 37°C for 1 h, and washed in 3.4 M NaCl solution overnight. The ECM was extracted from the pellet with 20 ml of 50 mM Tris buffer containing 4 M guanidine HCl and 2 mM dithiothreitol (pH 7.4), which is known to solubilize proteoglycans and other ECM components (11, 12). The extract was centrifuged at 1,500 g for 10 min and the supernatant

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1. **Abbreviations used in this paper:** CFU-GM, colony-forming unit-granulocyte/macrophage; CFU-Mk, colony-forming unit-megakaryocyte; ECM, extracellular matrix; EHS, Engelbreth-Holm-Swarm; Gdn/DTT, guanidine/dithiothreitol.

removed and dialyzed exhaustively against distilled water. The extraction procedure was repeated four times and the extracts pooled. 2 ml of this extract containing ~0.4 mg dry weight of insoluble material was pipetted onto 35-mm tissue culture plastic dishes (Becton-Dickinson & Co.) and allowed to dry overnight. The dishes were sterilized with 500,000 rad of ^{60}Co irradiation.

Fresh marrow cells were obtained from 4- to 6-wk-old male, syngeneic C57/BL6 mice (Charles River Breeding Labs, Inc., Wilmington, MA). Monodispersed nucleated cells (3×10^6) were suspended in 3 ml of Fisher's medium containing 5×10^{-7} M hydrocortisone (Sigma Chemical Co.), 40 $\mu\text{g}/\text{ml}$ of gentamicin, and 20% horse serum (HyClone Laboratories Inc., Logan, UT). Cultures were established on ECM-coated dishes or on control tissue culture dishes. The cultures were incubated at 33°C in a humidified atmosphere with 5% CO_2 . At 2 wk (week 0), all nonadherent cells were gently removed and the cultures recharged with 3×10^6 fresh nucleated marrow cells in 3 ml of Fisher's medium. At weekly intervals thereafter, medium and suspension cells were removed and replaced with fresh medium. At various intervals, the adherent layer was removed by incubation with trypsin-EDTA 1:250 (KC Biologicals) for 1 min at room temperature. Total nucleated cell counts were performed by hemocytometer and cell viability assessed by trypan blue exclusion. Cytoцентрифугed preparations of adherent and suspension cells were stained with Wright stain or acetylcholinesterase (13).

Progenitor cell assays. Short-term assays for granulocyte progenitor cells (CFU-GM) and megakaryocyte progenitor cells (CFU-MK) were performed as previously described (14, 15). Briefly, bone marrow cells were cultured as previously described using conditioned medium from WEHI-3 and mouse lung conditioned medium (16, 17). Before plating, the bone marrow cells were generally incubated at 37°C for 30–60 min at $\sim 5 \times 10^6$ cells/ml in McCoy's 5A medium containing 10% fetal calf serum (FCS). Marrow cells were cultured at $30\text{--}100 \times 10^4$ cells/ml in 35-mm petri dishes (Becton-Dickinson & Co.) in modified McCoy's 5A medium containing 10% FCS and 0.25% Bactoagar. After incubation (7 d, 37°C, 7% CO_2 , 100% humidity), petri dishes were removed from the incubator, dried, and stained in situ for acetylcholinesterase activity (18). Colonies were counted at a magnification of 40.

Electron microscopy. Representative matrix and control dishes were harvested at ~2 wk postfeeding and prepared for scanning and transmission electron microscopy, as described (19).

Protein analysis. SDS polyacrylamide gel electrophoresis, after the method of Laemmli (20), was performed on samples of bone marrow guanidine/dithiothreitol (Gdn/DTT) extract, high molecular weight standards (Sigma Chemical Co.), and Gdn/DTT extract of ECM was obtained from the Engelbreth-Holm-Swarm (EHS) tumor, which is known to produce large quantities of the ECM components laminin, type IV collagen, fibronectin, and entactin (12). All gels are run under identical conditions. The gels were stained using silver nitrate as described (21).

