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Original Article

Stromal cells from murine developing hemopoietic organs: comparison of colony-forming unit of fibroblasts and long-term cultures

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ABSTRACT The adherent stromal layer in long-term marrow cultures is essential to the proliferation and differentiation of hemopoietic cells. Adhering cells are heterogeneous and morphologically not adequately characterized. Comparative morphological studies were conducted on adherent cells in short-term clonal assays and long-term cultures derived from liver and bone marrow. Liver and bone marrow at different developmental ages have different hemopoietic activities *in vivo* and *in vitro*, as tested via CFU-GM recovery in long-term cultures. Adherent cells from each organ were recovered at an age with high hemopoietic activity (fetal liver and adult bone marrow) and at an age with low hemopoietic activity (neonatal liver and bone marrow). The presence of macrophages, alkaline phosphatase, acid phosphatase, myeloperoxidase, sulfated and non-sulfated glycosaminoglycans (GAGs) and fibronectin was compared. For a given organ, CFU-f colonies showed characteristics similar to those of the confluent adherent stromal layer in long-term cultures. The presence of macrophages and GAGs (sulfated and non-sulfated) in the adherent layer were directly related to the hemopoietic activity. The amount of alkaline phosphatase-positive cells and the amount of fibronectin showed no correlation with the hemopoietic activity of the cultures.

KEY WORDS: *CFU-f, confluent stromal layer, extracellular matrix, fetal liver*

Introduction

Hemopoietic organs like fetal and neonatal liver, spleen and bone marrow, are composed of a mixture of different hemopoietic and stromal cell populations. The latter are essential for hemopoietic cell proliferation and differentiation. A large amount of information is available regarding the migration, concentration and differentiation of hemopoietic stem cells (Metcalf and Moore, 1971). In contrast, little is known about the characteristics of the stromal cells apart from their histological features. Migration of blood stem cells suggests that the micro-environment changes during development and that circulating hemopoietic stem cells recognize and settle in the appropriate organ (Metcalf and Moore, 1971).

Hemopoietic organs (liver, spleen, bone marrow) at different development ages show different hemopoietic characteristics. Stromal cells in these developing hemopoietic organs are involved in the regulation of hemopoiesis. 1) Concentrations of stromal stem cells – CFU-f (= Colony-Forming Units of fibroblast-like cells) approximated by clonal growth resulting in adherent colonies of fibroblast-like cells – in liver, spleen and bone marrow are correlated with the course of hemopoiesis: high CFU-f numbers precede the onset of hemopoiesis (Klein *et al.*, 1983: Van den Heuvel *et al.*, 1987). 2) The CFU-GM number recovered in long-term cultures from hemopoietic organs at different developmental ages reflects the *in vivo* hemopoietic stem cell concentrations (Van den Heuvel *et al.*, 1988).

The requirements for hemopoiesis are complex and depend on a mixture of cell types, extracellular matrix (ECM) components and regulatory molecules (Dexter *et al.*, 1985). Long-term maintenance of hemopoietic stem cell proliferation and differentiation *in vitro* is strictly dependent upon the presence of adherent stromal cells (Dexter, 1977). The mechanisms and/or factors in the inductive micro-environment, underlying hemopoietic stem cell regulation are not elucidated. We investigated the relative contribution of

Abbreviations used in this paper: ACP, acid phosphatase; ALP, alkaline phosphatase; alpha-MeM, alpha-Modification of Eagle's Medium; BFU-E, burst-forming unit of erythrocytes; BM, bone marrow; BPA, burst-promoting activity; CFU-f, colony-forming unit of fibroblast-like cells; CFU-GM, colony-forming unit of granulocytes and/or macrophages; CSA, Colony-Stimulating Activity; ECM, extracellular matrix; ELISA, enzyme-linked immunoabsorbent assay; fn, fibronectin; GAGs, glycosaminoglycans; GM-CSF, granulocyte-macrophage colony-stimulating factor; IgG, immunoglobulin G; LTC, long-term cultures; MP, myeloperoxidase; PAP technique, peroxidase-anti-peroxidase technique; PBS, phosphate-buffered saline; TNF-alpha, tumor necrosis factor-alpha; TRAP, tartrate-resistant acid phosphatase.

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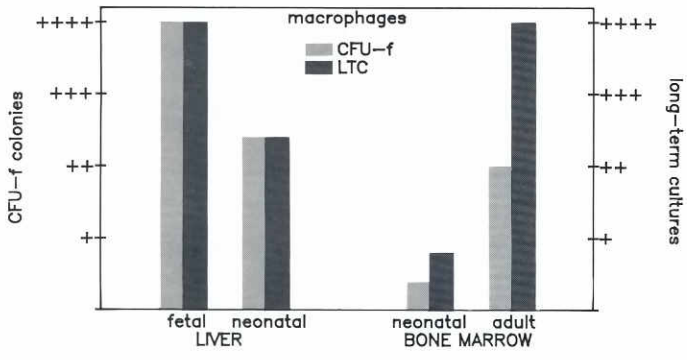

Fig. 1. Presence of macrophages in CFU-f colonies and in adherent stromal layers from long-term cultures. *Stromal cells are derived from fetal liver, liver and bone marrow of 2-day-old mice and adult bone marrow. Shown in the Y-axis are relative measures for the quantity of macrophages.*

components which may be involved in regulation of hemopoiesis. Their presence was compared in adherent cell populations derived from hemopoietic organs exhibiting different hemopoietic activities.

The adherent cells were studied for the presence of macrophages, extracellular components like glycosaminoglycans (GAGs) and fibronectin and cells which are positive for peroxidase, alkaline phosphatase and acid phosphatase. Evidence is present that these components may be important in the control of blood formation.

The observation that maturation of both granulocytic and erythroid cells occurs in close proximity to macrophages indicates a potential role for macrophages in hemopoiesis (Allen and Dexter, 1984). The regulation of hemopoiesis probably takes place (1) through the production of humoral factors, including erythropoietin, GM-CSF and burst-promoting activity (Zipori *et al.*, 1984; Rich and Kubanek, 1985; Sullivan *et al.*, 1985; Rich, 1986a, b; Sieff *et al.*, 1987), and (2) by recruiting other cells of the microenvironment to produce growth factors (Broudy *et al.*, 1986).

GAGs are a component of the hemopoietic extracellular matrix. There is experimental evidence for a role of GAGs in the regulation of hemopoiesis. GAGs play a role in growth factor binding and in mediating the adhesion of immature hemopoietic progenitors (Del Rosso *et al.*, 1981; Spooncer *et al.*, 1983; Zuckerman and Wicha, 1983; Zuckerman and Rhodes, 1985; Wight *et al.*, 1986; Gordon *et al.*, 1987; Roberts *et al.*, 1988).

Peroxidase is present in differentiated cells of the myeloid series. If this enzyme is detected in the stromal layer, it is probably present in adhering hemopoietic cells (granulocytes, progenitors of granulocytes and monocytes) in the cultures (Yam *et al.*, 1971).

Fibronectin is a matrix-located glycoprotein, present in many tissues and synthesized by reticulum cells as well as endothelial cells (Reincke *et al.*, 1982; Zuckerman and Wicha, 1983; Fridman *et al.*, 1985). Fibronectin has been shown to be a component of the extracellular matrix in human and mouse bone marrow cultures (Castro-Malaspina *et al.*, 1980; Hocking and Golde, 1980; Bentley *et al.*, 1983; Liesveld *et al.*, 1989). Fibronectin is associated with adhesion, migration and differentiation of cells (Critchley *et al.*, 1979; Hynes and Yamada, 1982; Yamada and Olden, 1978; Yamada, 1983; Allen and Dexter, 1984; Tsai *et al.*, 1987).

