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IN VITRO REGULATION OF FETAL BOVINE ERYTHROPOIESIS

by

QINGGANG LI

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Department of Experimental Medicine

McGill University

Montreal, Canada

ABBREVIATIONS

APO	Apolipoprotein
BFLS Cells	Bovine fetal liver stromal cells
BFU	Burst forming unit
BM	Bone marrow
BPA	Burst promoting activity
CFU	Colony forming unit
CM	Conditioned medium
CSF	Colony-stimulating factor
ECM	Extracellular matrix
EM Cells	Embryo stem cells
Epo	Erythropoietin
FBS	Fetal bovine serum
Fn	Fibronectin
HPLC	High performance liquid chromatography
HSC	Hematopoietic stem cells
GAG	Glycosaminoglycan

IGF	Insulin like growth factor
IL	Interleukin
KL	*c-kit* ligand
kD	Kilodalton
LTC	Long term marrow culture
PAS	Periodic acid-Schiff reaction
Tf	Transferrin
TGF	Tranforming growth factor
TNF	Tumor necrosis factor
VLA	Very late antigen

ABSTRACT

Fetal bovine serum (FBS) is one of the most important supplements for cell culture, and is a rich source of both defined and unknown factors required for proper cell growth. A serum-free bioassay system was developed to facilitate the purification and characterization of the heparin-binding growth factors in FBS. Three factors with different effects on erythropoiesis were isolated and identified with the combination of several chromatographic techniques. An 8 kd heparin-binding peptide which stimulated thymidine incorporation into fetal erythroid cells had an N-terminal sequence identical to insulin-like growth factor (IGF II). The growth promoting effect of this peptide was potentiated by heparin in culture. It was also found that the relative affinity of IGFs was in the order of IGF II > IGF I > insulin. The second heparin-binding erythroid regulating factor isolated was a 46 kd protein. The N-terminal sequence of this protein was identical to that of apolipoprotein H (Apo H). It inhibited thymidine incorporation into fetal erythroid cells with an ED_{50} of 36 nM. A 100% inhibition of thymidine incorporation and a 40% decrease in cell numbers in culture were observed at 840 nM. The third factor identified was an 11 kd peptide with an N-terminal sequence similar to C4a,

a fragment of complement C4. This peptide was a potent cytotoxic agent and was not species-specific, lysing not only bovine fetal erythroid cells, but also human adult red blood cells at very low concentrations.

A clonal assay system for bovine fetal liver cells was developed to further characterize the erythropoietic effects of IGF II, the most important of the isolated factors. It was found that bovine fetal erythroid colonies could not be developed at low concentrations of FBS, unless they were grown over stromal cells. Bovine fetal liver stromal cell lines could support erythroid growth through secreting soluble factor(s) and by direct contact to erythroid cells. It was clear that IGFs stimulated erythropoiesis in this system.

RÉSUMÉ

Le sérum foetal bovin (FBS) est un des plus importants suppléments nécessaires à la culture de cellules. C'est aussi une source riche en facteurs, soit bien définis, soit inconnus, indispensables à une bonne croissance cellulaire. Un système de culture sans sérum a été développé pour faciliter la purification et la caractérisation des facteurs de croissance, contenus dans le FBS, qui ont comme propriété de se lier à l' héparine. Trois facteurs avec des effets différents sur l'érythropoïése ont été isolés et identifiés grâce à la combinaison de plusieurs techniques de chromatographie.

Un peptide de poids moléculaire de 8kd qui se lie à l'héparine et qui stimule l'incorporation de la thymidine dans les cellules érythroïdes foetales a une séquence amino-terminale identique à celle du facteur de croissance similaire à l'insuline II (IGF II). La capacité de ce peptide de stimuler la croissance cellulaire a été augmentée avec l'addition de l'héparine en culture. On a aussi trouvé que l'affinité des IGF vers l'héparine était dans l'ordre IGF II > IGF I > insuline. Le deuxième facteur de régulation des cellules érythroïdes à être isolé est une protéine de poids moléculaire de 46kd. La séquence amino-terminale de cette protéine est identique à celle de l'apolipoprotéine H (Apo H). Elle inhibe

l'incorporation de la thymidine dans les cellules érythroïdes foetales avec une dose médiane de 36 nM. A une concentration de 840 nM, on a observé une inhibition de 100% de l'incorporation de la thymidine, tandis que la diminution du nombre des cellules a été seulement de 40%. Le troisième facteur identifié est un peptide de poids moléculaire de 11kd, avec une séquence N-terminale similaire à C4a, une partie du complément C4. Ce peptide est un agent cytotoxique très puissant, mais non-spécifique à une espèce en particulier, puisqu'il détruit, à des concentrations très basses, non seulement les cellules érythroïdes foetales bovines, mais aussi les érythrocytes adultes de l'humain.

Un système de clonage pour les cellules foetales de foie bovin a été développé pour caractériser davantage les effets érythropoïétiques de l'IGF II, puisque celui-ci est le plus important des trois facteurs isolés. On a trouvé que des colonies de cellules bovines foetales ne se développaient pas en présence de basses concentrations de FBS, sauf si elles étaient cultivées sur des cellules oestromales. Les lignées de cellules oestromales du foie bovin utilisées pouvaient supporter la formation des clones érythroïdes soit par contact direct, soit par la sécrétion dans le milieu de culture de facteurs solubles. IGF II a stimulé d'une façon très évidente l'érythropoïése dans ce système.

PREFACE

There has been consistent indication of the existence of erythropoietic growth factors different from erythropoietin in fetal bovine serum (FBS). When I started my Ph.D. training in Dr. L.F. Congote's laboratory, he had found that some of erythropoietic growth factors in FBS could bind to heparin, and might be isolated by heparin affinity chromatography. We decided to identify this heparin-binding factor(s) as the first step of my Ph.D. Project. This led to some important discoveries described in this thesis. First, we developed a unique serum-free bioassay system, which greatly facilitated identification and characterization of the heparin-binding factors in the FBS. We have found that the major heparin-binding erythropoietic growth factor is insulin like growth factor II, and the affinity of IGFs to heparin may be determined by their internal structure. We have purified two erythropoietic inhibitory factors which may have influence in the outcome of cell growth in vitro. We have also established a fetal liver stromal cell line which could support erythropoiesis in vitro through direct contact or by secreting soluble factor(s).

Most of the studies were carried out under the supervision of Dr. L.F.

Congote. It would be impossible for me to finish the project without his generous support, constructive criticism, and continuous encouragement. I would like to express my sincere gratitude to him for his understanding, kindness and patience.

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Most studies described in this thesis have been published as follows:

A. Papers

1. Congote, L.F., J. Theberge, L. Mazza and Q. Li. Assay for thymidine incorporation into anchorage dependent- and independent cells without filtration or cell harvesting. *J Tissue Culture Meth* 12:47, 1989

2. Li, Q., R. Blacher, F. Esch and L.F. Congote. An heparin-binding erythroid cell stimulating factor from fetal bovine serum has the N-

terminal sequence of insulin-like growth factor II. *Biochem Biophys Res Commun* 166:557, 1990

3. Li, Q., R. Blacher, F. Esch and L.F. Congote. Isolation from fetal bovine serum of an apolipoprotein H-like protein which inhibits thymidine incorporation in fetal calf erythroid cells. *Biochem J* 267:261, 1990

4. Li, Q. and L.F. Congote. Isolation and characterization of three inhibitors of thymidine incorporation into bovine fetal liver cells. *Ann N Y Acad Sci* 628:241, 1991

5. Congote, L.F. and Q. Li. Accurate processing and secretion in the Baculovirus expression system of an erythroid cell stimulating peptide consisting of a chimera of IGF II and an insect insulin-like peptide. *Biochem J* 299:107, 1994

6. Li, Q. and L.F. Congote. Bovine fetal liver stromal cells support erythroid colony formation: enhancement by insulin-like growth factor II. *Exp Hematol* 23:66, 1995

B. Abstracts

1. Li, Q. and L.F. Congote. Isolation and characterization of three inhibitors of thymidine incorporation into bovine fetal liver cells. The 2nd International Conference on Negative Regulators of

Hematopoiesis. Providence, Rhode Island, USA. August 1990

2. Li, Q. and L.F. Congote. Bovine fetal erythroid colony formation is enhanced by stromal cells isolated from fetal liver. XXI Annual meeting of International Society for Experimental Hematology. Providence, Rhode Island, USA. July, 1992

3. Congote, L.F. and Q. Li. Production in the baculovirus expression system of erythroid cell stimulating factors consisting of chimeras of bombyxin, IGF II and fragments of FGF. Exp Hematol 22:113, 1994

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CHAPTER 1

INTRODUCTION

The hematopoietic system is characterized by constant turnover of cells. Consequently, continuous replacement is necessary to maintain stable population of leukocytes, platelets, and erythrocytes. The rate of cell production is adjusted to actual needs and can go from a minimum, just to replace the aging cells, to a several fold increase in response to appropriate stimuli, such as hypoxia, bleeding, or infection. This expansibility and focused responsiveness of the hematopoietic system are attributable to a population of stem cells and other progenitors which are able to either self-replicate or produce a mature progeny, and the responsiveness of the system to specific promoters in a suitable milieu called hematopoietic microenvironment.

1.1 HEMATOPOIETIC PROGENITOR CELLS

Blood cell formation was thought to take place in the lymph nodes, liver, or spleen. The concept that the bone marrow was the major source of blood cells was first proposed and accepted at the end of nineteenth century (1). During the

following decades, with the development of in vivo marrow sampling and better staining methods, considerable information about the morphology of immature blood cells and a dynamic understanding of their proliferation, differentiation, and relation to disease was obtained.

It was postulated, at the beginning of this century, that blood cells were derived from a single class of progenitors (2). This hypothesis was later expanded by adding the concept of hierarchies of pluripotent cells, the progeny of which were progressively more committed to a single lineage (3). This hypothesis remained largely untestable until 1961, when Till and McCulloch demonstrated that single cells were capable of establishing nodules of hematopoietic growth in the spleen of irradiated mice and that such colonies displayed multilineage or pluripotent differentiation (4). Subsequent studies have shown that cell lines consist of differentiated end cells with a finite functional life-span, capable of limited proliferation when young but without the capacity for self-renewal. Sustained cellular production, on the other hand, depends on the presence of pools of primordial cells capable of both differentiation and self-replication (5).

1.1.1 Stem Cells

A stem cell is defined as a cell with the ability to renew itself, as well as to produce progeny destined to differentiate (6). The morphologic identity of stem cells is largely undefined because of lack of cell markers. However most evidence

suggests they have the appearance of a transitional lymphocyte (7, 8). Functionally the earliest stem cells are pluripotential and capable of differentiating to either lymphoid or myeloid multipotential stem cells. The lymphoid stem cells leave the marrow for subsequent differentiation into B or T cells in the lymph nodes or thymus, respectively. The myeloid stem cells proliferate and, as a result of response to one of a number of differentiating stimuli, differentiate at random into self-renewing cells with multiple options for further development(9). Eventually they become responsive to growth and differentiation factors, lose most of their differentiation options, and develop to lineage specific progenitor cells.

The existence of hematopoietic repopulating cells was established in the early 1950s (10, 11), but a quantitative and functional assay became available only when the in vivo colony-formation assay was developed (4). In their studies, Till and McCulloch demonstrated that normal syngeneic bone marrow cells injected into irradiated mice could form hematopoietic colonies on spleens. These colonies, called colony-forming unit-spleen (CFU-S), consisted of one cell lineage (erythroid, granulocytic, or, occasionally, megakaryocytic) or a mixture of them (12, 13). Cytogenetic experiments based on the presence of unique radiation-induced chromosomal abnormalities in erythroid, megakaryocyte, and granulocyte lineages within splenic nodules confirmed that the CFU-S were clonal in origin and each colony was derived from a single cell (14, 15). They also contained cells that themselves were capable of establishing new multilineage colonies in other mice,

and retransplantation of the cells from colonies of a single cell type led to colonies with the same distribution of pure and mixed cell populations (15, 16, 17). These landmark experiments established for the first time the existence of a stem cell for hematopoiesis and provided a conceptual base of our present understanding of hematopoiesis.

Subsequently, it was demonstrated that CFU-S constituted a cell population that was heterogeneous with regard to adherence, density, cell size, self-renewal, and differentiation potential (18, 19, 20, 21, 22). In addition, various differences in position within the cell cycle, renewal, and differentiation exist among marrow, blood, and splenic CFU-S, suggesting that they may not represent the hematopoietic stem cell (23, 24). Studies of marrow with specific markers indicate that a single cell could repopulate the myeloid and lymphoid systems and that this cell might give rise to CFU-S (25. 26). Other studies found that 5-fluorouracil (5-FU) injection kills almost all CFU-S but not the long-term repopulating stem cells (27). This finding suggests that stem cells, but not CFU-S, can enter G_0 phase of the cell cycle, since 5-FU is toxic to proliferating cells.

A variety of techniques, including immunological and physical methods, have been applied to isolate the hematopoietic stem cells. Although they have been enriched into a small population of hematopoietic cells, hematopoietic stem cells have yet to be purified to homogeneity. The difficulty for the purification of stem cells is that these cells and their more mature progeny overlap in phenotype

for cell morphology and surface markers (6). However, by using highly enriched stem cells, it has become clear that hematopoiesis in a transplanted mouse may actually derive from a limited number of stem cells, and different clones may sequentially dominate hematopoiesis in an individual mouse (26).

1.1.2 Committed Progenitor Cells

Under suitable conditions the hematopoietic stem cells lose their ability of self-renewal, and differentiate into more mature cells. Although factors that influence commitment of stem cell are poorly understood and generally undefined, there are several theories proposed to address their commitment into a specific differentiation pathway (28). According to the stochastic theory, commitment is a random event that progressively restricts the potential for differentiation (29). In this theory, regulatory factors facilitate this process, and act only at later stages of hematopoiesis. The second theory of hematopoietic inductive microenvironment proposes that commitment of stem cell progeny to a specific pathway depends on the environment that surrounds each hematopoietic stem cell (30). The third theory proposes that commitment depends on humoral factors that compete among themselves at the stem cell level in promoting differentiation toward one specific pathway (31). While each of these theories focuses on somewhat different aspects of the stem cell function, they are not necessarily exclusive of each other.

Because of the complexity of the in vivo system and the relative inefficiency with which CFU-S form colonies in the spleen, the physiology of hematopoietic progenitors was difficult to explore in the intact animals. The introduction of in vitro assays for hematopoietic progenitor cells is another major breakthrough in the study of hematopoiesis. It was reported, in the mid-sixties, that hematopoietic colonies could be grown in semisolid medium (32, 33). Critical features of these culture systems are a semisolid matrix provided by agar, methylcellulose, or a plasma clot plus standard enriched culture media. These systems have allowed detailed studies of both the progenitor cells and their regulators (34). If these culture systems were supplemented by conditioned medium initially, only colonies of granulocytes or macrophages were produced. However, with the addition of plasma, erythropoietin, special conditioned media, or specific hematopoietic growth factors to the system, all of the combinations of erythroid, granulocytic, macrocytic, and megakaryocytic lines were found in single colonies (34). The cells responsible for these colonies vary in potentiality from the highly committed neutrophil-macrophage colony-forming units (CFU-GM) to the more primitive granulocyte-erythrocyte-macrophage-megakaryocyte-forming unit (CFU-GEMM) and the even more primitive blast-like cells (CFU-Blast) (9). These cells represent a continuum of differential stages of hematopoietic maturation. A single, highly differentiated CFU apparently can generate both macrophages and neutrophils (CFU-GM), while others generate eosinophils (CFU-Eo), basophils (CFU-Baso), and megakaryocytes

(CFU-Meg). At least two broad categories of committed erythroid progenitors exist: the relatively primitive burst-forming units (BFU-E) and the more mature erythroid colony-forming units (CFU-E). A less differentiated progenitor may give rise to more than three cell lineages, i.e., CFU-GEMM.