Results

Morphologic characteristics of long-term cultures on matrix substrata. Bone marrow cells cultivated in control dishes (tissue culture plastic) developed islands of adherent cells within 7–10 d. In contrast, ECM supported a more rapid and extensive development of an adherent layer that became confluent within the same time period. ECM derived from the nonhematopoietic tissues EHS tumor and cortical bone (devoid of marrow) did not promote adherent layer formation or hematopoiesis (data not shown). This suggests organ specificity. The adherent layer that developed on marrow-derived ECM was composed of flat, branching cells that formed large areas having a cobblestone appearance. However, relatively few hematopoietic cells were seen in either cultures at this time. After recharging at week 0, the control dishes showed gradual

development of the adherent layer while ECM-coated dishes showed a striking increase in both hematopoietic and stromal cells (Fig. 1, A and B). This difference was even more evident when the cultures were examined by scanning electron microscopy. In ECM cultures, the adherent cell layer was composed of large branching cells that extended long filamentous projections which encircled hematopoietic cells (Fig. 1, C and D). Transmission electron microscopy demonstrated that the adherent phase of the ECM culture was composed of several cell layers (Fig. 1, E and F). The basal layer consisted of flat stromal cells in direct contact with the matrix extract. Hematopoietic cells were seen to overlie this cell layer. They were often in close apposition to the stromal cells.

Cellular proliferation. Although hematopoietic cells were produced throughout the culture period in both ECM and control dishes, there was an eightfold increase in the suspension phase of ECM cultures compared with controls (Fig. 2). In the adherent phase, there was an even greater difference between the cellularity of ECM and control cultures. Within 2 wk of recharging the cultures, there was a 30-fold increase in the adherent layer cellularity of ECM cultures compared with controls (Fig. 3). A significant difference in adherent cellularity persisted throughout the 6 wk of culture. After week 6, a decrease in total cellularity occurred in both ECM and control cultures. However, the adherent layer cellularity of the ECM based cultures remained significantly greater than that of controls. In order to establish whether this decrease in cellularity was due to senescence of the adherent layer or stem cell depletion, another set of cultures was recharged a second time at week 6. These cultures were capable of another round of proliferation which lasted ~3 wk (data not shown).

Granulocytic and megakaryocytic progenitor cell proliferation. ECM-based cultures sustained greater numbers of hematopoietic progenitor cells than did control cultures (Table I). By week 3, suspension phase CFU-GM were significantly higher in the ECM cultures. In ECM-based adherent layers there were 30-fold more CFU-GM than in the adherent layer of control cultures. Megakaryocyte progenitor cells were also significantly elevated in ECM adherent layers from week 3 to 4 (Table I).

Granulocytic and megakaryocytic differentiation. In order to assess the degree of hematopoietic differentiation, cytoцентрифугed preparations were stained for granulocyte morphology (Wright's stain) or for megakaryocytes (acetylcholinesterase stain) and differential counts were performed. The results are shown in Table II. Both suspension and adherent cells from ECM-based cultures showed a higher total number and higher proportion of mature granulocytes (metamyelocytes, bands, polymorphonuclears) than controls. Megakaryocytes were also elevated in both adherent and suspension phases of ECM-coated dishes. By week 3, ECM-based cultures contained >4,500 mature megakaryocytes (80% of which were in the adherent layer), whereas control cultures contained <100 megakaryocytes (data not shown).

Erythroid proliferation and differentiation. No evidence of significant terminal maturation of erythroid cells was seen on microscopic examination of Wright stained suspension or adherent cells. This is consistent with previous work (22, 23), which demonstrated that certain manipulations, such as addition of exogenous erythropoietin, anemic mouse serum, or mechanical agitation, are required to induced terminal erythropoiesis in long-term cultures.