Alkaline phosphatase (ALP) is a rather non-specific membrane-bound enzyme, associated in bone marrow with non-phagocytic fibroblast-like cells which are found adjacent to areas of granulopoiesis (Westen and Bainton, 1979). Its biological function is still unknown. ALP is also associated with osteoblasts and bone lining cells.

Lysosomal acid phosphatase in bone marrow stroma is detected in phagocytic fibroblast-like cells located close to areas of erythropoiesis (Westen and Bainton, 1979).

Results

Colony-forming units of fibroblasts (CFU-f)

Only adhering colonies composed of at least 50 cells were considered. CFU-f colonies were composed of two main cell types: fibroblastic cells and macrophages. All colonies contained both cell types.

After differential staining, the number of positive colonies was counted and expressed as a percentage of the total CFU-f number. Between 30 and 200 colonies were scored after each staining procedure.

Macrophages

Results are represented in Fig. 1.

Fetal liver cultures contained large numbers of phagocytic macrophages. Macrophages were seen within each CFU-f colony and between the colonies. CFU-f colonies from 2-day-old liver and adult bone marrow contained fewer latex-ingesting cells. The relative presence of macrophages in these stromal cell colonies varied from one colony to another. In neonatal liver cultures, CFU-f colonies without latex-ingesting cells were seen as well as colonies with a large number of macrophages. In adult bone marrow cultures, the majority of macrophages were scattered between the colonies, CFU-f colonies contained a moderate number of phagocyting cells (Fig. 5A, B). Bone marrow CFU-f from 2-day-old mice contained few macrophages.

Alkaline phosphatase (ALP)-positive colonies

Results are shown in Fig. 3.

ALP activity was present in some of the fibroblastic cells but absent in macrophages. Fetal liver CFU-f showed no colonies with ALP-positive fibroblast-like cells. Neonatal liver and bone marrow contained low numbers (respectively 20% and 9%) of ALP-positive colonies. In contrast, 67% of the colonies derived from adult bone marrow were ALP positive (Fig. 2C). The intensity of the ALP staining varied between colonies and between fibroblastic cells within a colony. Cells in the center stained stronger than the cells at the periphery. This could be due to a greater density of the cells.

Acid phosphatase (ACP)-positive colonies

Macrophages as well as fibroblast-like cells stained positive for ACP (Fig. 2D). Nearly all liver – fetal (100%) and neonatal (90%) – and adult bone marrow (100%) colonies contained ACP-positive cells (Fig. 3). Only 16% of 2-day-old bone marrow adherent colonies showed ACP-positive cells. Different staining intensities were seen between colonies.

A small proportion of the CFU-f colonies regardless of the organ of origin contained a few TRAP (tartrate-resistant acid phosphatase)-positive cells. In fetal liver cultures 3%, in neonatal liver cultures 5%, in neonatal bone marrow cultures 15% of the colonies contained

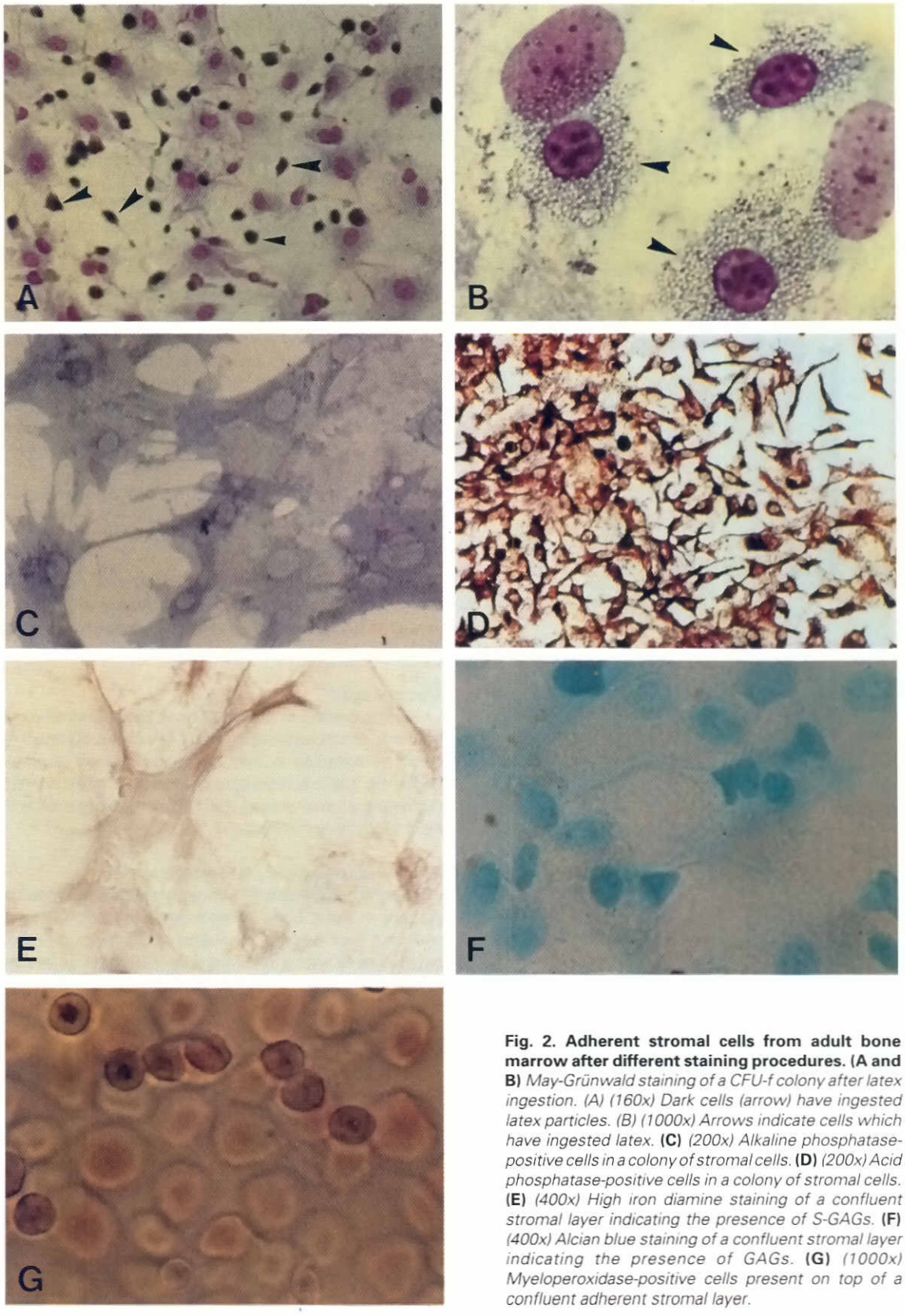

Fig. 2. Adherent stromal cells from adult bone marrow after different staining procedures. (A and B) *May-Grünwald staining of a CFU-f colony after latex ingestion. (A) (160x) Dark cells (arrow) have ingested latex particles. (B) (1000x) Arrows indicate cells which have ingested latex.* **(C)** *(200x) Alkaline phosphatase-positive cells in a colony of stromal cells.* **(D)** *(200x) Acid phosphatase-positive cells in a colony of stromal cells.* **(E)** *(400x) High iron diamine staining of a confluent stromal layer indicating the presence of S-GAGs.* **(F)** *(400x) Alcian blue staining of a confluent stromal layer indicating the presence of GAGs.* **(G)** *(1000x) Myeloperoxidase-positive cells present on top of a confluent adherent stromal layer.*