1.1.3 Hierarchy of Hematopoietic Progenitors

Further painstaking work over the following decades led to the evolving schema of hematopoietic stem cell development, differentiation, and maturation that provided the framework for our understanding of the control of normal and abnormal hematopoiesis, and bone marrow transplantation (Fig 1.1) (35). The

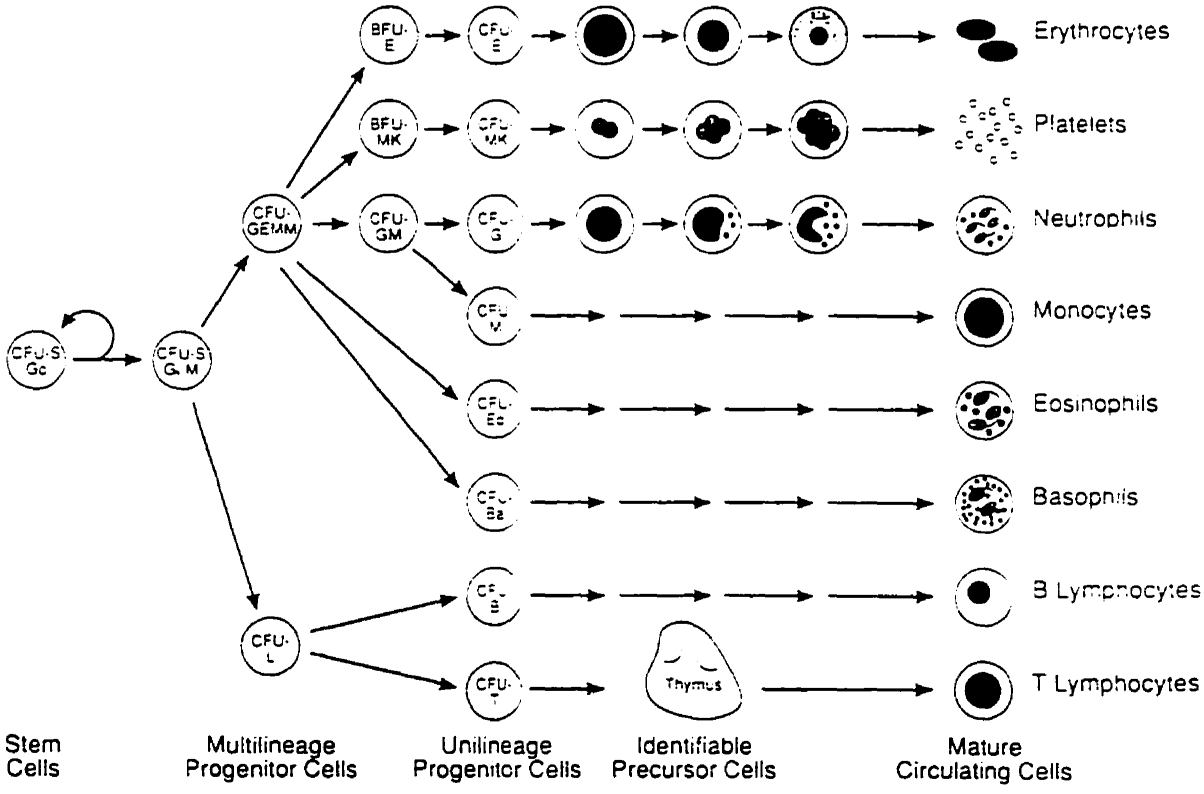


Figure 1.1. Stem Cell Model of Hematopoiesis (*from ref 35*)

most primitive progenitor, detectable after extreme hematopoietic damage, is a totipotent cell from which lymphocytes and pluripotent stem cells are derived. Committed progenitor cells derived from pluripotent stem cells loses their capacity for self-renewal, and follow the differentiation pathway to give rise to more mature precursor cells for granulocytes, macrophages, erythroid cells, and megakaryocytes. Commitment and differentiation are generally accepted as irreversible events. Once commitment occurs,differentiation proceeds fully to the stage of morphologically recognisable mature cells, most of which have limited life span.

1.2 HEMATOPOIETIC GROWTH FACTORS

The existence in the blood of a substance regulating the production of red blood cells was dimly perceived around the beginning of this century (36). In the early 1950's, there was evidence that a factor, later named erythropoietin, released by hypoxia stimulated red cell production in the bone marrow (37), and that such a factor was present in plasma of anemic and hypoxic animals (38). However further progress in isolation of hematopoietic growth factors was delayed until the development of in vitro hematopoietic assay systems. The requirement of appropriate feeder layers or conditioned media from certain cells containing critical elements for the proper growth of hematopoietic progenitors (32, 33) suggested the presence of humoral regulatory factors from the hematopoietic microenvironment for optimal hematopoiesis. It was soon clear that the type of

colonies and progenitor cells that could be produced would be determined by manipulating the contents of these elements. As these factors were discovered, they received a number of different names from different laboratories involved in their investigation. Most of the factors were named as "CSFs" for "colony-stimulating factors" because they could support the clonal growth of hematopoietic progenitor cells in vitro. These CSFs include granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF or CSF 1), granulocyte-macrophage-CSF (GM-CSF) and multi-CSF (also known as IL-3) (39). Using different culture systems, another group of soluble factors called interleukins, produced by macrophages and activated T or B cells and acting on T or B lymphoid cells, were identified. They were originally thought to have effects exclusively on lymphoid cells. Shortly afterwards, it was found that they were produced by a broad spectrum of cell types, and that these cytokines could act on, in addition to those of lymphoid lineage, a variety of cells (40).

With the development and application of molecular biology techniques and with the refinement of techniques for hematopoietic cultures, the numbers of identified hematopoietic growth factors have been greatly increased. Large amount of purified recombinant factors are now available for both in vitro and in vivo studies, which lead to dramatic expansion in our knowledge of the biological actions of these regulators. Recent studies have demonstrated that most hematopoietic growth factors are pleiotropic, potentiate the biological activities of

others, and have synergistic effects.

1.2.1 Molecular and Biochemical Characteristics of the CSFs

The molecular cloning of the cDNAs for the individual cytokines has greatly facilitated the analysis of the structure and functions of these growth factors. All of the hematopoietic growth factors identified so far are acidic glycoproteins with a polypeptide chain of similar length. Each of the factors is encoded by a single gene (41). Many of the hematopoietic growth factor genes are relatively small with the mRNA sequence around 1 kb in length, and this sequence is divided over four or five exons spread over 2 to 3 kb pairs of the genome. The gene for M-CSF is more complex and contains 10 exons spanning more than 20 kb, and the primary transcript can be alternatively spliced to yield several different mRNAs, coding both soluble and membrane bound M-CSF molecules (42). Studies on G-CSF, GM-CSF, and IL-3 sequences have also suggested the possibility of alternate splicing or initiation producing different molecular forms of these factors.

Genes for human IL-3, IL-4, IL-5, and GM-CSF have been located to the long arm of chromosome 5 (43), where the genes for M-CSF (44), the M-CSF receptor (the *c-fms* protooncogene), and the B isoform of the platelet-derived growth factor receptor are also found (45, 46). The genes for IL-3, IL-4, IL-5, and GM-CSF are all clustered to a 400 to 500 kb segment. Within this segment, the IL-3 and GM-CSF genes form a tandem array separated by only 9 kb. This tight

linkage, along with the similarity in gene structure, suggests that these genes evolved from a common ancestral gene (47).

Most of the cytokines are monomeric, but at least four of them are dimeric. M-CSF (48), IL-5 (49) and *c-kit* ligand (KL) (50) are homodimers linked by interchain disulfide bridges. IL-12 is a heterodimer consisted of subunits sharing homology with IL-6 and its receptor, respectively (51). One common feature of the hematopoietic growth factors is the glycosylation of these molecules. However, the extent of glycosylation is different, with up to 50% of the molecular mass contributed by carbohydrates (52). The length of the carbohydrate chain and the level of terminal sialation within the molecules are also variable. As a result, these peptides display significant size and charge heterogeneity (53). Studies with nonglycosylated or alternatively glycosylated recombinant hematopoietic growth factors have suggested that the main role of the carbohydrate is to enhance the solubility, stability, and resistance to proteolysis of the peptides (41, 54). Elimination of the carbohydrate present on G-CSF or on erythropoietin leads to reduced secretion, aggregation, and insolubility of each factor (55, 56, 57). On the other hand, change of the glycosylation status had little effect on the biological activity of recombinant M-CSF (58), GM-CSF and IL-3 (59). However, the large species of GM-CSF, due to heavy glycosylation, were less active than the less glycosylated forms because of decreased receptor association rates for the heavily glycosylated molecules (52).

Although hematopoietic growth factors have overlapping biological activities, there are not any similarities of the amino acid sequences among these factors, and there is a specific receptor for each of the cytokines (41). But recent studies on the structure-function relationship have demonstrated that a number of hematopoietic growth factors share many structural features (60). Information based on the structural analysis by X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) raveals that the tertiary structures of IL-2, IL-3, IL-4, IL-5, GM-CSF, M-CSF, and growth hormone (GH) are quite similar (53). They all contain a common structural feature of a left-handed four-helix bundle, presenting a hydrophobic face responsible for helix-helix packing stability and a hydrophillic face free to interact with the environment.

1.2.2 The Hematopoietic Growth Factor Receptors

The growth and development of hematopoietic cells is regulated by the binding of hematopoietic growth factors to their specific receptor on individual target cells. Recently, remarkable progress has been made in the molecular cloning of a large number of the receptors for the hematopoietic growth factors (41). In general, the numbers of receptors on the cell surface is low, typically in the range of 100-1000 per cell. The cell distribution of the receptors usually matches the known biologic action of each cytokine. However, the cytokine receptors are not restricted to cells of the hematopoietic system and have been

found to be expressed in tissues such as liver, placenta and some cancer cells (47, 61, 62, 63), suggesting a wider role of cytokines outside the hematopoietic system.

As an increasing number of cytokine receptors are identified it becomes apparent that many of the receptors are structurally related and form a new cytokine receptor gene family. These include the receptors for IL-2α, IL-2ß, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, Epo, GM-CSF, G-CSF, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), prolactin and GH (64, 65). All these cytokine receptors are transmembrane glycoproteins made up of an extracellular N-terminal ligand binding domain, a short hydrophobic transmembrane region and a C-terminal intracellular domain. They have a common structural motif in their extracellular domains (66). This motif consists of about 200 amino acid residues with two fibronectin type III modules. Each module consists of seven antiparallel ß-strands. There are four conserved cysteine residues in the N-terminal module and a Trp-Ser-X-Trp-Ser sequence just proximal to the transmembrane domain. The ligand-binding domains have a secondary α-helical structure which shows a significant degree of relatedness, suggesting a common ancestral origin of these receptors. The intracellular domains of these receptors are rich in proline and serine residues but there is little amino acid sequence homology. The intracellular domain of most of the cytokine receptors is required for signal transduction (67). However, The mechanisms involved in signal transduction are

largely unknown, and the nature of intracellular messengers remains to be elucidated. None of these receptors contains a kinase domain, although the phosphorylation of a number of the intracellular proteins is involved in the signal transduction (65). Both high-affinity and low-affinity cytokine receptors have been found, but when transfected into COS cells, only a single class of low-affinity receptor for GM-CSF and IL-3 was expressed (67). This and other studies have led to the realization that the receptors for a number of the cytokines are likely to be complex multi-unit structures where one or more of the subunits may be shared by a number of the cytokine receptors. For example, IL-3, IL-5, and GM-CSF receptors share a common ß subunit, which is important for the high-affinity binding and signal transduction (68, 69). Competition of binding of specific *α* subunits to a limited number of common ß subunits results in the cross-competition among IL-3, IL-5, and GM-CSF.

In contrast to the cytokine receptor family, the M-CSF receptor (c-*fms*) and product of c-*kit* gene belong to the tyrosine kinase receptor family (65). Both c-*fms* and c-*kit* genes were originally identified as the cellular counterparts of transforming genes carried by the feline sarcoma viruses SM-*fsv* (70) and HZ4-*fsv* (71), respectively. They are structurally similar to the receptors for PDGF and FGF, but shows less overall homology to other receptors of the protein tyrosine kinase gene family, such as EGF, insulin and IGF I receptors (47). The M-CSF receptor gene lies 0.5 kb downstream of the B isoform of the PDGF receptor (PDGF-R_B) on

chromosome 5, and the c-*kit* gene is similarly located downstream of the other PDGF receptor, namely PDGF-R_A on chromosome 4. These results imply that primordial gene for this family duplicated to yield a gene pair that has subsequently duplicated to yield the separate PDGF-R_B/*c-fms* and PDGF-R_A/*c-kit* gene pairs (40). Binding of M-CSF to its receptor results in rapid autophosphorylation on tyrosine residues as well as phosphorylation of several intracellular substrates involved in the signal transduction pathway (47).

1.2.3 Biological Activities of Hematopoietic Growth Factors

There are more than 20 well defined hematopoietic growth factors, and the number of the factors keeps expending rapidly. Most of these factors are pleotropic but each of them has its own distinct spectrum of biological activities. In early studies, the hematopoietic growth factors were classified into lineage-specific factors such as M-CSF, G-CSF and Epo, which could act on more mature progenitor cells, and those not lineage-restricted including IL-3 and GM-CSF, which were required throughout differentiation and important for self-renewal (72). However, with the advances in our understanding of normal hematopoiesis, it becomes apparent that most, if not all, cytokines have a broad spectrum of biological activities, and some of them originally thought to be lineage-specific actually have effects on both differentiated precursor cells and more immature progenitor cells. For example, G-CSF, first identified as a granulocyte stimulator

and leukemic differentiation factor, also acts on early stem cells and has some macrophage-stimulating ability (73). It also synergistically stimulates megakaryocyte colony formation, and has a pre-B inducing activity (73a, 74). The finding that the osteopetrotic mutation (*op*) in mice results from a failure to produce M-CSF and this defect could be corrected by injection of M-CSF indicates a role for M-CSF in the formation of osteoclasts (75). The identification of the M-CSF receptor in tissues other than hematopoietic cells also points to a broader range of possible biological activities of M-CSF (47).

The hematopoietic growth factors are required in normal process of hematopoiesis (39). They regulate the proliferation, differentiation and maturation of hematopoietic cells coordinately and synergistically (41). Factors such as IL-1, IL-3, IL-4, IL-6, IL-11, IL-12, GM-CSF, G-CSF, and KL, act on early stages of hematopoiesis to recruit dormant hematopoietic progenitors (76). Combinations of two or more of these factors are required for stem cell proliferation, and promote increased numbers of progenitor cells to proliferate, although a single factor might maintain the survival of these cells (77). After the dormant cells enter the active cell cycle, they begin to proliferate and differentiate in the presence of the cytokines, which synergistically stimulate the hematopoietic cells. As a result, the numbers of cells in the colonies are greater, when two factors are combined, than achievable by using twice the concentration of either factor alone (76, 77).

The presence of cytokines may also influence commitment to a particular

lineage, depending on the concentration and type of the factors used to stimulate the initial divisions (39). For example, stimulation with M-CSF tended to produce progeny committed exclusively to the formation of macrophages (78). In the late stages of hematopoietic process, the precursor cells differentiate and mature to granulocytes, macrophages, and erythrocytes under the stimulation of G-CSF, M-CSF, and Epo, respectively. Those cytokines acting on early progenitor cells, such as IL-1, IL-3, IL-6, IL-11, and KL, also have effects on these processes (76).

There are overlapping biological activities and functional similarities among the hematopoietic growth factors. Several factors have similar effects on the same target cells. Recent studies may have revealed a possible biochemical basis for the phenomena. Some of them, such as IL-6 and G-CSF may share a common ancestral gene (79). Others, such as IL-3, IL-5, and GM-CSF compete with the same subunit of their receptors, and all have the ability to stimulate eosinophil proliferation (67). Furthermore, a group of cytokines shares similar tertiary structural features and their receptors fall into same family because of structural homology (53, 65, 66). However, the loss of a factor may not be fully compensated for by others with similar activities. For example, defect in production of M-CSF causes major deficiencies in osteoclasts and partial deficiencies in other macrophage populations, demonstrating absolute requirement of M-CSF for the proper production of these cells (75, 80). In another case, dogs producing antibodies against G-CSF induced by injecting human G-CSF developed

neutropenia, which could not be compensated for by other factors such as GM-CSF or IL-3 (81). These results suggest the requirement of most, if not all, cytokines in normal hematopoiesis.

