OSTEOGENESIS AND HAEMOPOIESIS: A SYMBIOTIC MICROENVIRONMENT

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SUMMARY – Haemopoiesis is an important physiological event, in which proliferation, differentiation and interaction of many cell types are involved. Neutrophils, erythrocytes, platelets, eosinophils, basophils and lymphocytes are highly specialized cells present in the blood, body tissues and lymphoid system. The majority of myeloid cells have only a short life-span and must be continuously replaced by proliferation of stem cells residing in the bone marrow stromal system which is analogous to the haemopoietic system due to giving rise of stromal cell to committed progenitors of different cell lines. They include adipocytic, reticular, endothelial, osteogenic and fibroblastic cell lines. The integrity of haemopoietic-stromal system is maintained by extracellular matrix (laminin, collagens, proteoglicans and fibronectin) and factors (colony stimulating factors, CSF; interleukins 1, 2, 3, 4, 5, 6, IL-1, 2, 3, 4, 5, 6; tumor necrosis factor, TNF; tumor growth factor, TGF; stem cell factor, SCF; fibroblast growth factor FGF; and platelet derived growth factor, PDGF). Outside this surroundings, protected status of stem cells is lost.

Key words: osteogenesis, bone marrow stromal cells, haemopoiesis, interleukins, colony stimulating factors

INTRODUCTION

Haemopoiesis is an important physiological event, in which proliferation, differentiation and interaction of many cell types are involved. In adult mammals, bone marrow is the putative organ of haemopoiesis.

Neutrophils, erythrocytes, platelets, eosinophils, basophils and lymphocytes are highly specialized cells present in the blood, body tissues and lymphoid system. The majority of myeloid cells have only a short life-span and must be continuously replaced by proliferation of stem cells residing in the bone marrow. Within the bone marrow, stem cells are protected from differentiation-inducing stimuli.

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Among external influences maintaining integrity of the system are: extracellular matrix molecules, a variety of stromal cell types and various growth factors (1 - 6). Outside this environment, the protected status is lost and the stem cells cannot undergo differentiation into the various lineages (7).

Whether differentiation is caused by some random event in the genome of a stem cell, or is it determined by the cell/cell interaction or receptor binding of specific molecules that commit a stem cell to one or the other lineages, is the question which waits for clarification.

Access to this problem was opened by purification of several growth factors and molecules against such factors, and the evaluation of cloned lines of marrow stroma. At the same time, Owen (8, 9) has proposed a hypothesis for differentiation in the bone marrow stromal system that is analogous to that in the haemopoietic system where stromal stem cell gives rise to committed progenitors from different cell lines.

Their number and hierarchy, however, is not completely known. They include fibroblastic, reticular, adipocytic, osteogenic and possibly other lines (10 - 12).

Stromal system is part of the microenvironment which has an important role in haemopoiesis and which also includes other cell types, like macrophages and endothelial cells (13). The characteristics and regulating in haemopoiesis of each of these cell types are not fully understood.

STROMAL CELL SYSTEM OF THE BONE MARROW

Evidence exist for the presence of stromal stem cells in the soft connective tissues associated with marrow and bone surfaces which can give rise to a number of different cell lines. The major cells of the stromal fibroblastic system of marrow and bone surface include: osteogenic cells (preosteoblasts, osteoblasts and bone-lining cells), fibroblasts, reticular cells and adipocytes (12). Other candidates of the network are cells of mesenchymal origin which are associated with the walls of small blood vessels including pericytes, smooth muscle cells (14) and spindle-shaped fibroblastic cells called in past undifferentiated mesenchymal cells.

Fibroblastic colonies, each derived from a single colony forming unit fibroblastic (CFU-F), are formed when bone marrow cells are cultured in vitro. Each colony is probably raised from a single cell. Their clonal origin has been proposed on the basis of experiments using thymidine labeling, time-lapse photography and chromosome markers (12). A definite answer to the question of whether stem cells are present in the stroma of bone and marrow must come from an analysis based on differentiation capacity of individual CFU-F.

OSTEOGENIC POTENTIAL OF THE BONE MARROW STROMA

The best characterized cells of the marrow stromal system are osteogenic cells. High levels of alkaline phosphatase activity, synthesis of type I collagen and osteocalcin, and the presence of parathyroid hormone receptors make them all easier to identify (15,16).