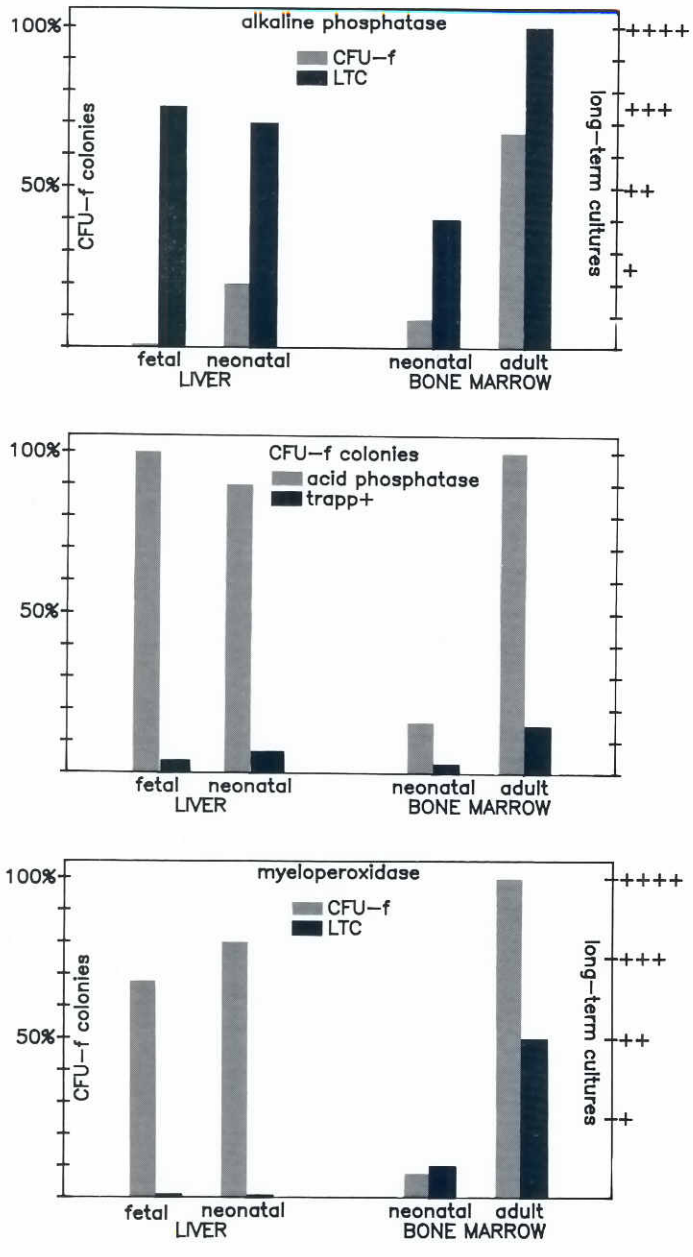

Fig. 3. Characterization of stromal cells grown from fetal liver, neonatal liver, neonatal and adult bone marrow. *The number of colonies containing alkaline phosphatase, acid phosphatase, and myeloperoxidase-positive cells is expressed as a percentage of the total number of colonies examined. Relative amounts for the presence of alkaline phosphatase and myeloperoxidase positive cells in the adherent layers of long-term cultures are shown.*

TRAP-positive cells. Positive cells showed no fibroblastic morphology and were scattered throughout the colony.

Presence of GAGs in the colonies

Results are shown in Fig. 4.

Bone marrow cultures (adult and neonatal) contained, apart from negative colonies, only colonies which after the combined staining of alcian blue and high iron diamine were positive for both S-GAGs (purple) and non-S-GAGs (blue). In liver cultures, 4 types of colonies were present: blue stained, purple stained, mixed colonies (stained blue and purple) and negative colonies. More than half of the CFU-f colonies originating from fetal liver (69%) and adult bone marrow (87%) contained GAGs. Both liver (29%) and bone marrow (8%) of 2-day-old mice showed a considerable number of colonies positive for the presence of GAGs.

In fetal liver, more colonies stained positive for non-S-GAGs than for S-GAGs. In neonatal liver and bone marrow, the presence of sulfated and non-sulfated GAGs in CFU-f colonies was comparable. In adult bone marrow the mixed colonies stained stronger for S-GAGs (purple) than for non-S-GAGs (blue).

Myeloperoxidase (MP)-positive colonies

Results are represented in Fig. 3.

Myeloperoxidase is an indicator for the presence of granulocytic cells in the adherent layer. The number of MP-positive cells per colony was very low and MP-positive cells were mainly present in the central part of the colony. Only a few (8%) CFU-f colonies from neonatal bone marrow contained MP-positive cells, while all CFU-f colonies (100%) from adult bone marrow contained MP-positive cells.

A large proportion of fetal liver and neonatal liver colonies contained MP-positive cells (respectively 68% and 80%).

Confluent stromal layer

Confluent stroma layers in cultures of different origins were evaluated. Stromal layers were compared with each other for the presence of a given component. Results were expressed in relative amounts.

Macrophages

Fetal liver stroma and adult bone marrow stroma contained high amounts of macrophages (Fig. 1). Considerably fewer macrophages could be detected in liver and bone marrow stromal layers from neonates. The macrophages in adult bone marrow LTC had a colony-like distribution whereas in the LTC of other organs the macrophages were dispersely distributed.

Alkaline phosphatase (ALP)-positive cells

Many focal areas with intensely stained ALP-positive cells were present in adult bone marrow adherent stromal layer. Fetal and neonatal liver stroma displayed a disperse distribution of ALP-positive cells with a low intensity. Neonatal bone marrow stroma contained some small foci of ALP-positive cells with moderate intensity (Fig. 3).

Presence of GAGs

Fetal liver and adult bone marrow stromal layers contained a large number of GAGs (Fig. 2E and F), while neonatal liver and bone marrow stroma expressed lower quantities of this ECM component (Fig. 4). GAG-positive areas were correlated with areas of high cell density. Similar quantities of sulfated and non-sulfated GAGs were found in the adherent layers.