Some growth factors, such as M-CSF and KL, are produced in different isoforms, namely membrane bound form and soluble form, raising the question of the relative biological role of the two distinct forms of these factors in the regulation of cell growth and differentiation (42,82). The observation that the Dickie allele encodes a soluble form of KL, which is functional, is a direct evidence to demonstrate the need for the membrane-bound ligand for a normal phenotype because Steel-Dickie homozygotes are black-eyed white, sterile, and profoundly anemic (82). The studies on stromal cells expressing different forms of KL also showed their distinct functions in maintenance of hematopoiesis (83). Hematopoiesis can only be maintained transiently on the stromal cells secreting soluble KL. In contrast, the stromal cells making membrane-bound KL support the long-term production of primitive hematopoietic progenitors in vitro (84, 85). The membrane-bound KL is also more effective than soluble KL in supporting the growth and survival of primordial germ cells in culture. These results demonstrate the distinct roles of these two forms of KL and suggest that the membrane-bound factor is absolutely required for normal growth and development.

1.2.4 Hematopoietic Inhibitors

Control of hematopoiesis under normal steady state conditions requires not only the regulation of growth factors with stimulating activities, but also the modulation of factors with inhibitory activities. The dynamic interaction of growth factors with stimulatory and inhibitory activities is believed to be the fundamental mechanism of hematopoiesis (86). Although the effects of growth factors with stimulatory activities on hematopoiesis are well-known, the convincing evidence for the influence of hematopoiesis by factors with inhibitory activities emerges only recently. A variety of molecules with hematopoietic inhibitory activity have been identified. They include some well-known growth factors such as TGF-ß and TNF-α, and some newly identified small peptides. The effects of hematopoietic inhibitors may be environment dependable and be variable in the presence of different hematopoietic stimulators (87). This is because the inhibitors could work by blocking, downmodulating, or reducing the function of receptors for stimulators. They may interfere with signal transduction pathways, transcriptional factors, and mRNA production or stability, involving genes activated by stimulators. They may also act indirectly to block synthesis of positive regulators.

TGF-ß was first identified by its ability to induce a reversible transformation of rat fibroblasts (88). It has since been shown that, depending on the nature of the target cells, TGF-ß can induce and inhibit cell proliferation and differentiation. Structurally, three isoforms of TGF-ß (TGF-ß1, TGF-ß2, and TGF-ß3) have been

isolated , and are the result of the homodimeric and heterodimeric combination of the subunits ß1 and ß2 (89). TGF-ß is a potent inhibitor of the hematopoietic cells, and its action is not lineage specific(86). It inhibits the growth factor stimulated proliferation and differentiation of the progenitor cells, but has less prominent effects on the unstimulated cells (90). It was demonstrated to strongly inhibit BFU-E in the presence of GM-CSF and Epo. Human CFU-E was also inhibited, but murine CFU-E was unaffected under similar conditions (91). The action of TGF-ß on myelopoiesis is more complex. Day 14 human and early murine bipotent and multipotent CFU have been reported to be inhibited, while day 7 human CFU-GM is potentiated (92). TGF-ß may also exert negative effects on mature hematopoietic cells (89). TNF-α is another potent hematopoietic inhibitor, and has been shown to have preferential effects on erythroid cell proliferation (93). However, TNF-α may, depending on the cytokines present in culture, have both positive and negative effects on human marrow colony growth (94). Analysis of stem cell proliferation using the CFU-S assay led to the identification of another inhibitor of hematopoietic stem cells and early progenitor cells (95). This inhibitor was found to be identical to macrophage inflammatory protein 1α (MIP-1α). Later studies found that MIP-1α inhibited the proliferation of primitive hematopoietic progenitor cells while it stimulated the more mature cells (96). Two small peptides, pEEDCK (97) originally isolated from human granulocytes, and AcSDKP (98) first purified from fetal calf bone marrow, were demonstrated to be inhibitory on

hematopoietic progenitor cells. Their actions were more restricted to early progenitor cells at low concentrations (99). The inhibitory effects diminished with increased peptide concentrations. The reason for this phenomenon is not clear, but may be due to, in the case of pEEDCK, the dimerization of the peptide at high concentrations (100). Recent data indicates that AcSDKP may also have indirect effects on progenitor cells through the modulation of the local microenvironment (101). There are increasing data indicating the involvement of other well-known and newly identified inhibitory factors in the regulation of hematopoiesis (102). However, more studies are required to detail the regulatory mechanisms of these factors under physiological conditions.

1.3 ERYTHROPOIESIS

1.3.1 Erythroid Progenitor Cells

Erythropoiesis involves a great variety and number of cells at different stages of maturation starting with the first stem cell progeny committed to erythroid differentiation and ending with the mature circulating red blood cells. This process is divided, for descriptive purposes, into several stages, including the commitment of pluripotent stem cell progeny into erythroid differentiation, early phase of erythropoiesis (relatively Epo-independent), and late phase of erythropoiesis in which erythroid precursor cells rapidly differentiate into morphologically recognisable erythrocytes under the influence of Epo (103). The erythroid

progenitor cells cannot be identified morphologically. However, the development of semisolid culture in vitro for cloning of hematopoietic progenitor cells has led to the recognition of at least two erythroid progenitors, CFU-E and BFU-E.

CFU-E is an erythroid cell population closely related to the proerythroblast (104). It gives rise to colonies of 8 to 49 hemoglobin-containing erythroblasts. Most of CFU-Es are in a phase of active DNA synthesis (S-phase) as demonstrated by a 70 to 90% killing of cells after short exposure to ^{3}H-thymidine in vitro or administration of cycle-specific chemotherapeutic agents in vivo (105, 106). Extensive studies have demonstrated that CFU-E carries the highest density of Epo receptors on its surface and is the most Epo-sensitive cell (107, 108, 109, 110). Moreover, the survival of CFU-E is also dependent on the presence of Epo (111). On the other hand, BFU-E is an erythroid progenitor with extensive proliferative potential requiring a long culture period. It is more closely related to the multipotent hematopoietic stem cell (103), and requires additional factors, termed burst-promoting activity (BPA) as well as a high concentration of Epo, for its optional in vitro growth (106). It has been well established that the early stage of BFU-E proliferation and differentiation is Epo independent, and its survival in vitro does not require Epo. However, the presence of other factors with burst promoting activities, such as IL-3, IL-4, IL-11, GM-CSF, and KL, are needed for adult BFU-E growth and survival (112, 113, 114, 115, 116).

BFU-E and CFU-E are erythropoietic progenitors at two recognisable stages

of a continuum process of differentiation, and CFU-E can be formed by culturing blood BFU-E in vitro (107). A variety of cells with the properties between BFU-E and CFU-E have been detected (105). For example, a subclass of erythroid progenitors termed "mature" BFU-E shares properties from both BFU-E and CFU-E (105, 117). There is also evidence for an erythroid progenitor cell more primitive than the conventional BFU-E, with a high proliferative and differentiative capacity in murine species (118). Clearly, during erythroid development, early progenitors of high proliferative potential but a relatively low cycling status, dependent on BPA and relatively independent on Epo, differentiate progressively through various stages into later progenitors of low proliferative potential and a high cycling status that are totally Epo dependent. The biological events that occur at the stem cell progeny during its commitment to erythroid differentiation are not very clear. The same holds true for the earlier identifiable erythroid progenitors (BFU-E). These cells are IL-3 dependent and show a small number of Epo receptors (110). Within 72 hours in culture, BFU-Es become fully dependent on Epo and proliferate and differentiate into CFU-Es (107, 110).

The first morphologically recognizable erythroid precursor cell is called pronormoblast, which develops to basophilic erythroblast. Other morphologically characterized erythroid precursors with increased maturity include polychromatophilic erythroblast, orthochromatic erythroblast, and reticulocyte. All identifiable erythroid progenitors and the morphologically recognizable erythrocyte

precursors are functionally destined to mature (103). Therefore, they are not capable of self-renewal. Maintenance of the erythrocyte population and its expansion on demand are functions of the stem cell compartment.

1.3.2 Regulation of Erythropoiesis

There is a well-balanced mechanism that maintains the number of erythrocytes and the hemoglobin levels within normal limits and mediates the response to a variety of normal and abnormal situations. Early studies demonstrated that hypoxia produced an increase in erythropoiesis, while hyperoxia resulted in reduced erythropoiesis (111). Later studies demonstrated that the oxygen tension did not exert its effects directly on bone marrow. Instead reduced oxygen tension induced the elaboration of Epo (103). More recently, studies in vitro have demonstrated the requirement of other hematopoietic growth factors with BPA, in addition to Epo, for optimal erythropoiesis (106). Other physiological factors, such as sex, are also important. Within the normal range of hemoglobin, men have a larger red cell mass than women, even though their plasma Epo levels do not differ (112a). This gender-related difference in red cell mass is due to androgen production in men since castration causes the red cell mass to fall to the level found in women without significant change in the plasma Epo level (113a).

1.3.2.1 Erythropoietin

Erythropoietin (EPO) is a glycoprotein hormone produced mainly by the kidney in vivo and is the major humoral regulator of red cell production (111). The gene for Epo has been cloned and expressed in mammalian cells, from which large quantities of recombinant human Epo have been produced. Epo availability has greatly contributed to improve research on erythropoiesis and on the clinical applications of Epo.

The initial attempt to purify Epo was performed with plasma from anemic sheep (114a), which proved to be extremely difficult because only minute amounts were present in the serum. Human Epo was originally purified from the urine of anemic patients (115a). This natural Epo was found to be a glycoprotein with a molecular weight of 34,000 determined by SDS-PAGE, and had a specific activity of 74,000 U/mg protein. It contained 30% carbohydrate consisting of 11% sialic acid, 11% total hexose, and 8% N-acetylglucosamine (116a).

Although the natural Epo obtained was never in sufficient supply for extensive use, its purification and sequencing led to the isolation and cloning of the Epo gene (117a). The gene encoding human Epo exists as a single copy in a 5.4 kb region of the genomic DNA on human chromosome 7 (117a, 118a). It contains four introns and five exons for the 193-amino acid protein. A leader sequence of 27 amino acids at the N-terminal is cleaved during Epo secretion, leaving a mature protein of 166 amino acids with a calculated molecular weight of 18,398 (119). The

C-terminal arginine is lost in both natural and recombinant hormone, possibly due to posttranslational processing by a carboxypeptidase (120). Like the natural hormone, the recombinant Epo produced in CHO cells is heavily glycosylated. It has a molecular weight of 30,400 estimated by sedimentation equilibrium and contains 39% carbohydrate (121). However, the two forms of Epo are almost indistinguishable except for a slight difference in sialylation (121, 122).

Glycosylation of Epo is necessary for its biosynthesis, secretion and in vivo activity. There are three N-linked and one O-linked glycosylation sites in human Epo (119, 122). When N-linked glycosylation was inhibited by tunicamycin, the secretion of recombinant Epo from COS cells was impaired (57). With site-directed mutagenesis, it was identified that N-linked glycosylation at positions 38 and 83 were required for proper Epo secretion (56). Prevention of O-linked glycosylation also jeopardized Epo secretion (56). When natural Epo was treated enzymatically or chemically to remove the carbohydrate, the protein aggregated and such preparation lost biological activity (116a). Enzymatically deglycosylated natural Epo, and Epo made in *E coli* that is not glycosylated has a decreased in vivo activity, although its in vitro activity is largely preserved (123). The mechanism of this phenomenon is not clear but can be attributed in part to rapid clearance by the liver, because galactose residues newly exposed after desialation bind to galactose receptors of hepatic cells, which rapidly sequester and metabolize the desialated Epo (114a, 124). Oxidation of the galactose residues, or simultaneous

administration of asialo-orosomucoid, could partially restore the in vivo activity of the asialated Epo (114a).

The amino acid sequence of Epo from several species has been identified (111). A very high degree of conservation is evident, with a 94% sequence homology between human and monkey (125). There are four cysteine residues, forming two intromolecular disulfide bonds: Cys^{7}-Cys^{161} and Cys^{29}-Cys^{33} (126, 127). Reduction or alkylation of these residues lead to the loss of biological activity. Reoxidation of the reduced molecules restores the activity. The recent results has demonstrated that breaking the disulfide bridge Cys^{29}-Cys^{33} did not alter the secretion and biological activity of Epo, indicating that only the disulfide bridge Cys^{7}-Cys^{161} is crucial for the preservation of the molecular structure of Epo (128). A predicted tertiary structure of Epo with four anti-parallel α-helical bundle has also been tested to demonstrate the proper folding of Epo into its native tertiary structure is necessary for stability and biological function. The mutants with short deletions inside predicted α-helical were not processed and did not exhibit biological activity. In comparison, when deletions were created in predicted interconnecting loops, the mutants, to varying degrees, could be secreted and detected with bioassay (128).

The site of Epo production was indicated by studies in which animals with bilateral nephrectomy no longer increased Epo production in response to phlebotomy or cobalt administration (129). Patients with end-stage renal failure

were also found to be anemic and had low serum Epo levels, which could be restored to normal after renal transplantation (130). However, Epo could not be extracted from normal kidneys or from various normal kidney fractions despite the evidence that the kidney is the site of Epo production (123). Trace amounts of Epo mRNA were detected in normal kidneys when polyadenylated RNA was analyzed (131, 132). Induction of anemia led to a rapid increase of Epo mRNA, and a rise of more than 200-fold could be achieved by the end of 4 hours (131). Epo mRNA was also detected in the anemic liver, but not in other tissues. The changes in plasma Epo concentrations paralleled the changes in Epo mRNA levels, implying Epo produced in response to anemia represented *de novo* synthesis rather than the release of preformed hormone (133).

The cells synthesizing Epo mRNA were found in the interstitium of the renal parenchyma, outside the tubular basement membrane, mostly in the inner cortex and outer medulla (134, 135), and they were identified as the fibroblast-like type I interstitial cells (136). The liver cells can also synthesize Epo and is the extrarenal source of Epo in anephric patients (123). The adult liver contributes to 10% to 15% of total Epo production with severe hypoxia (137). Two types of Epo-producing liver cells have been identified (138). The hepatocytes are the major source of Epo production, and account for 80% of Epo-synthesizing cells in the liver of severely anemic mouse. The other 20% of the Epo-producing cells are nonepithelial, and are located in or adjacent to sinusoids. The nonparenchymal

cells have recently been found to be Ito cells (139). During fetal life, the liver is the major organ for Epo production, with a switch to renal production first occurring around birth (140).

It has been known for a long time that hypoxia stimulates Epo production, but the mechanism of Epo synthesis is still poorly understood. Several factors such as prostaglandins, cAMP, and calcium have been proposed to be involved in the regulation of Epo production, but inhibitors of prostaglandin synthesis or calcium channel blockers have never been associated with changes in Epo production in man (111). Studies employing the hepatoma cell line, Hep 3b, which produces erythropoietin in response to hypoxia, suggest that a heme-containing protein that changes from its oxy to deoxy form is the intermediate regulator of transcription of the Epo gene (141). The sequences in the Epo gene that are sensitive to oxygen and involved in the regulation of Epo gene expression have been identified (142). These oxygen-sensitive sequences can confer to cells the ability to respond to hypoxia by an increase of the protein encoded by the reporter gene. With hypoxia, transcription of the erythropoietin gene is activated and its mRNA is stabilized by a cytosolic binding protein (143, 144). Other studies have demonstrated that a ribonucleoprotein capable of binding to a segment -61 to -45 relative to the start site of transcription in the Epo gene may negatively regulate Epo mRNA transcription (145). These results suggest that hypoxia apparently affects gene transcription through one or more mediators.