Long periods of subculturing stromal cells in vitro and subsequent implantation under the renal capsule or within diffusion chambers result in differentiation of bone, cartilage and conjoined haemopoiesis (17, 18). These studies proved that the
precursor cells determined in an osteogenic direction were present in marrow stroma. Tissue formed within the chambers was generated by a small number of precursor stromal cells with a high capacity for proliferation and differentiation, implying cells with stem cell characteristics (19). The sequential expression of different collagen types, laminin and fibronectin during the development of osteogenic tissue in the diffusion chambers was similar to that seen during embryonic bone and cartilage development or during matrix-induced cartilage, bone and bone marrow formation (20). This suggests that the formation of tissues by precursor cells derived from adult marrow resembles the normal developmental osteogenic process (21). Moreover, clear symbiosis between osteogenic and haematopoietic tissue thus exists, with mutual need for both stromal and haemopoietic cells.

HAEMOPOIESIS IN LONG-TERM BONE MARROW CULTURE (LTBMC)
RESEMBLES IN VIVO EVENTS

Successful tissue culture of long term bone marrow allowed investigations of fine details of the mechanisms of haemopoiesis (22, 23). One of the most significant findings from the studies on mouse long term bone marrow culture (LTBMC) was the functional importance of bone marrow stromal tissue in the growth of developing haemopoietic cells (5).

In the absence of an adherent cell layer, sustained stem cell proliferation would not occur. Only a bone marrow derived stromal adherent cell layer appears to be effective in supporting prolonged haemopoiesis. Adherent cells from the liver, spleen or embryonic fibroblasts did not maintain stem cell proliferation and differentiation (24).

To date, many studies have been performed using various methods to analyze adherent layers in LTBMC. Under appropriate in vitro conditions, adherent cells derived from bone marrow finally contain endothelial cells, adipocytes, macrophages and reticular cells (22, 23, 25). When inoculated with fresh marrow cells continuous stem cell proliferation and differentiation results in the long term production of committed granulocytes/macrophage progenitor cells (GM-CFC) (22, 26), primitive erythroid progenitor cells (BFU-E) (27, 28) megakaryocyte precursors and cells with lymphoid potential (29, 30). At the beginning of the culture (4 days - 10 days), the individual adherent cells were primarily macrophages, sinus endothelia and reticular cells. No adipocytes were apparent and deposition of extracellular matrix was limited.

At 10 days of culture, stromal cell aggregation began to fuse with each other, and blanket cells with widely extended, thin cytoplasmic extensions were apparent (31). In LTBMC extensive extracellular matrix deposition was apparent from day 10 and gradually increased throughout cellular areas of the cultures, characterized as fibronectin, laminin, collagen types I,III,IV and V, and factor VIII rAG (31). However, in vivo basement lamina- like materials are restricted to specific areas, such as those around sinus endothelia, reticular cells, Schwann cells, periarterial adventitial cells and arterial smooth muscle cells (32). Within 2-3 weeks after establishing the primary cultures, the following morphologically distinct cell types could be recognized: endothelial cells, reticular cells with large branching and adipocytes (33). All three cell types usually occur in association with macrophages.

The morphology of the adherent layer is culture medium dependent, e.g., when fetal calf serum is substituted for horse serum, different cells appear. The appearance
of adipocytes correlated with substantial haemopoiesis (34). Indeed, the addition of hydrocortisone to fetal-calf serum supplemented cultures stimulates adipocytes to appear and cell layer becomes competent to support stem cell proliferation and differentiation. In normal conditions of long-term marrow culture, development of alloreactive T-cells did not occur, which means that the cultures can supply a source of stem cells for marrow transplantation in the absence of graft-versus-host disease (34).

The addition of erythropoetin (27) to the cultures will facilitate the progression of the BFU-E to more mature cells, while mechanical shaking (27) or adding anaemic mouse serum to the cultures will lead to the production of haemoglobinizing cells (35). Erythroid cell development in long-term cultures normally proceeds to the level of primitive progenitor cells on days 10 and 14 BFU-E. Cells grow in an intimate membrane contact with macrophages which have also been observed in vivo (36).