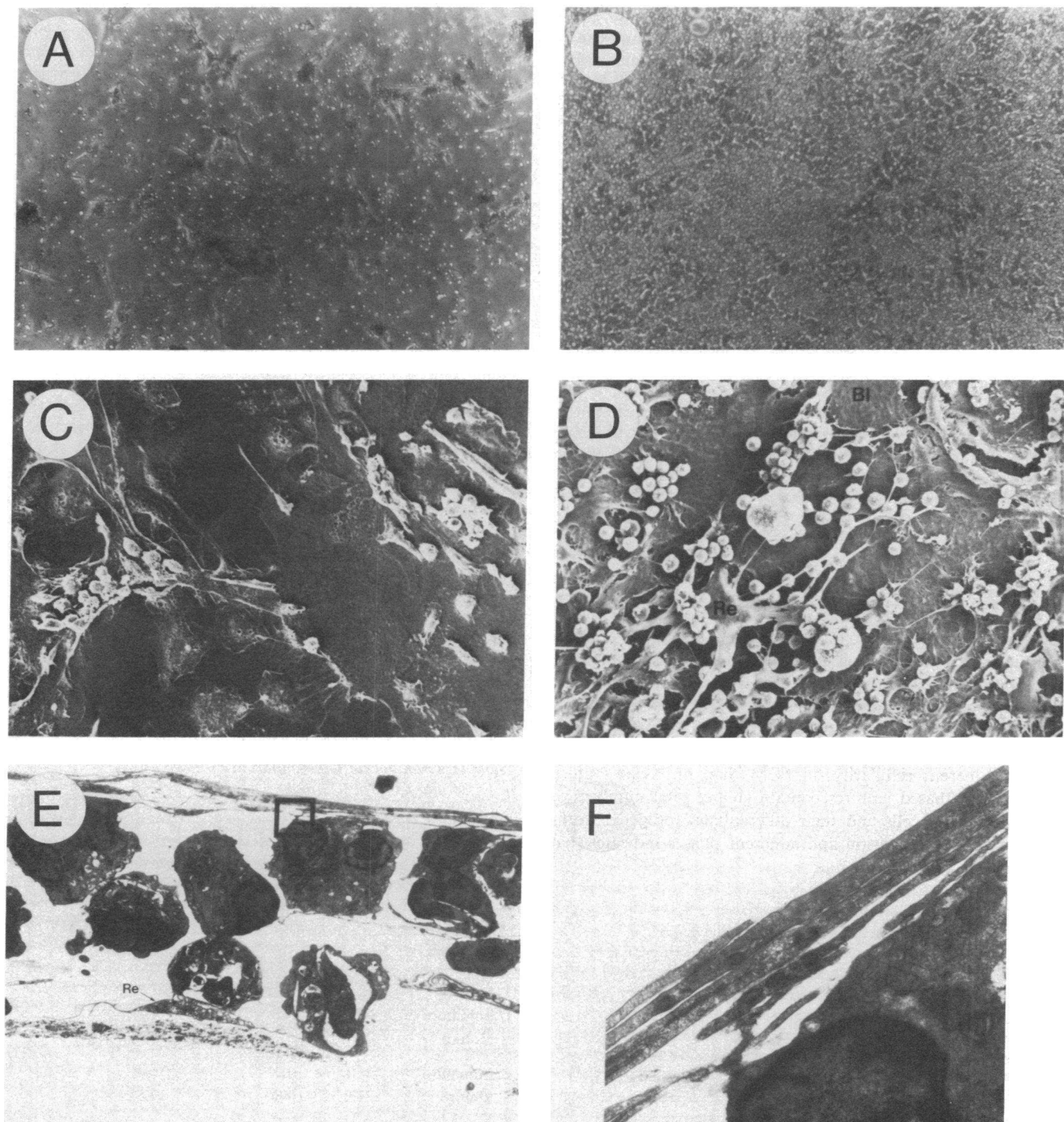


Figure 1. Photomicrographs of long-term cultures. (A, B) Phase-contrast photomicrograph of cultures at week 2. (A) Tissue culture controls. Note relatively sparse hematopoietic islands. $\times 100$. (B) ECM-based cultures. Note dense confluent layer of well spread, flattened, adherent cells underlying large aggregations of round hematopoietic cells. $\times 100$. (C, D) Scanning electron microscopy of cultures at week 2. (C) Tissue culture controls. Note scant adherent layer plus small aggregations of hematopoietic cells. $\times 517$. (D) ECM substratum. The ECM is covered by a confluent layer of adherent cells. Associated with these layers are large reticular (Re) and blanket (Bl)