Epo is the primary regulator promoting the viability, proliferation and differentiation of mammalian erythroid progenitor cells (146). It acts on the bone marrow to selectively increase erythropoiesis while not significantly affecting the production of other blood cells (123). CFU-Es are considered as the primary target of Epo and the amplification of this pool of cells directly effect erythropoiesis (147). Plasma Epo fluctuations have been correlated with changes in the size of the CFU-E compartment. The number of CFU-Es was found to be reduced in plethora and to be increased in anemia (148, 149). Hence, it was proposed that an increased influx from the pool of BFU-Es was responsible for the expansion of the compartment of late erythrocytic progenitors during rapid erythroid regeneration (150). Nevertheless, there has been evidence that Epo may act at early stages of erythropoiesis. It was demonstrated that early erythroid differentiation was hindered by using antisense oligodeoxynuceotides to either Epo or the Epo receptor (151), which was interpreted as Epo playing a role in erythroid differentiation near the stage of commitment by an autocrine mechanism. In addition to the stimulation of erythropoiesis, Epo may also act on other cell lineages. Specific high-affinity binding sites for erythropoietin were found on rat and mouse megakeryocytes (152). Treatment of anemic patients with recombinant Epo increased numbers of the CFU-GEMM and CFU-Meg as well as CFU-E and BFU-E in bone marrow (153, 154). Epo receptors have also been identified in non-hematopoietic cells such as endothelial cells (155, 156) and Leydig cells (157),

suggesting the involvement of Epo in the regulation of some non-erythroid target cells.

1.3.2.2 IGFs as Hematopoietic Growth Factors

Insulin-like growth factors(IGF I and IGF II), also called sometomedins, belong to a family of single chain peptides with structural homology to proinsulin. These two peptides have about 70% identity in their amino acid sequence and are highly conserved during evolution. They also share about 50% structural homology to proinsulin, suggesting a common evolutionary precursor (158). In addition to the major structure, a number of variant forms of IGF II have been identified or predicted (159, 160 161). IGF I and IGF II are produced by most tissues of the body and are abundant in the circulation (162). Like other growth factors, the IGFs are secreted constitutively but not stored (163). They have a wide range of biological activities and regulate proliferation and differatiation of a variety of cells (162, 164). While the activities of IGF I are primarily regulated by the action of growth hormone, the production of IGF II is less dependent on the presence of growth hormone. Also, the circulating level of IGF II is much higher than that of IGF I. Although the liver is the major source of circulating IGFs, local production is considered to be important in the regulation of growth and differentiation, and both paracrine and autocrine actions have been documented (162). The major biological activities of the IGFs are thought to be conducted

through the IGF I receptor, even though it has been demonstrated that IGF II receptor, which is identical to the cation-independent mannose-6-phosphate receptor and functions as a lysosomal enzyme targeting protein, is functional in mediating some biological actions of IGF II (162, 165). The regulation of the biological functions of the IGFs also involves the IGF-binding proteins (IGFBPs). Six IGFBPs (IGFBP-1 to IGFBP-6) have been described in both human and rats (166, 167). The majority of IGFs in the circulation is associated with a 150 kd complex that is composed of glycosylated IGFBP-3, an 85 kd acid-labile subunit and IGF I or II (168). IGFBP-3 has very high affinity for both IGF I and IGF II, and binds greater than 95% of the IGF I and II in the circulation (162). The others are minor binding proteins and usually contain most of the unsaturated IGF binding sites in serum. The binding of the IGFs to IGFBPs protects them from proteolytic degradation and prolongs their half-lives (166). The truncated form of IGF I with reduced affinity for IGFBPs is cleared more rapidly (169). The presence of IGFBP can potentiate or enhance the action of IGFs, although the mechanism is not clear (162). On the other hand, the binding to IGFBPs may limit the availability of bioactive IGFs. Thereby, IGFBPs in some system may inhibit the action of IGFs (170, 171, 172).

The accumulation of information about IGFs has led to the recognition of these growth factors as important regulators of hematopoiesis. In early studies, administration of GH to hypophysectomized rats or to hypopituitary dwarfs resulted

in increased erythropoiesis (173, 174). The Effects of GH on erythropoietic system was later found to be indirect and was mediated through the local secretion of IGF I (175). However, it was difficult to study the role of the IGFs on hematopoiesis as most culture systems required fetal bovine serum (FBS), which contained high concentrations of the IGFs (164). With the development of serum-free culture system, the effects of the IGFs on erythropoiesis became evident. An activity in FBS that stimulated CFU-E formation in mouse fetal liver was found to be IGF I (176). In addition, one of the non-erythropoietin factors with erythropoietic activity in FBS and in bovine serum albumin (BSA) named erythrotropin was found to be IGF II (177, 178). Moreover, a peptide capable of stimulating erythropoiesis isolated from the serum of an anephric patient with normal red blood count was identical to IGF I (179). Other in vitro studies have also demonstrated the stimulatory effects of the IGFs on erythropoiesis (109, 180, 181).

How IGFs effect erythropoiesis is not very clear. However, several lines of evidence point to the direct action of IGFs on erythroid precursors. The IGF receptors have been identified on normal and abnormal erythroid cells (182, 183, 184, 185, 186). While both high affinity and low affinity IGF I receptors were found on immature erythroid cells, only low affinity receptors seemed to be present on mature erythrocytes (182, 184). Furthermore, it has been reported that IGF II could bind to type I IGF receptors on erythroid cells (187). Addition of monoclonal antibodies against IGF I receptor abrogated the effects of both IGF I and IGF II in

vitro(188). In addition to the studies that demonstrated direct actions of IGFs on erythropoiesis in the presence of Epo (109), a recent study has shown that IGF I could stimulate BFU-E formation in the absence of Epo in an improved serum-free medium (189). This result suggests the possible involvement of IGFs as an alternative pathway of erythropoiesis independent of Epo. Although the mechanism of action of IGFs is not fully understood, it has been shown that IGF I as well as Epo and KL reduced apoptosis of highly purified early erythroid progenitor cells (190). Further detailed studies have revealed that the effects of IGF I were different from those of Epo, and both Epo and IGF I were needed for erythropoiesis (190). Epo maintained viability of erythroid progenitors and stimulated erythroid proliferation and maturation. However, the erythroid cell maturation was somehow incomplete in the presence of Epo alone in culture. and this defect could be corrected by addition of IGF I, which greatly enhanced nuclear condensation and enucleation in the late erythroblasts. IGF I also led to enhanced DNA synthesis, moderate cell proliferation, and heme synthesis (190, 191). Thus, Epo alone is not sufficient for complete erythroid maturation. The presence of IGF I is needed for cellular proliferation and maturation at a late stage of erythropoiesis. Although the effects of both IGFs on erythropoiesis have been well documented, their role seems to be somehow different. While the potency of both IFG I and IGF II are similar in adult erythroid progenitors (188), recent studies indicate that IGF II may be the predominant regulator of Epo-independent neonatal erythropoiesis

(192).

The effects of IGFs in vitro are in agreement with experiments in vivo. Administration of IGF I to neonatal rat resulted in significant increases in bone marrow erythroid precursor cells (193). In addition, injection of IGF I to hypophysectomized rats, which were in growth arrest and with decreased erythropoiesis, resulted in both body weight gain and red blood cell production (194). In the study that examined the correlation between activity of erythropoiesis and serum concentrations of Epo and IGF I, it was found that red blood cell production increased almost linearly during the period of accelerated growth in both male and female rats (195). While the concentrations of Epo fell during this period, the serum levels of IGF-I rose initially and correlated linearly with red blood cell production during this period. The administration of IGF in vivo not only promotes normal erythropoiesis, but also increases the hematopoietic progenitor cell content (196). These findings indicate that IGF I rather than Epo regulates erythropoiesis during accelerated growth in rats.

1.3.2.3 Other Erythroid Regulators

In addition to Epo, full erythropoietic development in culture requires the presence of other growth factors. Early studies demonstrated a second factor called burst-promoting activity (BPA) was needed for the growth of early erythroid progenitor cells (106). Several factors have been identified to have this activity

(197). IL-3 was one of the first factors to be recognised to possess BPA (198). IL-3 has multiple activities on virtually all of the myeloid lineages, as well as lymphoid cells. It acts synergistically with Epo to stimulate BFU-E formation in culture. It may also promote erythropoietic proliferation independently (199). Another factor with BPA is the *c-kit* ligand (KL), also called stem cell factor. This factor stimulates the growth of a wide spectrum of hematopoietic and non-hematopoietic cells (200). It exists in both soluble and membrane-bound forms, which may play distinct role in regulation of cell growth. This factor is believed to be important in the early development of hematopoiesis. IL-11 is a recently identified multifunctional growth factor derived from the hematopoietic microenvironment. It can, in combination with KL and Epo, promote erythropoiesis and have effects on BFU-Es and CFU-Es. It could stimulate BFU-E growth in the presence of IL-3 in culture, even in the absence of exogenous Epo (201). Other well-known hematopoietic growth factors with erythropoietic effect include GM-CSF, G-CSF, IL-1, IL-4 (197).

1.3.3 Influence of the Hematopoietic Microenvironment on Erythropoiesis

Effective hematopoiesis is the result of the interplay among hematopoietic stem/progenitor cells, hematopoietic growth factors and the local microenvironment in specific organs. Bone marrow is the predominant hematopoietic organ in normal adult mammals and, in fact, is the only site where

myelopoiesis, erythropoiesis, and lymphopoiesis proceed simultaneously (202). The in vivo studies demonstrating that a single stem cell could differentiate to the different lineages in different hematopoietic organs led to the use of the term hematopoietic microenviroment to emphasize the local stromal influence on hematopoietic progenitor cells within an organ (203, 204, 205). Histological studies of bone marrow have also revealed that hematopoietic cells were in close association with stromal cells (206, 207, 208), suggesting that stromal cells nurture hematopoietic cells. However, the hematopoietic microenvironment is not easy to define because of the involvement of various types of cells and other components participating in the regulation of hematopoiesis. Nevertheless, it could be considered as the local network of stromal cells, accessory cells and their products capable of influencing the self-renewal and differentiation of hematopoietic stem/progenitor cells (209). Due to the heterogeneity of the hematopoietic tissues, the nature of the interactions between hematopoietic cells and their environment has been difficult to analyze. The development of in vitro systems, especially the long term marrow cultures (LTC), has been crucial for defining the cells and signals that are important in these interactions (210, 211).

1.3.3.1 Long Term Marrow Cultures (LTCs)

The first culture system that allowed the analysis of stromal cells in vitro was the fibroblast colony-forming unit (CFU-F) assay (212). The cells defined by the

CFU-F assay could support hematopoiesis upon transplantation in vivo (213) and for a limited time in vitro (214). The murine LTC developed by Dexter and colleagues permits long-term maintenance of in vitro hematopoiesis (210, 215)). The stromal components of this system consists of macrophages, fibroblasts, endothelial cells, and reticular cells as well as hematopoietic cells (216). Although this LTC does not allow differentiation or proliferation of lymphoid cells, precursors for both B and T lymphocytes, even pluripotent stem cells are maintained by the stroma (209).

The Whitlock-Witte culture system, originally designed to obtain stromal layers devoid of hematopoietic cells, is a modification of the Dexter LTC and is found to be optimal for the growth of B lymphocytes and their progenitors (211). Unlike the Dexter cultures, which are maintained in a rich medium containing high concentrations of serum and hydrocortisone, the Whitlock-Witte TLC contains 5% fetal calf serum without addition of cortisone. The stromal layers established from bone marrow cells appears less heterogeneous than those in Dexter conditions and have been classified as either macrophages or stromal cells (217). However, the stromal cells in these cultures lack any detectable phagocytic activity. The lymphoid cells can either sit on the surface of these cells or become enveloped by them, and it is common to observe the association of multiple lymphoid cells with one stromal cell (218). Myeloid cells and their precursors are depleted a few weeks after initiation of the culture.

Both the Dexter and the Whitlock-Witte LTC systems contained all stromal cells necessary to support hematopoiesis, and one type of LTC could be converted to the other. By exchanging the composition of the medium, mouse Dexter LTC could be switched to Whitlock-Witte LTC (219). However, the switch was not reversible, indicating that certain elements in either the hematopoietic compartment or the microenvironment were depleted under Whitlock-Witte conditions. Subsequent studies showed that purified stromal layers initiated in Whitlock-Witte culture could be switched to Dexter conditions and then could sustain myelopoiesis (220, 221). These studies also demonstrated that stromal layer produced higher levels of colony-stimulating factors in the Dexter than in the Whitlock-Witte culture conditions (220).

1.3.3.2 Composition of the Hematopoietic Microenvironment

Isolated cell lines provide obvious advantages over primary stromal cell cultures for analysis of the cellular products of the stromal cells and the effects of each component of the microenvironment on hematopoiesis. Consequently, many laboratories have generated cloned stromal cells from fresh bone marrow or from the adherent layers of long-term bone marrow cultures. A variety of stromal cells, including fibroblasts, adipocytes, reticular, endothelioid cells has been described (222). It remains to be clarified whether the cells with different morphology represent distinct cell types, because there is not always a clear-cut correlation

between the morphological features of the stromal cell lines and their functions (222, 223). Stromal cells can also change their appearance according to the culture conditions. It is clear, however, that all established cell lines supporting hematopoiesis produce one or more hematopoietic growth factors.

Initial attempts to isolate these cytokines in long-term bone marrow cultures were unsuccessful and led to the conclusion that they were not produced (210). This was most likely due to their sequestration in the adherent layers, possibly by ECM components, and to their utilization by hematopoietic cells in the cultures (223). It is now known that stromal cells produce multiple cytokines, including soluble and membrane-bound factors. A noticeable exception is the lack of detection of IL-3 in stroma or in isolated cell lines. However, by using more sensitive techniques such as reverse transcriptase PCR, IL-3 mRNA was found in stromal cells (224). In addition, the ability of the stromal cells to support IL-3 dependent cell lines was blocked by anti-IL-3 antibody, indicating constitutive IL-3 production by stromal cells.

Extracellular matrix (ECM) is another important product of stromal cells (225, 226). The ECM consists of a heterogeneous mixture of molecules such as collagen, fibronectin, laminin, and proteoglycans. The molecules in the ECM can interact with each other via glycosaminoglycans (GAG) and GAG binding sites, forming a mesh that embeds stromal and hematopoietic cells (227). Hematopoietic cells at distinct stages of differentiation selectively attach to specific stromal cells

or components of the ECM (228, 229, 230), indicating diverse functions for each element of the ECM. The GAG molecules, such as heparan sulfate, chondroitin sulfate, dermatan sulfate and hyaluronic acid, may not only participate in the interaction with hematopoietic cells, but also be involved in the regulation of local concentrations of the cytokines (231, 232).

1.3.3.3 Origin of Stromal Cells

The origin of the cells of the hematopoietic microenvironment has been a subject of great controversy. Early theories of hematopoiesis regarded stromal cells as forming a fixed framework for blood cell development and undifferentiated cells within that population were able to generate hematopoietic cells (233). More recent studies have demonstrated that hematopoietic stem cells originate in an extramedullary, intraembryonic site and seed into the marrow cavity following establishment of the stroma (223). Bone marrow macrophages, circulating monocytes, and other mature myeloid cells, as well as lymphocytes, are derived from these pluripotent hematopoietic stem cells (234). The origin of the stromal cells, on the other hand, is not so clear (209). Fibroblast cells were found from adherent, nonphagocytic progenitors capable of forming fibroblastic colonies (CFU-F) in vitro, and these cells were different from hematopoietic progenitors (235). A cell-surface marker recognized by a monoclonal antibody STRO-1 has been found to be expressed on the CFU-F and cells giving rise to adipocytes and endothelial

cells but not on the hematopoietic progenitor cells, suggesting these two types of cells are not closely related (236). However, other studies have implied the existence of a pluripotent cell in the bone marrow that gives rise to hematopoietic cells and their microenvironment (237). This hypothesis has been supported by the finding that the cells comprising the microenvironment in long term marrow culture were derived from the same progenitor as the neoplasmic hematopoietic cells in leukemic patients (238), and that the marrow stroma grown in culture contained cells with hematopoietic potential in rats (239).