There is also an association between lipolysis of the adipocytes from adherent layer and increased erythropoiesis. Developing granulocytes are usually found in association with the adherent layer, and granulopoiesis is especially intense in the areas of adherent layer that are undergoing adipogenesis (33). It has been shown that the stem cells and committed progenitor cells are very similar to the corresponding cells produced in vivo (34), making the long-term culture system representative of haemopoiesis in vivo.

The injection of cultured cells into haemopoietically ablated mice resulted in reconstruction of both myeloid and lymphoid elements. In terms of mature cell proliferation, extensive granulopoiesis occurs so that for each colony forming unit-spleen (CFU-S) there are on an average, 10 GM-CFC, and for each GM-CFC there is the production of 500-1000 mature granulocytes. This is the same order of magnitude as that seen in vivo (34). Moreover, the mature granulocytes produced in long-term cultures appear to be identical with their in vivo produced counterparts (26). While mature (platelet producing) megakaryocytes are also present in culture, terminal maturation during erythroid development can be induced under appropriate circumstances (35). Thus, in vitro and in vivo evidence indicate that stromal cells play a determinative role in haemopoiesis; from the stem cell to the mature end cell.

MECHANISMS OF INTERACTIONS BETWEEN BONE MARROW STROMA, HAEMOPOIETIC CELLS AND BONE CELLS

As described, the proliferation and development of stem cells proceed in close association with marrow stromal cells (4, 6). Membrane complexes found between the adherent cells and the developing haemopoietic cells suggest a mechanism for the regulation through intimate cell/cell contact and the transmembrane passage of appropriate stimulating factors. This intimate cell contact presumably enables stem cells and their progeny to respond to stimuli present on the stromal cell surface. Recently, it was shown that the granulocyte/macrophage colony stimulating factor (GM-CSF) could be adsorbed out of solution by an extract of human marrow stromal extracellular matrix with retention of biological activity (6). Growth factors necessary for haemopoiesis have been synthesized by stromal cells (37) and then transferred to the stromal cell plasma membrane (38). The inability to detect the growth factors as secreted material in the culture medium implies that it must be bound to the surface of stromal cells and is only effective when the stroma and stem cells are in intimate contact
contact. Even metabolically dead stromal cells facilitated the development of attached stem cells into mature progeny (39). As growth factors are not released into the culture medium, Roberts and colleagues (38) have been able to demonstrate that the major sulphated glycosaminoglycan of mouse marrow stroma, heparan sulphate (40), adsorbs both GM-CSF and the multilineage haemopoietic growth factor, interleukin-3 (IL-3). Once bound, this growth factor can be presented in the biologically active form to haemopoietic cells. Thus, the synthesis and secretion of growth factors just as their binding and presentation through extracellular matrix define the regulatory domains in haemopoiesis. Apart from the haemopoietic growth factors, other growth factors like fibroblast growth factor (FGF), tumor growth factor (TGF-), platelet derived growth factor (PDGF) and osteogenin also bind to extracellular matrix suggesting a general mechanism by which matrix-bound regulatory factors (that are not found in the circulation) are protected from proteolytic degradation and in this sense are an efficient natural delivery system in the bone and bone marrow.

The matrix may aid in the local release of these factors in response to physiologic stimuli or during repair and wound healing. The fact that haemopoietic stem cells do not possess their own cell surface heparan sulphate and thus cannot bind growth factors through their surface polysaccharides, explains the importance of contact-dependent physiologic processes. However, these cells do produce chondroitin-4-sulphate proteoglycan (38), but this molecule is unable to bind growth factors.

Other macromolecules, like collagen type I and III, are widely distributed throughout the reticular fibroblastic network and connective tissues of blood vessel wall (41, 42). Type IV collagen and laminin are localized in the walls of small blood vessels and associated with basement membrane (43).

We have recently shown that TGF- and osteogenin bind to type IV collagen, which allows it to serve as an additional important regulatory molecule in the bone marrow cellular interactions (45, 46). It is possible that stromal cells which synthesize these later components, are derived from perivascular mesenchymal cells. There is little understanding of the relationship of perivascular cells to other cells of the stromal system. The physiologic significance of growth factor binding to extracellular matrix is that it results in the formation of a repository of growth factors for the local regulation of cells, haemopoietic as well as osteogenic. Recently, we have shown for the first time that bone forming cells interact with specific molecular domains of laminin from the basement membrane, which could play a key role in the initiation of the osteoblastic lineage from progenitor stem cells (46).