Presence of fibronectin (fn)

Intra- and extracellular fn was determined in the adherent layer of LTC using PAP-immunohistochemistry (qualitative method) and ELISA (quantitative method) (Fig. 5). The latter technique was also

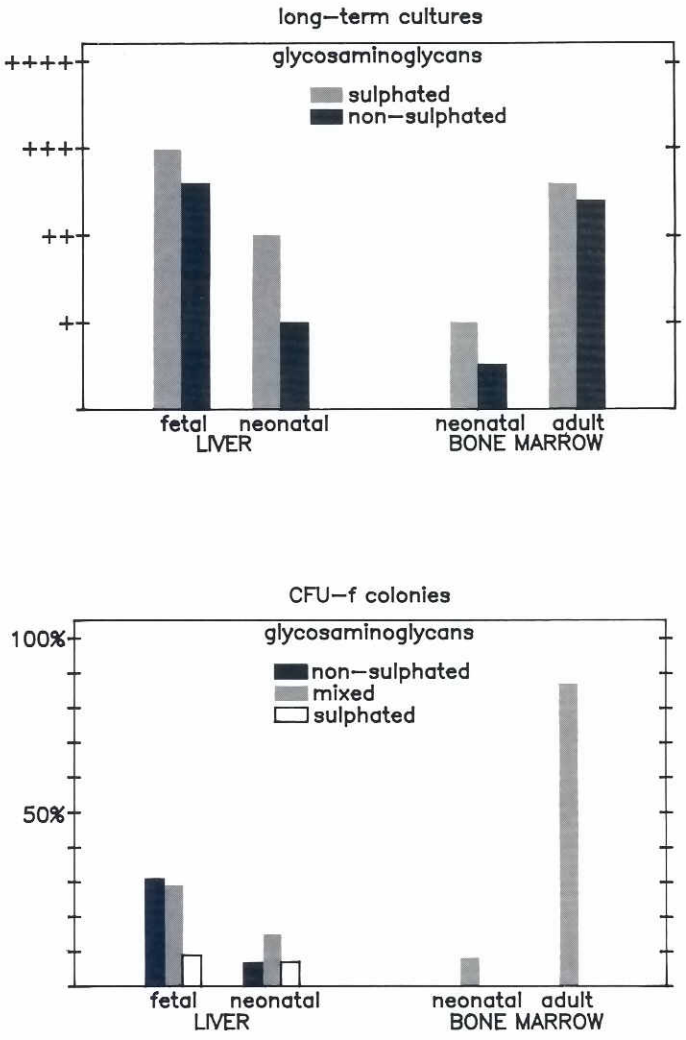

Fig. 4. Presence of glycosaminoglycans (sulfated and non-sulfated) in fibroblast colonies and stromal cells of long-term cultures derived from different hemopoietic organs. *The number of colonies which stain positive for GAGs is expressed as a percentage of the total colony number. After comparison of long-term cultures from different origin, the presence of GAGs in the adherent layer is expressed in relative amounts.*

used to examine the amount of fn in the liquid phase of the LTC.

Fn was present in all stromal layers regardless of their origin. Both methods showed that extracellular fn was present in higher amounts than intracellular fn. Extracellular fn was present in comparable amounts in the different LTC and was found in the marginal region of the fibroblast-like cells.

Using ELISA, a significant difference in the amount of extracellular fn was seen between fetal liver and 2-day-old liver and between 2-day-old bone marrow and adult bone marrow stroma. Intracellular fn differed (t-test, $P<0.05$) between liver (6±1 ng/well) and bone marrow (12±1 ng/well) LTC, the latter expressing a higher amount. The amount of intracellular fn was not significantly different (t-test, $P>0.05$) between liver cultures at different developmental ages or between adult and neonatal bone marrow cultures.

Fn was present in the liquid phase of the LTC. Neonatal and adult bone marrow cultures produced comparable amounts of soluble fn (respectively 120±50 ng/well and 180±70 ng/well). The amount of fn in fetal liver LTC (100±70 ng/well) was significantly lower (t-test, $P<0.05$) than in neonatal liver cultures (190±90 ng/well).

Myeloperoxidase-positive cells

Fetal liver stroma and liver stroma of 2-day-old mice contained no MP-positive cells. A few MP-positive cells were seen in bone marrow stroma of 2-day-old mice. Adult bone marrow stromal layers contained some colonies of MP-positive cells, present as colonies on top of the stromal adherent cells (Figs. 2G and 3).

Discussion

The CFU-f technique is the nearest approximation to a clonal assay for the early precursors of stromal cells (Owen, 1985). Prolonged cultivation of fibroblast colonies gives rise to confluent stromal layers which are considered responsible for maintaining hemopoietic cells *in vitro* (Dexter *et al.*, 1977).

In the present study, the distribution of certain cell types and extracellular matrix components was measured in the adherent cell layer of hemopoietic active and non-active LTC. The idea was to relate their presence to the hemopoietic activity of the cultures.

All components that have been investigated were present in both liver and bone marrow LTC. Two components showed a positive correlation with the hemopoietic activity of the organ of origin: macrophages and (sulfated as well as non-sulfated) GAGs were present in high numbers in those LTC which were hemopoietically most active (fetal liver and adult bone marrow).

Results indicated a remarkable heterogeneity between CFU-f colonies. The characteristics of individual colonies were preserved after cultures had grown to confluency. GAGs and macrophages were present in higher numbers in those cultures originating from active hemopoietic organs, while this correlation did not hold for ALP-positive cells.

The adherent layer in bone marrow cultures consisted of fibroblastoid cells, fat cells, endothelial cells and macrophages (Dexter *et al.*, 1977). Phagocytosis is an important function of macrophages (Alberts *et al.*, 1983). Macrophages will ingest latex particles supplemented to the cultures. This feature makes it possible to identify these cells by light microscopy. Several investigators distinguished between macrophages and bone marrow CFU-f, which is a non-phagocytic cell (Friedenstein *et al.*, 1970; Castro-Malaspina *et al.*, 1980)., on the basis of phagocytic capacity (Allen and Dexter, 1976; Castro-Malaspina *et al.*, 1980; Brockbank and van Peer, 1983; Song and Quesenberry, 1984; Zipori *et al.*, 1984; Rich, 1986a).

In this report we used phagocytosis as a criterion to detect macrophages in stromal cultures.

Macrophages in the long-term cultures were not of stromal origin, but can be considered as a «functional» part of the adherent microenvironment. They appear to be involved in granulopoiesis by their association with blanket cells just before these regions become granulopoietically active, and they are also involved in red cell maturation in cultures stimulated for erythropoiesis (Allen and Dexter, 1984) and in intact marrow (Westen and Bainton, 1979). Evidence exists that macrophages can produce a variety of soluble regulatory molecules. Bone marrow-derived macrophages are functionally capable of producing a colony-stimulating activity that promotes the formation of granulocyte and/or macrophage colonies.

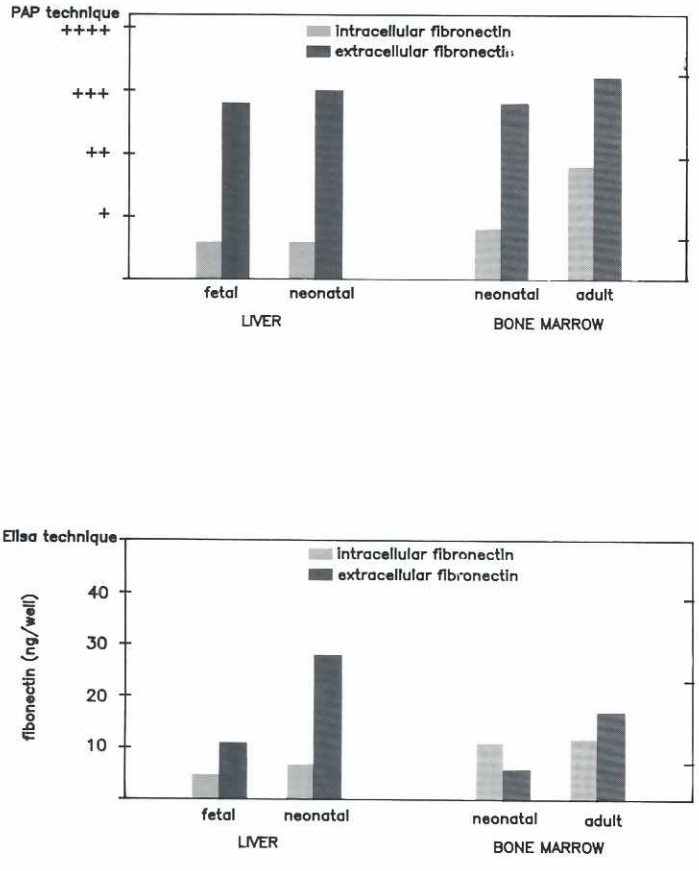

Fig. 5. The presence of intra- and extracellular fibronectin in LTC *is shown using* **(A)** *the PAP-technique: the presence of fibronectin is expressed in relative amounts;* **(B)** *the ELISA technique: the amount of fibronectin is expressed in ng per well.*