1.3.3.4 Interactions of Hematopoietic Cells and Their Microenvironment

Close association between hematopoietic cells and their environment is critical for homing during development, communication of regulatory signals, and compartmentalization of cells. Although the exact mechanisms by which the microenvironment influence hematopoiesis is not clear, direct cell-to-cell contact, production of ECM and secretion of cytokines by stromal cells are all involved in the control of the proliferation and differentiation of the hematopoietic cells (215).

Morphological studies using electron microscope have found intimate relationships between hematopoietic cells and the stromal cells (230). These studies showed that granulocytic and erythroid cells were closely associated to

distinct stromal cells. Direct cell to cell contact between stromal and hematopoietic cells has been demonstrated by the identification of an anatomical unit called erythroblastic island, where erythropoiesis occurs. This structure consists of a centrally located macrophage surrounded by maturing erythroblasts(206). Studies of hematopoietic differentiation in LTC have also shown that areas of erythropoiesis contained a central macrophage (240). Functional analyses have indicated that hematopoietic stem cells need to interact physically with stromal cells and are found preferentially in association with adherent stromal cells in LTC (231, 241, 242). Removal of stromal cells may interrupt the normal hematopoiesis (243). The intimate relationship between stromal cells and hematopoietic cells is the result of multiple interactions of the adhesion molecules and their receptors on the cell surface (227). The hematopoietic cells of different lineages may recognize distinct molecules on stromal cells. For example, fibronectin (Fn) interact with erythroid progenitor cells through VLA-5 (very late antigen) on their surface(244). Heparan sulfate on stromal cells could bind to Mac-1 and CD45, adhesion molecules of the β_2 integrin family, on hematopoietic progenitor cells (245). Another cell surface molecule of 30 kD has been identified to be responsible for the interactions of erythroid cells and macrophages (243). This molecule is found on both erythroid cells and macrophages, and the adhesion mediated by this molecule could be inhibited by heparin. Hematopoietic cells may also bind to the cytokines on the stromal cells. The hematopoietic growth factors in membrane-bound form, such

as M-CSF and KL, are also able to stimulate their target cells (246, 247). There is evidence indicating the membrane-bound factors are more potent than their soluble counterparts and may play a different role in the regulation of growth and differentiation (83, 248).

ECM molecules produced by stromal cells are important for cell adhesion. While granulocytic cells specifically bind to hemonectin (229), erythroid progenitors attach to Fn (244). Some of these molecules may also deliver proliferative signals to hematopoietic cells. This may occur directly or via intermediate molecules bound to the matrix. For example, Fn was found to stimulate erythropoiesis (249). Bone marrow GAGs could bind to growth factors and present them to hematopoietic cells locally (231). Heparin was later found to be the major component responsible for this binding (232).

Stromal cells in the microenvironment can produce a great number of hematopoietic growth factors, and the interactions of these factors may determine the outcome of hematopoiesis (209, 222). Since stromal cells are not only producers of hematopoietic cytokines, but are also regulated by them in an autocrine or paracrine fashion, the stromal-mediated control of hematopoiesis is far more complicated than originally thought. For example, when M-CSF is added to cultures, it acts directly on macrophage/monocyte precursors to stimulate their proliferation and differentiation (250). If high concentrations of this factor are used in LTMC, it stimulates macrophages to produce inhibitory activities such as TNFα,

which are capable of inhibiting the growth of multipotential, myeloid and erythroid progenitor cells (251). Thus, M-CSF acts both as a positive and negative regulator of hematopoiesis.

1.4 FETAL HEMATOPOIESIS

The course of ontogeny is associated with an orderly and predictable switch in the sites of hematopoiesis. In mammals the process begins in the yolk sac, migrates to the liver and spleen, and finally lodges in the bone marrow (BM), which remains hematopoietic throughout life (252). It is believed that the change in the sites of hematopoiesis is consequent to the migration of hematopoietic stem cells (HSC), via the blood stream, from one tissue to another (253). However, little is known of how one hematopoietic site in the embryo loses its hematopoietic potential and another site subsequently gains this potential. Recent studies demonstrated that the majority of the transplanted fetal HSC seeded in the developing bone marrow, while fetal liver was still the major hematopoietic organ (254). Although the HSC proliferated in the marrow, they did not participate actively in blood cell formation until the perinatal period, indicating the immaturity of marrow stroma for supporting differentiation and maturation of progenitor cells.

Yolk sac hematopoiesis is essentially extraembryonic and occurs in foci known as blood islands. The blood islands consist of outer cells that form a

network of vascular endothelium and inner cells that become free within the developing vessel lumen and develop into hematocytoblast and primitive normal blasts (233). The yolk sac-derived precursor cells produce megaloblastic nucleated red blood cells and appear insensitive to Epo (255). However, it is not clear whether this is due to Epo-independency or erythropoiesis is already maximally stimulated by Epo. The yolk sac environment is inductive only to erythropoiesis, and the development of other cell lines is not observed (252). Since there is evidence that yolk sac stem cells can differentiate into other lineages in vitro, regulatory factors from the yolk sac stroma are probably responsible for the erythroid preference (256, 257).

Hematopoietic activity in fetal liver is detectable in the mouse by day 10, and in human around weeks 5 to 6 of gestation (258, 259). Occurence of hepatic hematopoiesis is associated with the decline of hematopoiesis in yolk sac. Fetal liver becomes the major organ of erythropoiesis during mid-gestation, and erythropoietic precursor cells show increasing dependency on Epo. Fetal liver produces, in much lesser extend, some cells of granulocytic and megakaryocytic lineages as well (260). Other developing organs, such as thymus, spleen, kidney, and lymph nodes, also assume a hematopoietic function in fetal life.

The final phase of hematopoiesis during fetal development takes place in the bone marrow. The vascular mesenchyme forms a reticular network in the developing bone, where HSC can seed and proliferate. Hematopoiesis is heralded

by the appearance of undifferentiated basophilic cells in dilated marrow sinuses (252). The seeding of HSC is presumed to occur by migration of HSC from the liver via the bloodstream. In the mouse embryo, marrow hematopoiesis is limited to granulopoiesis. However, human embryonic marrow is erythropoietic from the beginning.

The tissue of origin of all stem cells is generally believed to be the yolk sac. With development progressing these cells migrate from extraembryonic sites to the fetal liver and finally colonize the bone marrow. This view is based on the studies in mice that demonstrate the dependence of intraembryonic hematopoiesis on an intact yolk sac (253). However, recent studies have found that HSC may also rise intraembyonically (261, 262).

The existence of differences between fetal and adult HSC is well documented (263). Fetal HSC has higher proliferative potential and shorter doubling times than adult cells when cultured in vitro (264, 265). The serial transplantability of CFU-S derived from the bone marrow of young or old mice is decreased when compared to CFU-S from the fetal liver and yolk sac (253). Cytokine requirements seem to be different. In vitro differentiation of embryo stem (ES) cells into hematopoietic cells occurs without exogenous hematopoietic growth factors, and the addition of these factors does not alter the kinetics of hematopoietic differentiation, even though the numbers of hematopoietic cells is markedly increased and the growth of multilineage cells is induced by adding the

hematopoietic growth factors (266). Their survival in culture, however, is prolonged in the presence of these growth factors. In vitro growth of BFU-E from the fetal cells does not require IL-3, which is involved in the development of adult hematopoietic colonies in culture (267, 268). Studies of gene expression during ES cell differentiation leading to hematopoietic development found that IL-3 and GM-CSF gene were not expressed in early ES cell differentiation, casting doubt about their role in early hematopoietic development (269). Although the KL and its receptor are expressed in early development, its essential role in embryonic hematopoiesis is open to question. It has been reported that hematopoietic precursor cells can still grow in the absence of KL (270). On the other hand, the requirement of some unidentified factors in embryonic hematopoietic development is evident (269, 271, 272).

In the following chapters, the purification and characterization of several heparin binding growth factors from fetal bovine serum acting on liver erythroid cells will be described. The isolation and establishment of a fetal liver stromal cell line, and the evidence that this cell line regulates fetal erythropoiesis through direct cell-cell contact and secretion of a soluble factor(s) will also be presented.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Materials and Chemicals for General Purposes

All chemicals for preparations of buffers and other solutions were purchased from Fisher Scientific (Montreal, Quebec), and Sigma (St. Louis, MO). Centricon™ microconcentrators were obtained from Amicon (Oakville, Canada).

2.1.2. Tissue Culture Supplies

Most materials and media for tissue culture were purchased from Gibco-Canada (Burlington, Ontario). Corning twelve well cell culture plates were purchased from Fisher Scientific (Montreal, Quebec) and sterile Titertube micro test tubes were from Bio-Rad (Missisauga, Ontario). Ficoll-Paque was from Pharmacia (Dorval, Quebec).

2.1.3. Radioactive Isotopes

Thymidine, methyl[^{3}H], specific activity 50 Ci/mmol, no. 24043, solution

containing 70% (vol/vol) ethanol in water was obtained from ICN (Irvine, CA).

2.1.4 Proteins and Cell Growth Supplements

Fetal bovine serum (FBS) was obtained from Flow Laboratories (Rockville, MD). Bovine serum albumin (BSA, Cohn fraction V), delipidated BSA and bovine apotransferrin (Tf) were purchased from Sigma (St. Louis, MO). Bovine insulin was either a gift of Lilly (Indianapolis, IN), or was purchased from ICN (Irvine, CA). Recombinant human erythropoietin (Epo) and recombinant human *c-kit* ligand were from R&D Systems (Minneapolis, MN). Recombinant human insulin like growth factor I (rhIGF I) was from Kabigen (Stockolm, Sweden). Recombinant human insulin like growth factor II (rhIGF II) was from Bachem California (Torrance, CA). Human apolipoprotein H was from Behringwerke (Marburg, Germany).

2.1.5. Cell Lines

CHO and NIH-3T3 cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained in F-12 medium and DMEM with 10% FBS, respectively.

2.1.6. Materials for Chromatography

The HPLC system, semipreparative μBondapak C_{18} column, μBondapak gel permeation columns and Shodex heparin affinity column (AF pak HR-894) were

obtained from Millipore-Waters (Milford, MA). Brownlee reversed phase columns (Aquapore RP-300) were purchased from Technical Marketing Associates (Massasauga, Ontario). Heparin-Sepharose was purchased from Pharmacia (Dorval, Quebec). ZetaChrom™ 250 QAE cartridges, and ZetaChrom™ 60 QAE disks, ZetaChrom™ 60 DEAE disks and ZetaChrom™ 60 SP disks were from Chemical Dynamics Corporation (South Plainfield, NJ).

2.2. METHODS

2.2.1. Affinity Chromatography on Heparin-Sepharose Columns

Heparin-binding proteins from fetal bovine serum were prepared by affinity chromatography on Heparin-Sepharose columns using a modification of the method described (273). Heparin-Sepharose (2 gm) was hydrated in 20 ml of 0.01 M tris buffer (pH 7.0) for 2 hrs, degassed and added to a 1 x 16 column slowly. The column was washed with 0.1 M NaCl in 0.01 M tris buffer (pH 7.0) for 6 hrs at a flow rate of 30 ml/hr with a Pharmacia pulsatile pump at a speed of 30 ml/hr. After washing, the column was ready for chromatography. The equilibrated heparin-Sepharose column can be reused many times.

FBS (100 ml) was loaded at 4° C to the equilibrated heparin-Sepharose column at a flow rate of 30 ml/hr, and unbound material was washed out with the tris buffer containing 0.1 M NaCl for 1 hr. The heparin-bound proteins were eluted using a linear gradient from 0.1 M to 1.6 M NaCl in 0.01 M tris buffer (pH 7.0) for

6.5 hrs at same flow rate. Fractions of 3 ml/tube were collected and aliquots of the fractions were taken for thymidine incorporation bioassay. The fractions showing stimulating activity in the bioassay were used for further purification.

2.2.2 Ion-Exchange Chromatography with ZetaChrom™ SP, DEAE and QAE Disks and Cartridges

2.2.2.1 Chromatography with ZetaChrom™ SP Disk

A ZetaChrom™ SP disk was equilibrated with 200 ml loading buffer (0.02 M ammonium acetate, pH 4.1) at a speed of 20 ml/min. 10 ml of FBS diluted with 100 ml loading buffer, pH 4.1 were pumped to the disk at a speed of 5 ml/min followed by washing of the disk with 50 ml of the loading buffer. The proteins retained were eluted with a buffer containing 5 mM HEPES and 0.5 M NaCl for fractions 1 to 5, 0.75 M NaCl for fraction 6 to 10, and 1.0 M NaCl for rest of fractions at a speed of 10 ml/min. Fractions of 10 ml were collected. Aliquots of 30 μl from each fraction were taken for the thymidine incorporation bioassay.

2.2.2.2 Chromatography with ZetaChrom™ QAE and DEAE disks

A ZetaChrom™ QAE disk was equilibrated with 200 ml loading buffer (0.015 M sodium phosphate, pH 7.0) at a speed of 20 ml/min. 10 ml of FBS diluted with 100 ml loading buffer, pH 7.0 were pumped to the disk at a speed of 5 ml/min followed by washing of the disk with 50 ml of the loading buffer. The proteins

retained were eluted with a buffer containing 5 mM HEPES and 0.5 M NaCl for fractions 1 to 5, 0.75 M NaCl for fraction 6 to 10, and 1.0 M NaCl for rest of fractions at a speed of 10 ml/min. Fractions of 10 ml were collected. Aliquots of 30 μl from each fraction were taken for bioassay.

The procedures for protein isolation with ZetaChrom™ DEAE disks were same as with QAE disks except that the pH of the loading buffer and elution buffer was adjusted to 6.3 instead of 7.0.

2.2.2.3 Chromatography with ZetaChrom™ QAE Cartridge

The samples isolated from heparin-Sepharose chromatography were pooled, diluted with 10 volumes of 0.01 sodium phosphate buffer (pH 7.0) and passed through a ZetaChrom™ 250 QAE anion-exchange cartridge, which had been equilibrated with the same buffer, at a flow rate of 20 ml/min. The cartridge was washed with 150 ml of the phosphate buffer, and the bound proteins were eluted with a linear gradient from 0 to 0.6 M NaCl in 5 mM Hepes-HCl buffer (pH 6.0) at a flow rate of 15 ml/min for 40 min. Fractions (15 ml/each) stimulating thymidine incorporation into liver cells were subjected to HPLC purification.

2.2.3 Reversed-Phase HPLC and Gel-Permeation HPLC

The samples obtained from chromatography with ZetaChrom™ QAE Cartridge were pooled, acidified with concentrated TFA (final concentration of TFA

in the samples was about 0.1%) and applied to a semipreparative Waters μbondapak C_{18} column with a Milton-Roy pump at a speed of 2-3 ml/min. The proteins were eluted with a gradient prepared with solution A [1% (v/v) acetonitrile in 0.1% TFA] and solution B (80% acetonitrile in 0.1% TFA). The percentage of solution B was increased from 20 to 55% in 30 min and then from 55 to 100% in 20 min with a constant flow rate of 2.5 ml/min. Fractions of 2.5 ml were collected. The bioactive fractions were then applied to a 10 cm Brownlee reversed-phase column (Aquapore RP-300) with a 3 cm pre-column of the same material. The proteins were eluted with a gradient prepared with solutions A and B as described above. The percentage of solution B was maintained at 0% for 5 min and was increased successively from 0 to 35% in 10 min, from 35 to 80%in 50 min and from 80 to 100% in 10 min. The flow rate was 0.3 ml/min. This separation step was repeated twice until the fractions with thymidine-incorporation-stimulating activity were separated from the thymidine-incorporation-inhibiting fractions. Further purification was obtained by gel-permeation HPLC (274). The samples from reversed-phase HPLC were evaporated with a gentle stream of N_2 to a volume about 50 to 100 μl and applied onto two I-125 Waters columns connected in series. Then the samples were eluted with 40% acetonitrile containing 0.1% TFA isocratically at a flow rate of 1.0 ml/min. BSA, ribonuclease and insulin were used as molecular weight markers. The final purification step employed reversed-phase HPLC using Aquapore columns as described above.