Bone marrow-derived stromal cells and osteoblasts influence the proliferation, differentiation and recruitment of osteoclast progenitors (47). IL-6 plays an important role in this process (48). System hormones and paracrine cytokines that promote bone resorption stimulate production of IL-6 by bone marrow stroma and osteoblasts. These include parathormon (PTH), IL-1 and TNF (48, 49). IL-1 and TNF induce IL-6 production by murine stromal cells and osteoblasts which could be inhibited by 17 beta-estradiol (49). Loss of estrogens in mice caused an increased osteoclast number and could be prevented by estrogen replacment and by an IL-6 neutralizing antibody both in vivo (49) and in vitro (47). There is also evidence that IL-6 is produced locally by human osteoblasts under the direction of other cytokines, which could have implications in bone remodeling and haemopoiesis (50).
GROWTH FACTORS AND HAEMOPOIESIS

The colony stimulating factors (CSFs) which regulate the survival, proliferation and differentiation of haemopoietic cells have been purified and recently molecularly cloned (51, 52). The CSFs are produced by a variety of cell types including bone marrow stromal cells, T lymphocytes (53 - 55), endothelial cells (56 - 58) and fibroblasts. The release and production of haemopoietic growth factors by fibroblasts and bone marrow cells is modulated by factors from mononuclear phagocytes, like interleukin-1 (IL-1) (59 - 61) and tumor necrosis factor (TNF) (62). Fibroblasts from sites other than bone marrow, like human fetal liver fibroblasts (63) or human lung fibroblasts (61, 62), also release growth factors which support erythroid, granulocyte and macrophage colony growth. Interleukin-3 (IL-3) activate T-lymphocytes in vitro. In vivo it has not been detected (64, 65). The same applies for interleukin-2 (IL-2). IL-3 and GM-CSF are multi-lineage stimulating factors that promote the growth and development of multipotent stem cells, and of progenitor cells committed to the granulocyte/macrophage, eosinophil, erythroid and megakaryocyte lineages (66, 67). IL-3 will also facilitate the proliferation of mast cells.

Since both IL-3 and IL-2 are powerful stimuli for proliferation of stem cells and T-cells, it is not surprising that their production is limited and under local control in vivo (5). On the other hand, GM-CSF is detectable in serum. Many cell types in vitro, including T-cells, fibroblasts and endothelial cells, can be stimulated to produce GM-CSF probably emphasizing its important role in modulating the response of mature cells to invasion by foreign organisms. Granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF), on the contrary, are lineage-restricted growth factors that promote the proliferation and development of the granulocyte/macrophage lineages into neutrophils and macrophages, respectively (2, 68 - 71). Erythropoietin is also a lineage-restricted growth factor promoting proliferation and haemoglobinization of fairly mature erythroid progenitor cells (72, - 74). As far as it is known, the action of these growth factors is restricted to stem cells and committed myeloid progenitor cells. Growth factors can effect target cell populations by direct or indirect mechanisms. G-CSF, for example, stimulates the formation of mixed colonies which contain several myeloid lineages through indirect effects on regulatory populations in the bone marrow, but, as described above, stimulates only the formation of neutrophils when acting on purified populations of colony-forming cells (69). Other factors, which do not induce colony formation per se, may act indirectly in any of several ways. IL-1, for example, can potentiate the response to CSFs by inducing stem cells to become sensitive to their action (75). The network of interacting cytokines is very complex. These include the CSFs, IL-1, IL-2 and TNF that regulate haemopoiesis either directly, by interacting with target cells, or indirectly, by modulating the production of other cytokines. Some cytokines may do both: TNF-inhibits the effect of G-CSF on neutrophil production, but stimulates the production of GM-CSF (75). It is known that CSFs do not compete directly for the same binding sites and that there are specific receptors for each CSF. Thus, IL-3 receptors are found in several myeloid lineages in fairly low numbers per cell (but a small proportion of bone marrow cells have several thousands per cell). Receptors for GM-CSF are also found in the lineages which are also positive for IL-3 receptors. Interestingly, the number of receptors per cell may decrease with cell maturation.
Receptors for G-CSF are found in all the cells within the neutrophil lineage but, in contrast, the numbers of receptors per cell increase with cell maturation in murine cells. Very small numbers are also found in premonocytes and monocytes. Receptors for M-CSF have been found in all the cells of monocyte-macrophage lineage, in numbers which increase markedly with cell maturation. Thus, multipotent stem cells have receptors for most, if not all, growth factors, but there is not a single growth factor which could transduce an effective stimulus for the proliferation of these cells. But with a combination of growth factors, synergistic effect can be seen.