cells that are closely associated with hematopoietic cells. $\times 517$. (E, F) Transmission electron micrographs of week 3 cultures. (E) Section cut perpendicularly to adherent cell layer. ECM is seen at the lower left hand border. Re, Reticular adventitial cell attached to ECM and extending cytoplasmic processes toward a neighboring cell. Similar cytoplasmic processes are seen surrounding the hematopoietic cells in a manner reminiscent of blanket cells. $\times 1,650$. (F) Higher magnification view of the boxed area in E, showing several layers of cytoplasmic processes in contact with each other and covering the lower hematopoietic cells. $\times 16,500$.

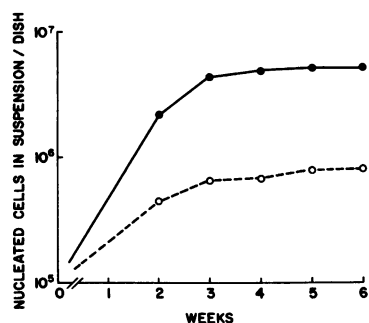


Figure 2. Cumulative suspension cell counts. Long-term marrow cultures were established as described in Methods, and weekly total nucleated suspension cells were determined. Values represent mean cumulative suspension counts of 3–5 individual cultures. ●, matrix extract; ○, tissue culture plastic.

Molecular characterization of ECM components. In order to characterize components possibly involved in the observed effects of ECM in long-term bone marrow cultures, marrow-derived ECM was subjected to electrophoretic separation on a 5% SDS polyacrylamide gel. A silver stained gel is shown in Fig. 4, and for comparison a gel of EHS-derived ECM is shown. In contrast to the EHS extracellular matrix, bone marrow extracellular matrix contained two major protein bands of M_r 116,000 and 135,000.

Discussion

Long-term bone marrow cultures established on a substrate of extracellular matrix show enhanced proliferation and differentiation compared with those established on plastic. These cultures are most striking for the rapid development of a densely cellular adherent layer. When these adherent layers are recharged, hematopoietic cells rapidly proliferate in the adherent cell layer, achieving greater cellularity than on plastic. Comparison of ECM with controls shows up to a 30-fold greater adherent cellularity on ECM than on tissue culture plastic. ECM-based cultures contain greater total numbers of both progenitor cells and their differentiated progeny. Comparison of the suspension and adherent phases indicates that

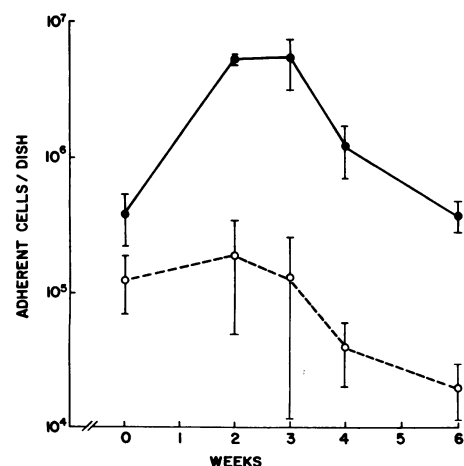


Figure 3. Weekly adherent cell counts. Cultures were established on tissue culture plastic or ECM, and at weekly intervals total nucleated cells in the adherent layer were counted as described in Methods. Values represent the mean \pm SD. At each time point, 3–5 individual cultures were harvested. At week 2 and thereafter the differences between ECM (●, matrix extract) and tissue culture plastic (○) were significant ($P \leq 0.05$).