2.2.4 Heparin Affinity HPLC

Each sample was injected to a high performance heparin affinity Shodex AF column, which was first equilibrated with 0.01 M NaCl in 0.01 M tris-HCl buffer, pH 7.5, and eluted with a linear gradient of 0.01 to 1.0 M NaCl in the same tris buffer in 40 min at a flow rate of 0.6 ml/min at room temperature. The column was purged with 1.6 M NaCl in same buffer. The column was stored at 4° C in 0.01 phosphate buffer, pH 7 with 0.2% (w/v) sodium azide, as indicated by the manufacturer.

2.2.5 Preparations of Bovine Fetal Liver Cells

Bovine fetal liver cells were prepared according to the previous described methods (274). Bovine fetuses of 90 to 120 days of gestation (between 3 to 20 cm crown to rump length) were obtained from a local slaughterhouse. The fetal tissues were handled under sterile conditions. Bovine fetal livers were pooled together, cut to small pieces with a pair of scissors, and suspended in 10 to 15 ml of HBSS; then the broken tissue was forced through 18 1/2 and 21 1/2 needles with a 50 ml syringe in order to release fetal liver erythroid cells. Connective tissue which can not pass through the needles was left behind. HBSS was added to adjust the cell suspension to a volume of about 40 ml. The suspension was centrifuged at 700 x g for 5 min; the supernatant was removed and fresh medium was added to resuspend the cells. The cell suspension was stirred with a

magnetic stirrer at medium to high speed for 10 min, then filtered through four layers of cheese cloth to remove the dead cells and cell debris. Most dead hepatocytes ruptured and the resulting nucleoprotein fibers could be removed by this procedure. The filtrate was centrifuged; a yellow layer of non-erythroid cells on top of erythroid cell pellet was removed with a pasteur pipet. The cells were used immediately for the thymidine incorporation bioassay or colony formation assay, or were frozen for later use as indicated below.

2.2.6 Preparations of Frozen Fetal Liver Cells

Bovine fetal liver cells prepared as describe above were suspended in IMDM or F-12 medium at a concentration of 3.3 x 10^6/ml containing 50% of FBS (v/v) and 10% of dimethylsulfoxide (DMSO). DMSO was mixed with the medium first and cooled off on ice before slowly adding it to the cells suspended in FBS. Aliquots of 1.8 ml in cryovials were frozen with a Cole-Palmer Mini-Cooler for 40 min at speed 5 followed by 60 min at speed 10. The frozen cells were stored in liquid nitrogen for later use.

2.2.7 Thymidine Incorporation Bioassay

Fetal liver cells were suspended in FBS-free medium (F12K or a mixture of 75% F12 + 25% IMDM) containing 2 mM of glutamine, 30 μg/ml of Tf and 10 μg/ml of gentamycin. They were counted using trypan blue as a vital stain and

dispersed in Bio-Rad microtubes at 4 x 10^6 cells/0.1 ml/tube (fresh cells) or 2 x 10^6 cells/0.1 ml/tube (frozen cells). The samples to be tested (volume adjusted to 0.1 ml) were added to each tube. The final incubation volume was of 0.2 ml/tube. The cells in the microtubes were mixed thoroughly with a vortex and incubated in a humidified incubator at 37° C and with 5% CO_2 overnight. A [^{3}H]thymidine solution was prepared by evaporating the ethanol present in the [^{3}H]thymidine stock solution with a gentle flow of N_2 and subsequently adding the serum-free medium to give a final [^{3}H]thymidine concentration of 50 μCi/ml. 50 μl of this solution were added to each tube. The cell suspension was vortexed and incubated for 1 hr. The reaction was stopped by adding 0.5 ml ice-cold HBSS. Then the cells were centrifuged for 4 min a 700 x g. The supernatant was removed and the cells were washed 3 times. The cells were lysed with trichloroacetic acid. The cold acid-insoluble materials were hydrolysed and [^{3}H]thymidine was counted with a scintillation counter (275).

This method can also be applied to anchorage dependent cells (3T3 cells) with modifications (275). The 3T3 cells were trypsinized and suspended in RPMI 1640 medium containing 10% FBS (v/v)at a concentration of 10^5 cells/ml. 200 μl of the cell suspension were plated in each well of the 96-well plate (2 x 10^4 cells/well) and incubated overnight. Then the medium was removed, and the wells were washed with FBS-free medium for at least 2 times to eliminate residual serum components. Samples to be tested (100 μl) were added to the wells and

incubated for 16-18 hrs. Then the test solution was removed and the cells were incubated with the [^{3}H]thymidine solution (100 μl/well) for 1 hr. Subsequently, the wells were washed with cold HBSS 5 times and the TCA precipitated materials were hydrolyzed, mixed with scintillation fluid and counted. The radioactivities of the samples were counted.

2.2.8 Preparation of Fetal Liver Stromal Cells

Fresh bovine fetal liver cells (BFLS cells, 10^8 cells/ml) prepared as described above were cultured in a T75 flask (10 ml/flask) in Iscove's modified Dulbecco's medium (IMDM) supplemented with 30% fetal bovine serum (FBS) and gentamycin (10 μg/ml) at 37° C in a fully humidified incubator with 5% CO_2 in air. The supernatant was discarded after 4 days and replaced with fresh medium. After two weeks, non-adherent cells of the supernatant were collected and transferred to a new flask and cultured for 14 days. After two more identical passages, they formed a monolayer with a cobblestone morphology. The cells then were maintained in IMDM with 10% FBS.

2.2.9 Preparation of Hematopoietic Progenitor Cells

Frozen bovine fetal liver cells were quickly thawed and suspended in warm IMDM. Then the cells were centrifuged for 5 minutes at 200 x g and suspended briefly in a NH_4Cl solution to selectively destroy the mature erythroid cells (276).

Subsequently cells were suspended in 4 ml of IMDM and were carefully laid on top of a cushion of 3 ml Ficoll-Paque in a 15 ml plastic tube and centrifuged in a swinging bucket rotor at 400 x g for 20 min at 4° C. The mononuclear cells at interface between Ficoll-Paque and medium were collected, washed once, and suspended in serum free IMDM for clonogenic assays.

In early experiments, the fetal liver cells were separated over Ficoll-Paque without treatment with NH_4Cl. There were still some differentiated erythroid cells remained at interface. As a result, more cells were required to obtain the same number of erythroid colonies than those cells previously treated with NH_4Cl.

2.2.10 Preparation of BFLS Cell Conditioned Medium (CM)

Confluent BFLS cells were trypsinized, transferred to new T75-flasks at a density of 2 x 10^6 cells/flask, and cultured in 10 ml IMDM containing 10% FBS at 37° C and 5% CO_2 overnight. Then the BFLS cells were washed with 10 ml of serum free IMDM once, and cultured in 10 ml of serum free IMDM under the same conditions. Conditioned medium was collected after 1 day to 5 days of incubation, and the supernatant after centrifugation was kept at -20° C. Control medium incubated without cells was similarly prepared.

2.2.11 Preparation of Stock Culture Solutions

Methylcellulose stock solution (3%) was prepared according to Caro (277).

Deionized distilled water, 50 ml, was boiled and 3 g methylcellulose powder was slowly added with constant stirring. When the mixture was cooled down to room temperature, an equal amount of double-strength (2 x) IMDM containing gentamycin (20 μg/ml) and α-thioglycerol (2 x 10^{-4} M) was slowly added. The resulting mixture was stirred continuously at room temperature overnight and then stored at 4° C. 10% fatty acid free BSA (Cohn fraction V; Miles, Naperville, IL) was prepared according to Sawada et al (109). The lipid mixture of cholesterol, oleic acid and L-α-phosphatidylcholine was prepared according to Akahane et al (181) with exception that F-12 medium was used instead of IMDM, and $NaHCO_3$ was not added. Cholesterol (Fisher Scientific) was recrystallized in methanol before use. Bovine Tf was saturated with iron and diluted with F-12 medium to a final concentration of 3 mg/ml (109). Epo was diluted with IMDM containing 0.1% BSA to 10 U/ml. For stock solutions of vitamin A and E, 19 mg of Vit E were dissolved in 100 μl of ethanol. Then 10 μl of this solution was mixed with 1.82 mg Vit A and the volume was adjusted to 100 μl with ethanol. 10 μl of this mixture was mixed with 58 μl 10% BSA and adjusted to 5.8 ml with IMDM. For the hemin stock solution, 65 mg hemin were dissolved in 4 ml 0.5 M NaOH. 6 ml of 1 M Tris buffer (pH 7.8) were then added. The solution was filter sterilized and stored at 4° C.

2.2.12 Erythroid Cell Clonal Assays

2.2.12.1 Clonogenic Assay with High Serum Concentrations

Mononuclear cells (1 x 10^5 cells/ml) prepared without NH_4Cl treatment were cultured in 30-mm tissue culture plates in culture medium containing 0.8% methylcellulose, 30% FBS, 1% BSA, 1 U/ml Epo, and 1 x 10^{-4} M α-thioglycerol. Alternatively, NH_4Cl treated cells (1 x 10^4/ml) were cultured in 12-well tissue culture plates in the same conditions as described above.

2.2.12.2 Clonogenic Assay with Low Serum Concentrations

NH_4Cl treated fetal liver mononuclear cells separated by Ficoll-Paque were diluted to 1 x 10^4 cells/ml in IMDM containing 0.8% methylcellulose, 2% FBS, 1% BSA, 60 μg/ml transferrin, 8 μg/ml cholesterol, 5.6 μg /ml oleic acid, 8 μg/ml L-α-phosphatidylcholine, 0.3 μg/ml vitamin A, 0.33 μg/ml vitamin E, 130 μg/ml hemin, 30% F-12 medium, 1 x 10^{-4} M α-thioglycerol, and variable concentrations of Epo and/or IGF II. 0.5 ml of this cell suspension were cultured in 12-well culture plates. CFU-Es were counted after 3 days and BFU-Es were counted after 9 days of culture.

2.2.12.3 Coculture of Fetal Hematopoietic Progenitor Cells with Bovine Fetal Liver Stromal Cells

The bovine fetal liver stromal cells (BFLS cells) in the T-flask were trypsinized and cultured for two days in IMDM with 10% FBS in 12 well plates at a concentration of 2 x 10^4 cells/well. Then the wells were washed with FBS-free

IMDM once, and 0.5 ml of the fetal liver mononuclear cells (1 x 10^4/ml) prepared as described above were cultured onto the stromal cells in the same condition as indicated in section 2.2.12.2.

In some experiments, the stromal layer was overlaid with 0.4 ml culture medium containing 0.3% agar. After solidification of the agar layer, the mononuclear cells were cultured on top of the layer.

2.2.13 Carbon Particle Uptake

BFLS cells were cultured in 1:1000 India Ink in IMDM with 10% FBS. As control, CHO cells and NIH-3T3 cells were cultured under the same conditions except that F-12 medium and DMEM with 10% FBS were used, respectively.

2.2.14 Immunocytochemistry

BFLS cells were cultured on coverslips for 3-5 days, and fixed with cold acetone. Then the coverslips were treated with anti-vimentin (1:50) or anti-cytokeratin AE1/AE3 (1:10) monoclonal antibodies (Boehringer Mannheim Biochemica), followed by staining with the secondary anti-mouse Ig-Texas Red antibody according to the manufacture's instructions. 4',6'-diaminide-2'-phenylindole dihydrochloride (DAPI) was used to localize the cells.

2.2.15 Cytochemical Staining

BFLS cells were cultured either in the tissue culture plates for 3 to 5 days or on microslides overnight and fixed with cold methanol or cold acetone. Staining for acid phosphatase, alkaline phosphatase, glycogen by PAS, and lipids by oil red O was done following the published procedures (278).

2.2.16 Determination of the Molecular Weight of the Erythroid Factor(s) present in CM

BFLS cell conditioned medium (2 ml) was applied to Centricon™ microconcentrators with MW cutoffs of 100 kD, 10 kD and 3 kD and centrifuged in a fixed-angle centrifuge at speeds of 1000 x g, 5000 x g, and 7000 x g respectively until minimum volume of the retentates was achieved. The volume of the retentates was restored to the original by adding plain IMDM. The biological activities of the retentates and filtrates were tested with the erythroid clonal assay described above.

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF ERYTHROID CELL REGULATORS FROM FETAL BOVINE SERUM

Fetal bovine serum (FBS) is one of the most widely used growth supplements for tissue cultures. It contains a number of characterized and unknown growth factors which can either stimulate or inhibit cell proliferation and differentiation. Preliminary experiments carried out in our laboratory indicated that heparin-Sepharose affinity column could retain some erythroid cell-stimulating activities from FBS. As a consequence, my first project was to isolate and characterize the heparin-binding erythropoietic factor(s) in FBS.

3.1 THE DEVELOPMENT OF THE ISOLATION PROTOCOLS

The strategy to isolate the heparin-binding growth factors was to use the combinations of different chromatographic techniques followed by a bioassay method to trace the activities in the serum as shown in figure 3.1. After each step of purification by chromatography, a large number of fractions of samples have to be tested to trace the interested activities. This demands a simple, less time

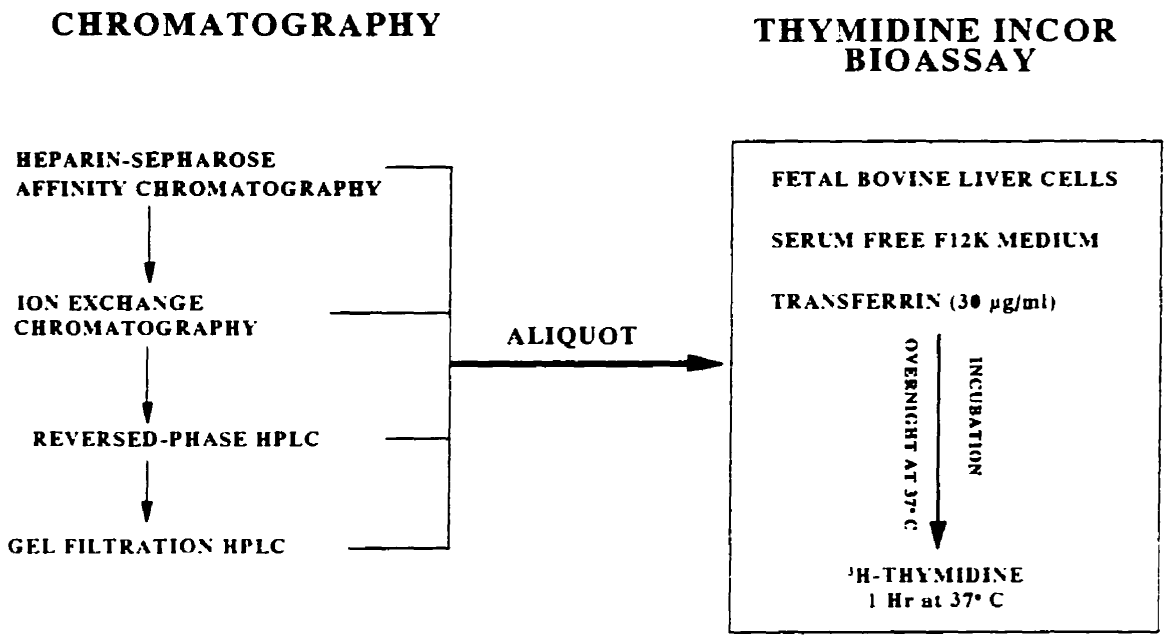


Figure 3.1. A working flow chart for the purification of heparin-binding factors. Several chromatographic techniques were combined to purify the heparin-binding factors from FBS. After each step of purification, aliquots from the fractions collected were tested using a serum-free thymidine incorporation bioassay method.

consuming, and sensitive bioassay system. In order to isolate the heparin-binding activities, a chemically defined serum free thymidine incorporation bioassay system was developed, which was described in detail in chapter 2.2.7. In this system, interference of FBS or other unknown factors with the sample tested was avoided because FBS was not required and all other components were chemically defined. Therefore, it is possible to quantitate the concentration of erythroid cell stimulating activities of FBS by measuring the incorporation of [^{3}H]thymidine into erythroid cells of bovine fetal liver (fig. 3.2).