They also produce erythropoietin and burst-promoting activity (BPA) that stimulates the proliferation of early erythroid progenitor cells (BFU-E). Macrophages have also been shown to produce factors, monokines (interleukin-1 and TNF-alpha) that can stimulate other cells including endothelial cells, skin fibroblasts, marrow fibroblasts and T-lymphocytes, to produce growth factors (GM-CSF, BPA and megakaryocyte CSA) (Broudy *et al.*, 1986; Rich 1986a, b; Bagby *et al.*, 1987; Sieff *et al.*, 1987). Oblon *et al.*, (1983) fractionated the adherent layer in LTC and found that the fraction enriched with monocytes/macrophages produced GM-CSF.

Macrophages were abundantly present in hemopoietic active organs like fetal liver and adult bone marrow. Liver and bone marrow of 2-day-old mice contained considerably fewer phagocyting cells. The functional role of macrophages in our cultures is not known but these observations might be an indication of the importance of macrophages in the regulation of hemopoiesis.

The presence of GAGs in the adherent layer of human (Keating and Singer, 1983; Wight *et al.*, 1986; Gordon *et al.*, 1987) and murine (Gallagher *et al.*, 1983; Spooncer *et al.*, 1983; Roberts *et al.*, 1988) bone marrow LTC has been demonstrated. In murine LTC, heparan sulfate (sulfated GAG) is mainly associated with the adherent cells, while hyaluronic acid (non-sulfated GAG) and chondroitin sulfate (sulfated GAG) are mainly found in the culture medium (Gallagher *et al.*, 1983).

Experimental evidence exists for a role of the GAGs in the regulation of hemopoiesis. Hemopoietic progenitor-cell proliferation occurs in close association with the stromal layer in LTC (Del Rosso *et al.*, 1981; Gordon *et al.*, 1987).

The possible function of GAGs in hemopoiesis is the ability to retain growth factors synthesized locally by stromal cells or produced elsewhere and to present the growth factors in biologically active form to hemopoietic cells. Gordon *et al.* (1987) demonstrated in human bone marrow cultures that exogenous GM-CSF binds predominantly to hyaluronic acid and biologically active GM-CSF can be extracted from the ECM of cultured stromal layers. Similarly, in mouse marrow stroma, heparan sulfate possesses the ability to adsorb both GM-CSF and interleukin-3 and retain its biological activity (Roberts *et al.*, 1988). In contrast GAGs from cultured human fetal liver cells (liver at the 18th week of gestation) were ineffective in removing growth factors. At this developmental age, human liver is predominantly erythropoietic (Gordon *et al.*, 1987).

Stimulation of chondroitin sulfate (present in the culture medium) synthesis with beta-D-xylosides (Nagasaka *et al.*, 1989) increases the production of hemopoietic stem, progenitor and differentiated cells in the non-adherent fraction (Spooncer *et al.*, 1983).

The presence of fibronectin and alkaline phosphatase activity was not correlated with the hemopoietic activity of an organ. Evidence is available that fibronectin is present at adhesion sites of maturing granulocytes and monocytes to the stromal cells (Bentley and Tralka, 1983) and that erythroid progenitors bind strongly whereas CFU-GM progenitors bind weakly to fibronectin (Tsai *et al.*, 1987). Our finding that extracellular fibronectin is present in higher amounts than intracellular fibronectin is in agreement with results of Zuckerman and Wicha (1983). They could not detect any intracellular fibronectin in stromal cells of 4-week-old BM LTC. The presence of intracellular fibronectin in our adult BM LTC, although low, could be the result of a higher sensitivity of our method. A cell-free three-dimensional fibronectin matrix is unable to support *in vitro* hemopoiesis. Whether fibronectin is an essential component for hemopoietic maintenance or a common matrix component of cultured stromal cells needs further investigation.

Fibronectin is present in lower amounts in stromal layers from fetal liver and neonatal bone marrow than in 2-day-old liver and adult bone marrow. The diminished yield of these components corresponds with periods of a high proliferation capacity of the stromal cells (Versele *et al.*, 1987). Perhaps stromal cells from neonatal liver and adult bone marrow which exhibit a lower proliferation activity, tilt the balance towards differentiation and start to produce «excessive» proteins. High levels of fibronectin might be a sign of a more advanced differentiation.

The prominent cell type in the adherent layer is the stromal fibroblast-like cell (Dexter *et al.*, 1977). Alkaline phosphatase (ALP) is present in this cell type and is associated with granulocytic activity (Wilson *et al.*, 1981; Tavassoli *et al.*, 1983; Gualtieri *et al.*, 1984; Broudy *et al.*, 1986; Liesveld *et al.*, 1989). Our results do not show a clear correlation between ALP activity and the hemopoietic activity of an organ.

In the CFU-f colonies, the ALP activity is inversely related to the proliferation capacity of the stromal cells. Comparable with fibronectin, the presence of ALP might be related to the state of maturation of the cells. The observation that ALP was absent in fetal

liver CFU-f colonies but present in fetal liver LTC gives evidence that the differentiation state of the stromal cells might be involved.

The results indicate that in each of the considered organs the fibroblast-like cells form a heterogeneous population containing ALP-positive and -negative cells. This heterogeneity was also found when the distribution of the ALP-positive cells is compared between stromal layers of different origin.

The presence of ALP is often associated with osteoblasts during osteogenesis. The presence of ALP activity in liver cultures shows that ALP also has other biological functions. The presence of ALP in cells from fetal liver and fetal bone marrow has been described previously (Ahmed and Bainton, 1986).

CFU-f colonies were also checked for the presence of acid phosphatase activity. The high occurrence of acid phosphatase in liver colonies might be related to the relatively higher amounts of macrophages in liver cultures compared with bone marrow and to the erythropoietic function of the liver. The former was also observed by Riley and Gordon (1987). Piersma *et al.*, (1985) demonstrated that the ACP activity in fibroblastic cells in CFU-f colonies from adult bone marrow was very weak or absent. In contrast, we found strong acid phosphatase activity in non-phagocytic fibroblast-like cells from adult bone marrow. Positive staining for ACP has also been described by others (Gualtieri *et al.*, 1984; Broudy *et al.*, 1986). Differences in culture conditions might explain the difference.

The presence of myeloperoxidase (MP)-positive cells indicates the presence of hemopoietic cells adhering to the stromal layer.

Only the adherent layer of adult bone marrow LTC contained MP-positive cells, although differentiated granulocytes (demonstrated by May-Grünwald-Giemsa staining of cytospin) were found in the non-adherent fraction of all LTC. Perhaps mature cells are more easily released in the other cultures due to differences in the stromal layer.

In contrast with the LTC, CFU-f colonies derived from the liver contained MP-positive cells. Perhaps these cells were brought into the culture when cultures were initiated and survived during the short culture period.