Table I. Effect of ECM on Hematopoietic Progenitor Cells

Week	Adherent phase*		Suspension phase†	
	ECM	Tissue culture plastic	ECM	Tissue culture plastic
Granulocyte progenitor cells per culture‡				
0	NT	NT	1,780 \pm 329	1,274 \pm 496
2	3,632 \pm 60	109 \pm 78	496 \pm 109	408 \pm 142
3	3,606 \pm 1,541	105 \pm 10	828 \pm 286	227 \pm 31
4	1,344 \pm 353	78	626 \pm 217	114
Megakaryocyte progenitor cells per culture**				
0	NT	NT	78 \pm 64	24 \pm 38
2	169 \pm 81	10 \pm 3	23 \pm 18	27 \pm 9
3	190 \pm 164	8 \pm 3	77 \pm 96	18 \pm 4
4	206 \pm 249	1 \pm 1	45 \pm 19	5

Values represent mean \pm SD of 3–5 replicate cultures.

* Adherent phase refers to cells attached to the bottom of the long-term culture dish, either on ECM or plastic, and removed by trypsinization.

† Suspension phase refers to cells found suspended in the long-term culture medium.

‡ Colony-forming units/granulocyte macrophage were assayed by plating 50,000 cells from either the adherent phase or suspension phase of ECM based or control long-term cultures. 3–5 individual long-term cultures were harvested at each time point, and for each of these 3–5 replicate CFU-GM assays were done.

^{||} $P \leq 0.05$ (t test).

^{||} Values represent pooled data from 1 to 2 individual progenitor cell assays.

** Colony-forming units-megakaryocyte were grown in semisolid agar cultures modified as described (15) so that the cultures were biased toward the formation of megakaryocyte colonies. 3–5 individual long-term cultures were harvested at each weekly time point, and for each of these 3–5 replicate CFU-Mk assays were done.

Table II. Granulocyte Differentiation in ECM and Control Cultures

Total granulocytes per culture $\times 10^5$				
Week	Adherent phase		Suspension phase	
	ECM	Tissue culture plastic	ECM	Tissue culture plastic
0 Immature	0.6	0.1	NT	NT
Mature	1.4	0.1		
1 Immature	NT	NT	4.7	7.1
Mature			14.8	10.5
2 Immature	26.1	0.7	6.4	2.5
Mature	24.0	0.8	14.8	1.6
3 Immature	21.2	0.3	3.0	0.6
Mature	22.8	0.0	12.3	0.7
4 Immature	4.3	0.1	3.2	0.2
Mature	6.1	0.0	8.2	0.3

Cultures were established as in Table I. The degree of granulocytic maturation was determined by removing at each time point, adherent phase, or suspension phase cells from 3 to 5 ECM- or plastic-based long-term cultures. The cells were cytocentrifuged, Wright stained, and 400-cell differential counts were performed. Immature cells are defined as blasts, progranulocytes, or myelocytes, and mature cells as metamyelocytes, megakaryocytes, bands, and polymorphonuclears. NT, not tested.