Because the characteristics of the erythroid cell stimulating activity in FBS were unknown, pilot experiments were performed to determine which type of ion exchange chromatography should be used to purify the heparin-binding proteins. ZetaChrom™ DEAE disk (a weak anion exchanger), ZetaChrom™ QAE disk (a strong anion exchanger) and ZetaChrom™ SP disk (a cation exchanger) were tested for their ability to retain erythroid cell stimulating activity from FBS (fig. 3.3). 10 ml of FBS were diluted (1:10) with the loading buffers and applied to ZetaChrom™ disks. The proteins retained in the disks were eluted with buffers containing 0.5 M, 0.75 M, and 1.0 M of NaCl. Aliquots of each fraction of the samples separated with ZetaChrom™ DEAE disks (fig. 3.3A), ZetaChrom™ QAE disks (fig. 3.3B) and ZetaChrom™ SP disk (fig. 3.3C) were analyzed with the thymidine incorporation bioassay. The best result was obtained with ZetaChrom™ QAE disk, implying the erythroid cell stimulating factors are positively charged.

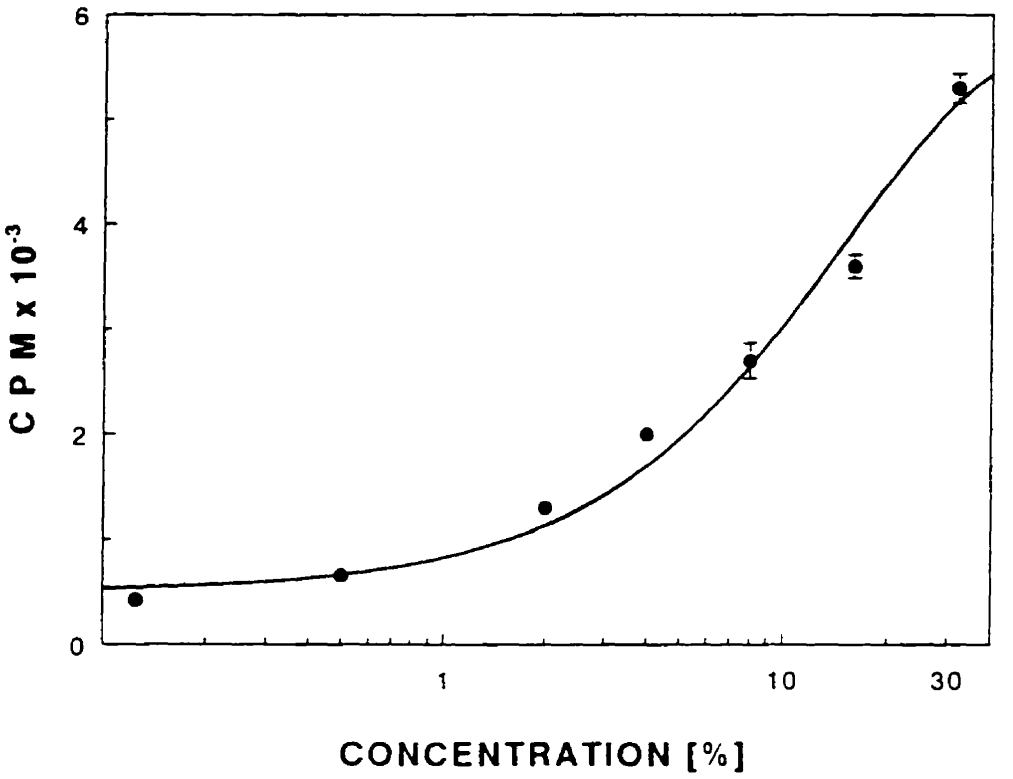


Figure 3.2. Effect of different concentrations of fetal bovine serum on thymidine incorporation into fetal liver erythroid cells. The cells were incubated in the presence of the indicated serum concentrations overnight, and [^{3}H]-thymidine incorporated into the cells was measured. Mean ± SE of triplicate determinations.

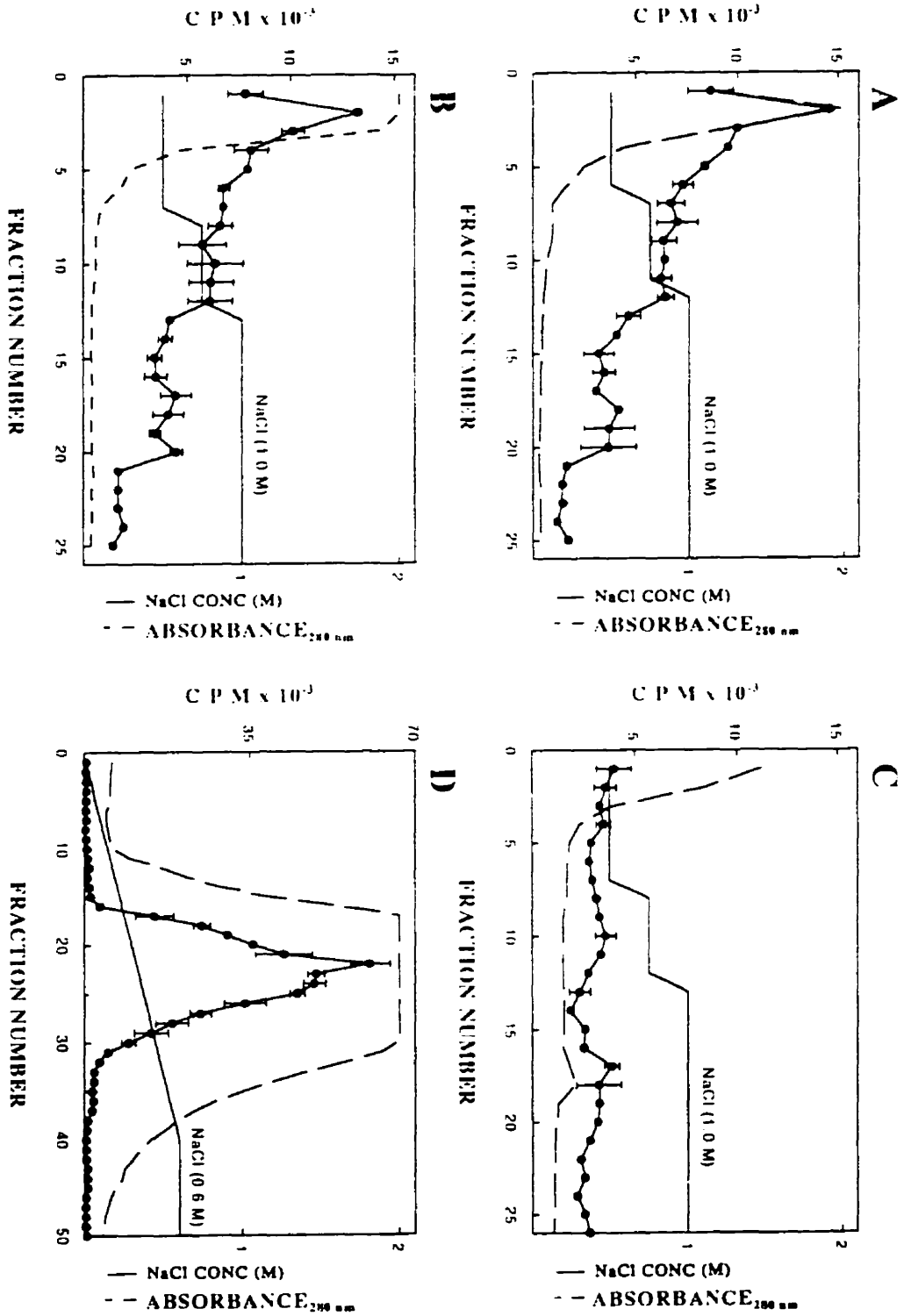

A
B
C
D
C P M x 10⁻³
FRACTION NUMBER
NaCl (1.0 M)
NaCl (0.6 M)
— NaCl CONC (M)
- - ABSORBANCE280 nm

Figure 3.3. Pilot experiments for the application of ion exchange chromatography. FBS (10 ml) was diluted and loaded to a ZetaChrom™ DEAE disk (A), a ZetaChrom™ QAE disk (B), or a ZetaChrom™ SP disk (C) respectively. The erythroid stimulating activity retained by each disk was determined by thymidine incorporation bioassay. Figure 3.3D shows the protein elution profile and the bioassay results of FBS applied to a ZetaChrom™ QAE cartridge as in (B) but eluted using a modified NaCl gradient. (---), absorbance. (●), thymidine incorporated into the cells. (———) NaCl concentration. Mean ± SE of triplicate determinations.

Subsequently, anion exchange chromatography with a ZetaChrom™ QAE cartridge (which contains the same material as a ZetaChrom™ QAE disk but has larger capacity to retain proteins) was chosen for the second step of purification. Because most of the bound proteins were eluted with 0.5 M NaCl (fig 3.3B), the concentration of NaCl of the elution buffer was adjusted to a continuous linear gradient of 0 to 0.6 M in elution buffer so that a better separation of the proteins could be achieved (fig. 3.3D).

3.2 ISOLATION OF ERYTHROID CELL STIMULATING ACTIVITIES

The components of FBS were first separated by heparin-Sepharose affinity chromatography as described in chapter 2. Most proteins bound to the heparin column eluted in fractions 31 - 50. These fractions stimulated thymidine incorporation into bovine fetal liver erythroid cells (fig. 3.4A).

The fractions containing erythroid cell stimulating activity after heparin-Sepharose chromatography were pooled together, and subject to ZetaChrom™ QAE chromatography to further purify the erythroid cell stimulating activities. Fig 3.4B shows that many fractions stimulated thymidine incorporation into erythroid cells. The fractions 25 to 27 from two batches of FBS, which contained the highest stimulating activities, were pooled together and further purified with reversed phase HPLC. Figure 3.5A shows the chromatographic profile and the thymidine incorporation activities of the sample purified by reversed phase HPLC with a

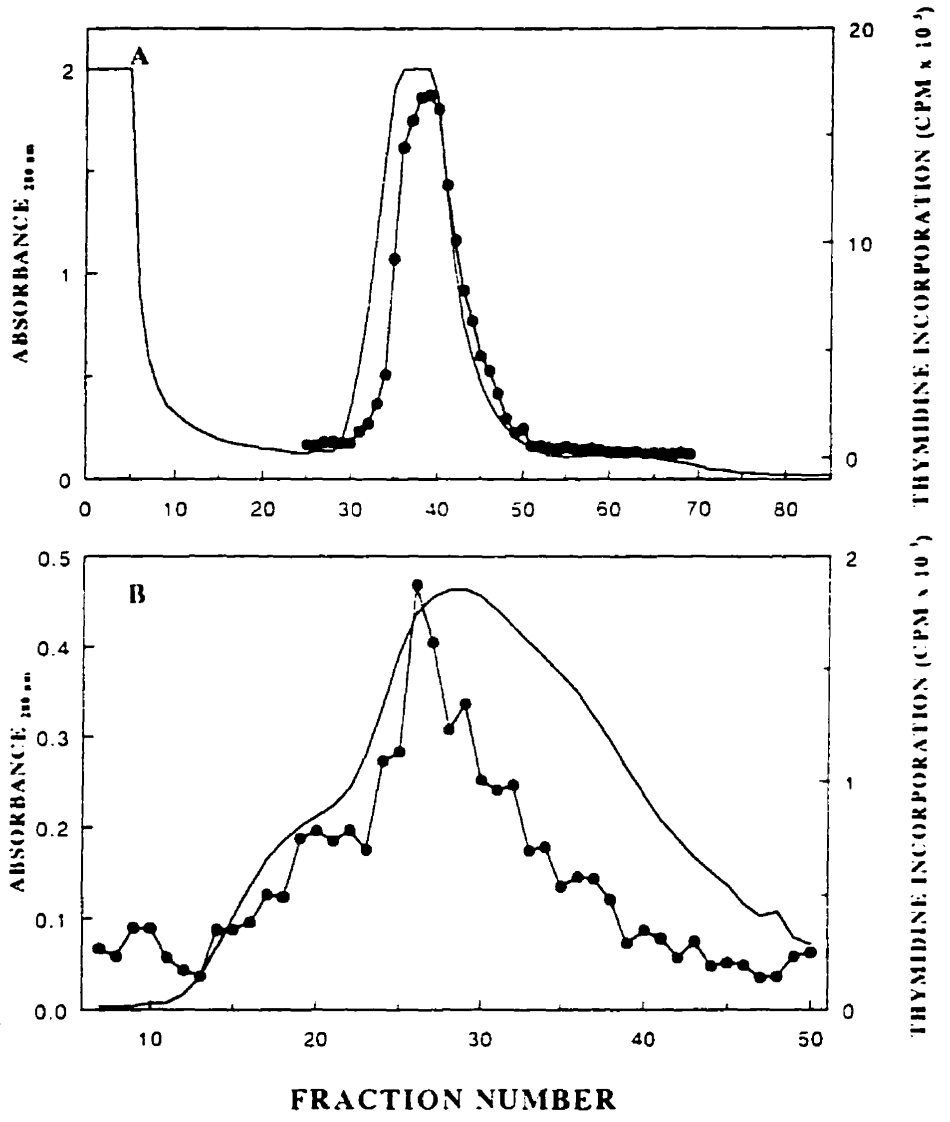

A
B
ABSORBANCE 280 nm
THYMIDINE INCORPORATION (CPM x 10⁻³)
FRACTION NUMBER

Figure 3.4. Initial purification of erythroid stimulating factors in FBS by heparin-Sepharose affinity chromatography followed by anion exchange chromatography. FBS was loaded onto a heparin-Sepharose affinity column, and the proteins bound were eluted with a linear gradient of NaCl. Aliquots from fractions of the sample were tested for thymidine incorporation activities (A). The fractions with erythroid stimulating activity were pooled together and further purified by ZetaChrom™ QAE anion chromatography (B). (———), absorbance. (●), thymidine incorporated into the cells.