The product of the murine S1 locus has recently been identified as a growth factor termed stem cell factor (SCF), kit ligand, or mast cell growth factor. SCF can be produced in both membrane-bone and soluble forms and is a ligand for the receptor encoded by the c-kit proto-oncogene, a member of a receptor tyrosine kinase family which also includes the M-CSF receptor. Mutations in the W locus encoding c-kit, or the S1 locus encoding SCF, lead to defects in germ cell development, neural crest-derived melanocytic development, and haemopoiesis. Characterization of the deletion found to occur in the Steel-Dickie mutation indicates that the membrane-bound form of SCF is perhaps more important than the soluble form in bone marrow stromal cell-mediated development of haemopoietic cells. However, haemopoietic stem cells can clearly respond to soluble SCF, since injection of SCF can ameliorate the defect in hematopoiesis seen in the Steel-Dickie mice (76).

When stem cell factor was used in combination with other growth factors, it allowed the survival of stem cells, while the other growth factor (GM-CSF) stimulated the stem cells to develop normally (77).

Various growth factors can elicit response in vivo. Administration of IL-3 to adult mice results in rapid recruitment of CFU-S into DNA synthesis (78, 79). Control infusion of IL-3 leads to an increase in the number of CFU-S and myeloid-committed progenitor cells in the spleen (67, 80). Both migration of stem cells from the bone marrow and in situ splenic proliferation of cells occurred place. GM-CSF stimulates splenic haemopoiesis and increases the levels of circulating leukocytes in mice and monkeys (71, 81). Recent results suggest that GM-CSF or G-CSF may also be useful in enhancing recovery of the haemopoietic system following marrow transplantation (81).

However, some can change the response to other CSFs by modulation of their receptors in bone marrow cells. IL-3 may transmodulate GM-CSF and M-CSF receptors, and GM-CSF the receptors for M-CSF. In contrast, G-CSF and M-CSF will primarily down-modulate their own receptors. Here again exists a hierarchical organization which is reminiscent of the relative specificity for different cell lineages (52, 82). These concepts highlight the complex regulation which maintains a stable output of mature cells in a normal steady state, and which is able to show a prompt, flexible and adequate response to haemopoietic injury. Recently, it has been shown that transfection of the human bcl-2 gene into an IL-3 dependent, multipotent haemopoietic cell line allowed these cells to survive in the absence of IL-3 (83). This indicated that exposure to growth factors may not be obligatory for the differentiation of stem cells (83).
OSTEOGENEZA I HEMOPOEZA:
SIMBIOTSKI MIKROOKOLIŠ

SAŽETAK – Hemopoëza je značajan fiziološki događaj pri kojem se odvija proliferacija, diferencijacija i interakcija mnogo različitih stanica. Neurofili, eritrociti, trombociti, eozinofili i limfociti visoko su specijalizirane stanice prisutne u krvi, ikivima i limfooidnom sustavu. Većina mjeloidnih stanica ima krtak životni vijek i moraju se trajno nadomješati proliferacijom matičnih stanica koje su dobro zaštićene u koštanoj moždini. Stromalni sustav koštane moždine analogan je hemopoëtskom po tomu što stromalne matične stanice proizvode različite linije potpornih stanica koštane moždine koje imaju presudnu ulogu u hemopoëzi. To su adipociti, retikuloce, endoteilije i osteogene stanice, te fibroblasti.

Integritet hemopoëtsko-stromalnog sustava održavaju izvanstanični matriks (laminin, kolageni, proteoglukan, fibronektin) i činitelji rasta (CSF; IL-1,2,3,4,6; TNF; FGF; TGF; PDGF i SCF). Izvan toga okruženja, zaštićeni status matičnih stanica je izgubljen i diferencijacija u različite hemopoëtske linije nije moguća.

Ključne riječi: osteogeneza, stromalne stanice koštane srži, hemopoëza, interleukini, čimbenici rasta kolonija

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