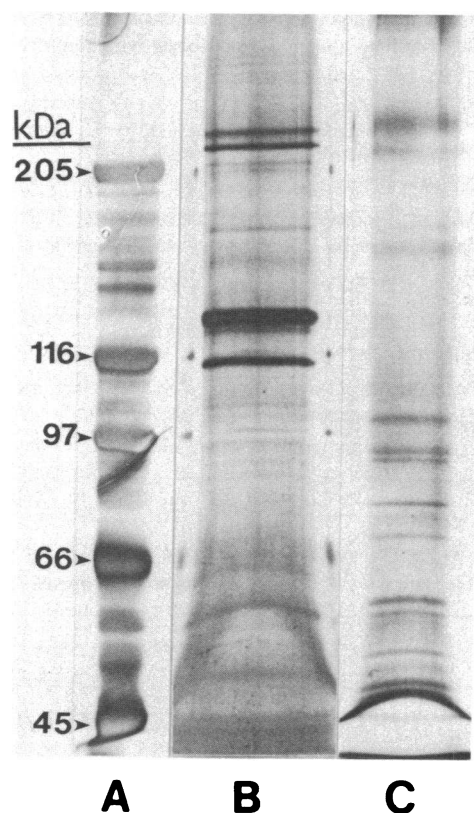


Figure 4. SDS polyacrylamide gel electrophoresis of ECM derived from bone marrow or EHS tumor. Bone marrow and EHS extracellular matrix were electrophoresed on a 5% polyacrylamide gel under reducing conditions, according to Laemmli (20), and stained with silver nitrate as described (21). The EHS tumor has previously been shown (12) to contain the basement membrane components laminin, type IV collagen, fibronectin, and entactin. (A) High molecular weight standards: myosin (M_r 205,000), β -galactosidase (M_r 116,000), phosphorylase b (M_r 97,400), bovine albumin (M_r 66,000), egg albumin (M_r 45,000), carbonic anhydrase (M_r 29,000). (B) Bone marrow extracellular matrix Gdn/DTT extract. Note major protein components at M_r 116,000 and M_r 135,000. (C) EHS extracellular matrix Gdn/DTT extract. Note absence of 116,000- and 135,000- M_r protein bands. kDa, molecular weight.

the ECM-induced response was primarily restricted to the adherent cell layer. We hypothesize that ECM is involved in the development of a stromal cell layer that promotes proliferation and differentiation of hematopoietic stem cells to a greater extent than stromal cell layers based on plastic. Also, we have found that the stromal cell layer induced by ECM can be recharged a second time with fresh marrow cells resulting in renewed hematopoietic cell proliferation.

Electrophoretic analysis of marrow-derived ECM shows the presence of major protein constituents which are not present in the basement membrane containing EHS ECM. The functional significance of these bands is currently the subject of investigation.

A major emphasis in the study of the hematopoietic microenvironment in long-term cultures has been the examination of stromal cells and soluble factors. Allen and Dexter (24) have recently emphasized the importance of blanket cells and macrophagelike cells in regulating stem cell proliferation

in culture. The former appear to be flat, large, well-spread cells that directly overlie developing hematopoietic cells. Song and Quesenberry (4) have shown that the adherent cell layer is capable of elaborating soluble factors that regulate hematopoiesis. Little is known about the role of ECM in the hematopoietic microenvironment. Sampath and Reddi (11) showed that ECM derived from cortical bone could induce the formation of a bony ossicle containing fully functional hematopoietic tissue when implanted subcutaneously in rats. This inductive property was lost when the ECM was extracted with 4 M guanidine, but regained again when the residue and extract were reconstituted. We previously demonstrated that in long-term cultures, stromal cells are capable of synthesizing and depositing extracellular components such as fibronectin, laminin, and type IV collagen (10). Furthermore, the addition of *cis*-hydroxyproline (which specifically inhibits collagen synthesis) resulted in a decrease of stem cell number in these cultures (8). Keating et al. (25) found that proteoglycans are also a major component of the adherent layer of long-term cultures. Spooner and co-workers (9) have shown that stimulation of glycosaminoglycan synthesis with β -xylosides results in increased total cellularity and stem cell numbers in long-term cultures. These studies emphasize the importance of extracellular matrix in the hematopoietic microenvironment.

Our data suggest the ECM is an important component of the hematopoietic environment. We hypothesize that the role of extracellular matrix is to stimulate soluble factor production by stromal cells. Thus, a complete understanding of the hematopoietic microenvironment must take into account its three major components: the ECM, cellular elements, and soluble factors. The use of extracellular matrix in our cultures represents a new system for the long-term culture of hematopoietic cells. This system provides a powerful tool to study the role of extracellular matrix in the hematopoietic microenvironment.

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