In this study, stromal cultures (CFU-f and LTC) from liver and bone marrow, corresponding to different hemopoietic activities, were used to investigate the importance of certain cell types and extracellular matrix components in the hemopoietic process.

Results indicated that culture conditions for CFU-f colony growth select a stromal cell population, the characteristics of which are maintained in the confluent stromal layers which support hemopoiesis *in vitro*.

Two components showed a positive correlation with the hemopoietic activity: macrophages and GAGs (sulfated and non-sulfated) were present in high amounts in fetal liver and adult bone marrow. In contrast, the presence of fibronectin and alkaline phosphatase was assumed to be related to the differentiation or proliferation stage of the stromal cells.

Our model using hemopoietic active and non-active cultures proved to be useful in the study of the stromal micro-environment. Further investigations are needed to improve our knowledge of the specific role of each component in hemopoiesis.

Materials and Methods

Mice

BALB/c mice from SCK/CEN breeding were used. Mice were mated in the evening. The next day is defined as day zero of gestation.

Collection of cells

Fetal mice at 13 days of gestation, 2-day-old neonatal mice and adult mice (12 weeks old) were used. Adult mice were killed by cervical dislocation and young mice with chloroform. The hemopoietic organs of interest were dissected out. Cell suspensions were prepared from fetal liver, neonatal liver and bone marrow and adult bone marrow (Van Den Heuvel *et al.*, 1987).

CFU-f: short-term clonal assays from stromal cells

This technique has previously been described in detail (Van Den Heuvel *et al.*, 1987).

Briefly, bone marrow or liver cells were suspended in alpha-MeM supplemented with 10% horse serum, 10% fetal calf serum and 100 µg/ml gentamicin (all from Gibco). 1 ml aliquots containing $2x10^6$ cells were inoculated in 35 mm petri dishes.

Replicate cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere for 10 days. Cultures were fed twice by total replacement of the medium.

Long-term cultures

Cells were inoculated ($2x10^6$ cells/ml/well) in 24-multiwell petri dishes. Alpha-MeM medium was used enriched with 10% horse serum, 10% fetal calf serum, 1% L-glutamine, 100 µg/ml gentamicin and 10^{-6} M hydrocortisone sodium hemisuccinate (all from Gibco, Belgium) (Van Den Heuvel *et al., 1988).*

Cultures were grown in a humidified 7% CO_2 atmosphere at 33°C. At weekly intervals the medium was completely removed and replaced. At 4 weeks of culture, confluency of the adherent layer was reached and the adherent cells were examined.

Cytochemical studies

Stromal CFU-f colonies and confluent adherent layers were examined for several cell types and some extracellular matrix components.

Macrophages

Macrophages could be functionally discriminated from fibroblastoid cells on the basis of phagocytic capacity. One hour prior to fixation latex particles (Dow Latex, 0= 0.81 µm) (15 µl latex solution/ml culture medium) were added to the cultures. Cultures were further incubated at 33°C or 37°C, washed several times with alpha-MeM and stained with May-Grünwald-Giemsa (Castro-Malaspina *et al.*, 1980; Wilson *et al.*, 1981). After incubation only a few particles were associated with fibroblastoid cells, while macrophages contained abundant latex.

Alkaline phosphatase

Cultures were fixed with cold 4% formaldehyde in absolute methanol (min). Adherent cells were stained for the presence of alkaline phosphatase using naphtol AS-BI alkaline and fast blue BB base (Sigma kit nº 86).

Acid phosphatase

Adherent stromal cells were stained for the presence of acid phosphatase (Sigma kit nº 387). The presence of two isoenzymes was separately investigated: tartrate acid-sensitive (TRAP) and resistant phosphatase. Cells which stain in the presence of tartrate (TRAP-positive cells) may be related to osteoclasts. This isoenzyme is characteristic for osteoclasts in the mouse (Van de Wijngaert, 1986).

Sulfated (S-GAGs) and non-sulfated glycosaminoglycans (non-S-GAGs)

Cultures were fixed with a solution of 4% para-formaldehyde and 0.2% sodium acetate. A Feulgen hydrolysis (1 N HCl, 60°C, 10 min) excludes possible DNA interference. Cultures were incubated for 18 h in a mixture of the meta- and para-isomeres of N,N-dimethyl-phenyleendiamine, which stains the sulfated GAGs. Subsequent staining for both sulfated and non-sulfated GAGs with Alcian Blue 8GX (3%) (pH 2.3) distinguished between sulfated (purple) and non-sulfated GAG's (light blue) (Scott *et al.*, 1964; Spicer, 1965). Positive controls involved the staining of femur ends of mice. GAGs have been demonstrated in bone marrow stroma in situ (Oguri *et al.*, 1987; Gordon, 1988).

Fibronectin

Adherent layers were immunohistochemically stained for intracellular and extracellular fibronectin (fn) by the 3-step peroxidase-anti-peroxidase (PAP) technique.

The medium was decanted from the confluent cultures and the adherent layers washed 3 times in PBS (Gibco). To demonstrate intracellular fn, cultures were fixed with ice-cold absolute methanol (1 min). To demonstrate extracellular fn, cultures were fixed by incubation with 4% paraformaldehyde for 15 min at room temperature. After fixation cultures were washed 3 times in PBS. Endogenous peroxidase activity was destroyed by incubation for 20 min with 0.3% hydrogen peroxide (H_2O_2) in PBS and cultures were washed again.

Rabbit anti-fn IgG was kindly provided by Prof. Dr. Foidart. The second and third antibody, goat anti-rabbit IgG and the rabbit antiperoxidase-peroxidase antisera were obtained commercially from Nordic. Optimal antibody concentrations were determined using serial dilutions. Adult bone marrow stroma, which contains fn (Bentley and Tralka, 1983) was used as a positive control. Empty dishes without adherent cells were used as negative control. The cells were washed (3x15 min) with PBS between antibody incubations. The presence of fn was visualized after the addition of 3.3'-diaminobenzidine tetrahydrochloride (0.2 mg/ml) in 0.03M H_2O_2 (15 min at room temperature) which is converted by the peroxidase reaction into a brown insoluble precipitate.

Quantitative amounts of intracellular, extracellular and soluble fibronectin were measure via inhibition ELISA. The method used is a modification of the procedure described by Rennard (1980, 1982) and Tyssen (1985). Briefly, 100 ng human plasma fibronectin was adsorbed in a 96-well plate. A standard concentration of fn and the various adherent layers of LTC were incubated with rabbit anti-fn serum. Afterwards, the supernatants containing unbound antibodies were added to the fibronectin-containing wells. Goat anti-Rabbit IgG, Rabbit antiperoxidase-peroxidase and orthophenylendiamine reagent (490nm) were added. With a photospectometer (490 nm) the number of antibodies which bound to the fibronectin in the coated wells could be measured and was thus inversely related to the amount of fibronectin antigen present in the adherent layers of the LTC.

Myeloperoxidase

Cultures were fixed with cold (4°C) glutaraldehyde-aceton for 1 min and stained for the presence of myeloperoxidase (Yam, 1971) (Sigma kit nº 391).

Quantification of results

Using CFU-f colonies, quantification of a feature is possible by counting the number of positive and negative colonies.

Using confluent stromal layers, long-term cultures from different organs are compared to one another. A feature is expressed then in relative amounts, indicated with +.

+: a small number of positive cells or staining of a small area
++: a moderate number of positive cells or staining of a moderate area
+++: a large number of positive cells or staining of a large area
++++: a very large number of positive cells or staining of a very large area

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References

AHMED, L.A. and BAINTON, D.F. (1986). Presence of alkaline-phosphatase-positive reticulum cells in fetal liver and bone marrow of mice and their absence in yolk sac. *Exp. Hematol. 14:* 705-709.

ALBERTS, B., BRAY, D., LEWIS, J., RAFF, M., ROBERTS, K. and WATSON, J.D. (1983). Membrane transport of macromolecules and particles: exocytosis and endocytosis. In *The Molecular Biology of the Cell.* Garland Publishing, Inc., New York & London, pp. 311-312.

ALLEN, T.D. and DEXTER, T.M. (1976). Cellular interrelationships during *in vitro* granulopoiesis. *Differentiation 6:* 191-194.

ALLEN, T.D. and DEXTER, T.M. (1984). The essential cells of the hemopoietic microenvironment. *Exp. Hematol. 12:* 517-521.

BAGBY, G.C. (1987). Production of multilineage growth factors by hematopoietic stromal cells: an intracellular regulatory network involving mononuclear phagocytes and interleukin-1. *Blood Cells 13:* 147-159.

BENTLEY, S.A. and TRALKA, T.S. (1983). Fibronectin-mediated attachment of hematopoietic cells to stromal elements in continuous bone marrow culture. *Exp. Hematol. 11:* 129-138.

BROCKBANK, K.G.M. and VAN PEER, C.M.J. (1983). Colony-stimulating activity production by hemopoietic organ fibroblastoid cells *in vitro. Acta Haematol. 69:* 369-375.

BROUDY, V.C., ZUCKERMAN, K.S., JETMALANI, S., FITCHEN, J.H. and BAGBY, G.C. (1986). Monocytes stimulate fibroblastoid bone marrow stromal cells to produce multilineage hematopoietic growth factors. *Blood 68:* 530-534.

CASTRO-MALASPINA, H., GAY, R.E., RESNICK, G., KAPOOR, N., MEYERS, P., CHIARIERI, D., McKENZIE, S., BROXMEYER, H.E. and MOORE, M.A.S. (1980). Characterization of human bone marrow fibroblast colony-forming cells (CFU-f) and their progeny. *Blood 56:* 289-301.

CRITCHLEY, D.R. ENGLAND, M.A., WAKELEY, J. and HYNES, R.O. (1979). Distribution of fibronectin in the ectoderm of gastrulating chick embryos. *Nature 280:* 498-500.

DEL ROSSO, M., CAPPELLETTI, R., DINI, G., FIBBI, G., VANNUCCHI, S., CHIARUGI, V. and GUAZELLI, C. (1981). Involvement of glycosaminoglycans in detachment of early myeloid precursors from bone marrow stromal cells. *Biochim. Biophys. Acta 676:* 129-136.

DEXTER, T.M., ALLEN, T.D. and LAJTHA, L.E. (1977). Conditions controlling the proliferation of haemopoietic stem cells *in vitro. J. Cell. Physiol. 91:* 335-344.

DEXTER, T.M., HEYWORTH, C. and SPOONCER, E. (1977). Studies of the self-renewal and differentiation of stem cells in long-term marrow cultures. In: *Hematopoietic Stem Cell Physiology* (Eds. E.P. Cronkite, N. Dainiak, R.P. McCaffrey, J. Palek and P.J. Quesenberry). Alan R. Liss, Inc., New York, pp. 235-246.

FRIDMAN, R., ALAN, Y., DOLJANSKI, F., FUKS, Z. and VLODARSKY, I. (1985). Cell interaction produced by endothelial cells and fibroblasts. *Exp. Cell Res. 158:* 461-476.

FRIEDENSTEIN, A.J., CHARLAKHYAN, R.K. and LALYKINA, K.S. (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet. 3:* 393-403.

GALLAGHER, J.T., SPOONCER, E. and DEXTER, T.M. (1983). Role of the cellular matrix in haemopoiesis. I. Synthesis of glycosaminoglycans by mouse bone marrow cell cultures. *J. Cell Sci. 63:* 155-171.

GORDON, M.Y. (1988). Extracellular matrix of the marrow microenvironment. *Br. J. Haematol. 70:* 1-4.

GORDON, M.Y., RILEY, G.P., WATT, S.M. and GREAVES, M.F. (1987). Compartmentalization of a haemopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. *Nature 326:* 403-405.

GUALTIERI, R.J., SHADDUCK, R.K., BAKER, D.G. and QUESENBERRY, P.J. (1984). Hematopoietic regulatory factors produced in long-term murine bone marrow cultures and the effect of *in vitro* irradiation. *Blood 64:* 516-525.

HOCKING, W.G. and GOLDE, D.W. (1980). Long-term human bone marrow cultures. *Blood 56:* 118-124.

HYNES, R.O. and YAMADA, K.M. (1982). Fibronectin: multifunctional modular glycoproteins. *J. Cell Biol. 95:* 369-377.

KEATING, A. and SINGER, J.W. (1983). Further characterization of the *in vitro* hemopoietic microenvironment. *Exp. Hematol. 11:* 144.

KLEIN, A.K., DYCK, J.A., STITZEL, K.A., SHIMIZU, J., FOX, L.A. and TAYLOR, N. (1983). Characterization of canine fetal lymphohematopoiesis: studies of CFU-GM, CFU-L and CFU-F. *Exp. Hematol. 11:* 263-274.

LIESVELD, J.L., ABBOUD, C.N., DUERST, R.E., RYAN, D.H., BRENNAN, J.K. and LICHTMAN, M.A. (1989). Characterization of human marrow stromal cells: role in progenitor cell binding and granulopoiesis. *Blood 73:* 1794-1800.

METCALF, D. and MOORE, M.A.S. (1971). Embryonic aspects of haemopoiesis. In *Hemopoietic Cells* (Eds. A. Neuberger and E.L. Tatum). North Holland Research Monographs, Amsterdam, pp. 171-271.

NAGASAKA, T., SOBUE, M., NIWA, M., YASUI, C., NARA, Y., FUKATSU, T., NAKASHIMA, N. and TAKEUCHI, J. (1989). Effects of beta-D-xylosides on proliferation and matrix formation of adherent fibroblastic cells in mouse bone marrow culture. *Exp. Hematol. 17:* 923-928.

OBLON, D.J., CASTRO-MALASPINA, H. and BROXMEYER, H.E. (1983). The production of colony stimulating activity by monocyte enriched fractions from murine continuous bone marrow culture adherent layers. *Br. J. Haematol. 54:* 291-299.

OGURI, K., OKAYAMA, E., CATERSON, B. and UKAYAMA, M. (1987). Isolation, characterization and localization of glycosaminoglycans in rabbit bone marrow. *Blood 70:* 501-510.

OWEN, M. (1985). Lineage of osteogenic cells and their relationship to the stromal system. In *Bone and Mineral Research* (Ed. W.A. Peck). Elsevier Science Publishers, Amsterdam, pp. 1-25.

PIERSMA, A.H., BROCKBANK, K.G.M., PLOEMACHER, R.E., VAN VLIET, E., BRAKEL-VAN PEER, K.M.J. and VISSER, P.J. (1985). Characterization of fibroblastic stromal cells from murine bone marrow. *Exp. Hematol. 13:* 237-243.

REINCKE, U., HSIEH, P., MAUCH, P., HELLMAN, S. and BO CHEN, L. (1982). Cell types associated with fibronectin in long-term mouse bone marrow cultures. *J. Histochem. Cytochem. 30:* 235-244.

RENNARD, S.L., BERG, M., MARTIN, G.R., FOIDART, J.M. and GEHRON ROBEY, P. (1980). Enzyme-linked immunoassay (ELISA) for connective tissue components. *Anal. Biochem. 104:* 205-214.

RENNARD, S.L. MARTIN, G.R. and CRYSTAL, R.G. (1982). Enzyme-linked immunoassay (ELISA) for connective tissue proteins: type I collagen. In *Immunohistochemistry of the Extracellular Matrix*, Vol. I (Ed. H. Furthmayer). CRC Press Inc., Florida, pp. 237-252.

RICH, I.N. (1986a). A role for the macrophage in normal hemopoiesis. I. Functional capacity of bone marrow derived macrophages to release hemopoietic growth factors. *Exp. Hematol. 14:* 738-745.

RICH, I.N. (1986b). A role for the macrophage in normal hemopoiesis. II. Effect of varying physiological oxygen tensions on the release of hemopoietic growth factors from bone marrow derived macrophages *in vitro*. *Exp. Hematol. 14:* 746-751.

RICH, I.N. and KUBANEK, B. (1985). The central role of the macrophage in hemopoietic microenvironmental regulation. In *Hematopoietic Stem Cell Physiology* (Eds. E.P. Cronkite, N. Dainiak, R.P. McCaffrey, J. Palek and P.J. Quesenberry). Alan R. Liss, Inc., New York, pp. 283-298.

RILEY, P. and GORDON, M.Y. (1987). Characterization of culture stromal layers derived from fetal and adult hemopoietic tissues. *Exp. Hematol. 15:* 78-84.

ROBERTS, R., GALLAGHER, J., SPOONCER, E., ALLEN, T.D., BLOOMFIELD, F. and DEXTER, T.M. (1988). Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. *Nature 332:* 376-378.

SCOTT, J.E., QUINTARELLI, G. and DELLOVO, M.C. (1964). The chemical and histochemical properties of alcian blue. I. The mechanism of alcian blue staining. *Histochemie 4:* 73-85.

SIEFF, C.A., NIEMEYER, C.M. and FALLER, D.V. (1987). The production of hematopoietic growth factors by endothelial accessory cells. *Blood Cell 13:* 65-74.

SONG, Z.X. and QUESENBERRY, P.J. (1984). Radioresistant murine marrow stromal cells: a morphologic and functional characterization. *Exp. Hematol. 12:* 523-533.

SPICER, S.S. (1965). Diamine methods for differentiating mucosubstances histochemically. *J. Histochem. Cytochem. 13:* 211-234.

SPOONCER, E., GALLAGHER, L.T., KRIZSA, F. and DEXTER, T.M. (1983). Regulation of haemopoiesis in long-term marrow cultures. IV. Glycosaminoglycan synthesis and the stimulation of haemopoiesis by beta-D-xylosides. *J. Cell Biol. 96:* 510-514.

SULLIVAN, R., LIPKIN, E.W., BELL, R., LARSEN, N.E. and McCARROLL, L.A. (1985). The kinetics of the production of granulocyte-monocyte colony stimulating activity (GM-CSA) by isolated human monocytes: response to bacteria endotoxin. In *Hematopoietic Stem Cell Physiology* (Eds. E.P. Cronkite, N. Dainiak, R.P. McCaffrey, J. Palek and P.J. Quesenberry). Alan R. Liss Inc., New York, pp. 173-187.

TAVASSOLI, P. and FRIEDENSTEIN, A. (1983). Hemopoietic stromal microenvironment. *Am. J. Hematol. 15:* 195-203.

TYSSEN, P. (1985). Practice and theory of enzyme immunoassays. In: *Laboratory Techniques in Biochemistry and Molecular Biology* (Eds. R.H. Burdon and P.H. Van Knippenberg). Elsevier, Amsterdam, pp. 449-487.

TSAI, S., PATEL, V, BEAUMONT, E., LODISH, H.F., NATHAN, D.G. and SIEFF, C.A. (1987). Differential binding of erythroid and myeloid progenitors to fibroblasts and fibronectin. *Blood 69:* 1587-1594.

VAN DEN HEUVEL, R.L., SCHOETERS, G.E.R. and VANDERBORGHT, O.L.J. (1988). Haemopoiesis in long-term cultures of liver, spleen and bone marrow of pre-and postnatal mice: CFU-GM production. *Br. J. Haematol. 70:* 273-277.

VAN DEN HEUVEL, R.L., VERSELE, S.R.M., SCHOETERS, G.E.R. and VANDERBORGHT, O.L.J. (1987). Stromal stem cells (CFU-f) in yolk sac, liver, spleen and bone marrow of pre- and postnatal mice. *Br. J. Haematol. 66:* 15-20.

VAN DE WIJNGAERT, F.P. and BURGER, E.H. (1986). Demonstration of tartrate-resistant acid phosphatase in un-decalcified, glycolmethacrylate-embedded mouse bone: a possible marker for (pre) osteoclast identification. *J. Histochem. Cytochem. 34:* 1317-1323.

VERSELE, S.R.M., VAN DEN HEUVEL, R.L., SCHOETERS, G.E.R. and VANDERBORGHT, O.L.J. (1987). Proliferation of stromal stem cells (CFU-f) from hemopoietic organs of pre- and postnatal mice. *Radiat. Res. 111:* 185-191.

WESTEN, H. and BAINTON, D.F. (1979). Association of alkaline-phosphatase-positive reticulum cells in bone marrow with granulocytic precursors. *J. Exp. Med. 150:* 919-937.

WIGHT, T.N., KINSELLA, M.G., KEATING, A. and SINGER, J.W. (1986). Proteoglycans in human long-term bone marrow cultures: biochemical and ultrastructural analyses. *Blood 67:* 1333-1343.

WILSON, F.D. TAVASSOLI, M., GREENBERG, B.R., HINDS, D. and KLEIN, A.K. (1981). Morphological studies on adherent cells in bone marrow cultures from humans, dogs and mice. *Stem Cells 1:* 15-29.

YAM, L.T., LI, C.Y. and CROSBY, W.H. (1971). Cytochemical identification of monocytes and granulocytes. *Am. J. Clin. Path. 55:* 283-290.

YAMADA, K.M. (1983). Cell surface interactions with extracellular materials. *Ann. Rev. Biochem. 52:* 761-799.

YAMADA, K.M. and OLDEN, K. (1978). Fibronectin-adhesive glycoproteins of cell surface and blood. *Nature 275:* 179-184.

ZIPORI, D., FRIEDMAN, A., TAMIR, M., SILVERBERG, D. and MALIK, Z. (1984). Cultured mouse marrow cell lines: interactions between fibroblastoid cells and monocytes. *J. Cell. Physiol. 118:* 143-152.

ZUCKERMAN, K.S. and RHODES, R.K. (1985). The hematopoietic extracellular matrix. In *Hematopoietic Stem Cell Physiology* (Eds. E.P. Cronkite, N. Dainiak, R.P. McCaffrey, J. Palek and P.J. Quesenberry). Alan R. Liss, Inc., New York, pp. 257-266.

ZUCKERMAN, K.S. and WICHA, M.S. (1983). Extracellular matrix production by the adherent cells of long-term murine bone marrow cultures. *Blood 61:* 540-547.

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