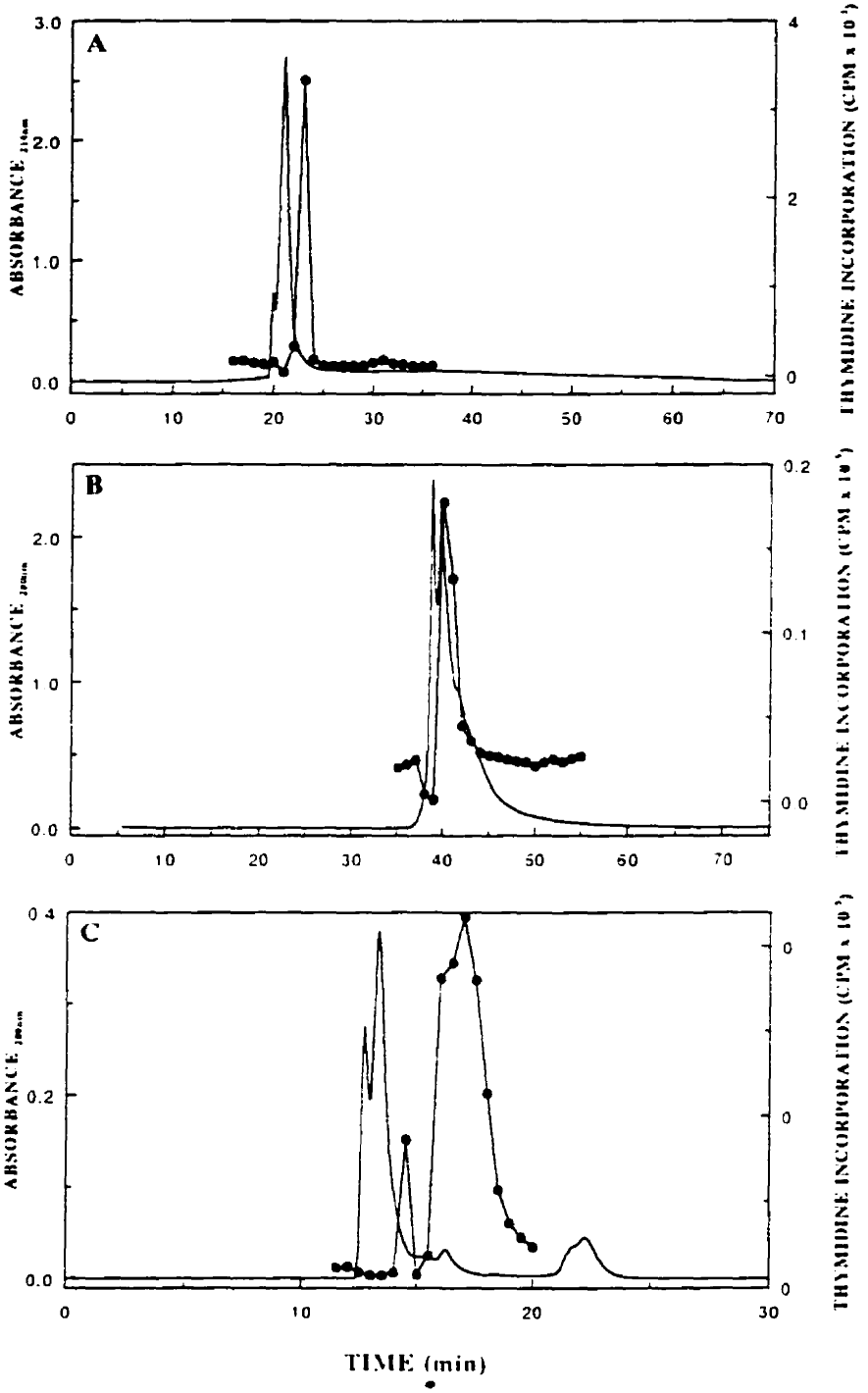

A
B
C
ABSORBANCE
THYMIDINE INCORPORATION (CPM x 10³)
TIME (min)

Figure 3.5. HPLC purification of the erythroid stimulating activity in the serum. The pooled sample from figure 3.4B was subjected to reversed phase HPLC by a semipreparative column as described in chapter 2 (A). The erythroid stimulating activity was further purified by Aquapore reversed phase HPLC (B) followed by gel permeation HPLC (C). (———), absorbance. (●), thymidine incorporated into the cells.

Waters μBondpak C_{18} semipreparative column. The highest thymidine incorporation activities fell in fraction 23. This fraction was well separated from other proteins, indicating the erythroid cell stimulating activities were highly enriched after this purification step. Fractions 21 and 22 of the sample seemed to inhibit thymidine incorporation. These fractions were kept and analyzed with other methods as will be discussed later. The fraction 23 was purified with Aquapore reversed phase HPLC. After this purification, erythroid cell stimulating and inhibitory activities were evident with the thymidine incorporation bioassay (fig. 3.5B). The fractions containing stimulating activities were pooled, concentrated with a stream of N_2 gas, and purified with gel permeation HPLC, which separates samples according to their molecular weights (fig. 3.5C). The apparent molecular weight of the erythroid cell stimulating factor(s) was smaller than 10 kD. Most proteins were excluded from the stimulating activity, which was enriched in fractions 32 to 35 (between 16 to 17.5 minutes of elution time). These fractions were further purified by Aquapore chromatography with a shallow gradient of acetonitrile (fig. 3.6A) followed by another round of gel permeation HPLC (fig. 3.6B). A single symmetric peak of sample with strong erythroid cell stimulating activity was obtained after the final step of purification. It had an apparent molecular weight of about 8 kD on gel permeation HPLC, as measured using bovine serum albumin, RNase and insulin as standards. The peptide stimulated thymidine incorporation into fetal liver erythroid cells in a dose

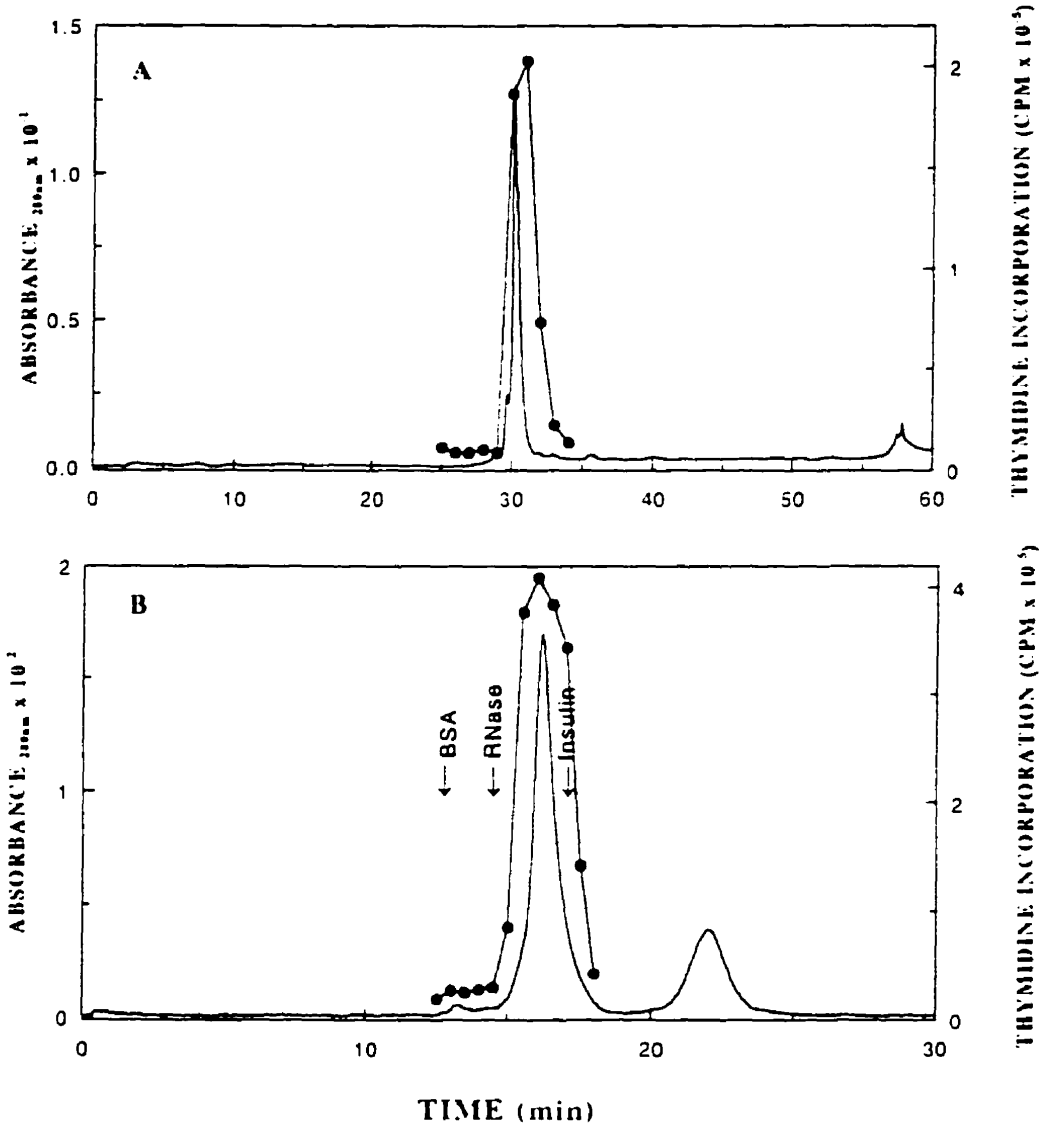

A
B
ABSORBANCE 280nm x 10⁻¹
ABSORBANCE 280nm x 10⁻²
THYMIDINE INCORPORATION (CPM x 10⁻⁵)
THYMIDINE INCORPORATION (CPM x 10⁻⁵)
BSA
RNase
Insulin
TIME (min)

Figure 3.6. Further purification of erythroid stimulating activity by reversed phase HPLC. The samples containing erythroid stimulating activity in figure 3.5B were loaded to Aquapore reversed phase HPLC column and eluted with a very shallow gradient of acetonitrile (A). The molecular weight of the purified peptide was determined by gel permeation HPLC with BSA, RNase, and insulin as standard molecular weight markers (B). (———), absorbance. (●), thymidine incorporated into the cells.

responsive manner (fig. 3.7). The N-terminal sequence of this peptide was A Y R P S E T L X G G E L V D T L Q F V X G D R G F Y F S R P, which was identical to the published N-terminal sequence of bovine IGF II/erythrotropin. This result was surprising because to the best of our knowledge there were no reports on the possible heparin-binding properties of IGFs. This result also raised the question that whether IGF II could bind to heparin directly or indirectly through IGF binding proteins, and whether other members of IGF family could also bind to heparin. The study on this question was carried out by using heparin affinity HPLC (fig. 3.8) with human apolipoprotein H as a reference. Insulin came out with the void volume, indicating its lack of affinity to heparin. Both IGF I and IGF II bound to heparin, but IGF II had higher affinity to heparin than IGF I. Because heparin enhances the biological activities of fibroblast growth factors (FGFs), which are well-known heparin-binding peptides (279), it was possible that this glucosaminoglycan may alter the biological activity of IGFs as well. This possibility was studied by adding different concentrations of heparin into cell culture with and without IGF II. The addition of porcine heparin alone did not stimulate thymidine incorporation into fetal liver erythroid cells. However, heparin enhanced the effect of IGF II (fig. 3.9). This result suggests the possible involvement of extracellular matrix components on the regulation of the effects of IGFs on cell proliferation.

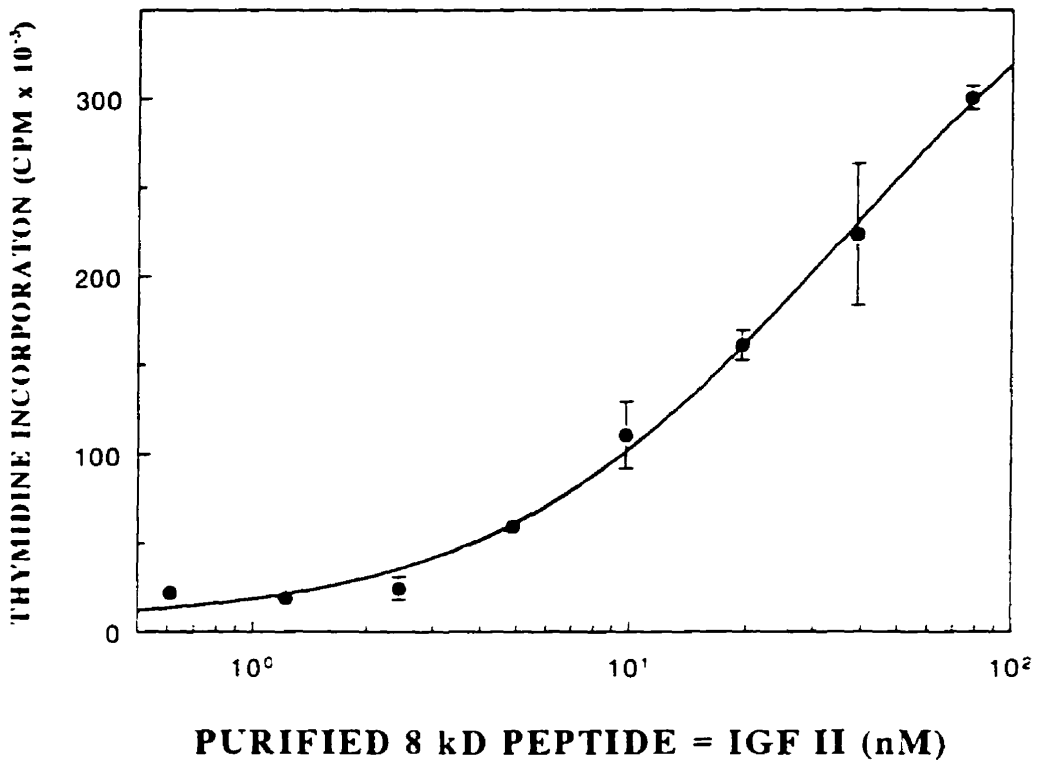


Figure 3.7. Effect of the purified IGF II-like peptide on thymidine incorporation into fetal liver erythroid cells. Different concentrations of the purified IGF II-like peptide were incubated with fetal liver cells overnight and [^{3}H]-thymidine incorporated into the cells were determined as described in chapter 2. Mean ± SE of triplicate determinations.

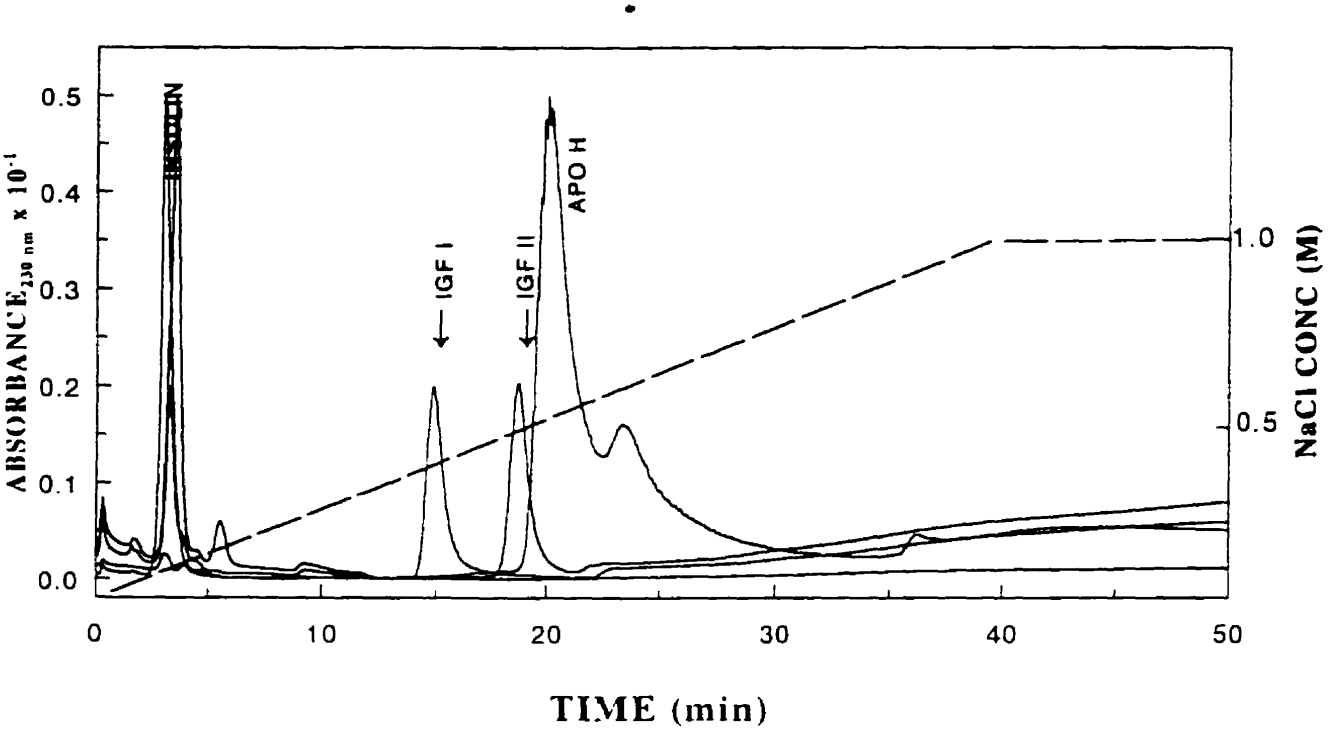


Figure 3.8. Heparin affinity chromatography of purified peptides. The samples (2 μg each) were applied to a Shodex heparin affinity column and eluted with a linear gradient of NaCl (- - -). The samples eluted were monitored at 230 nm.

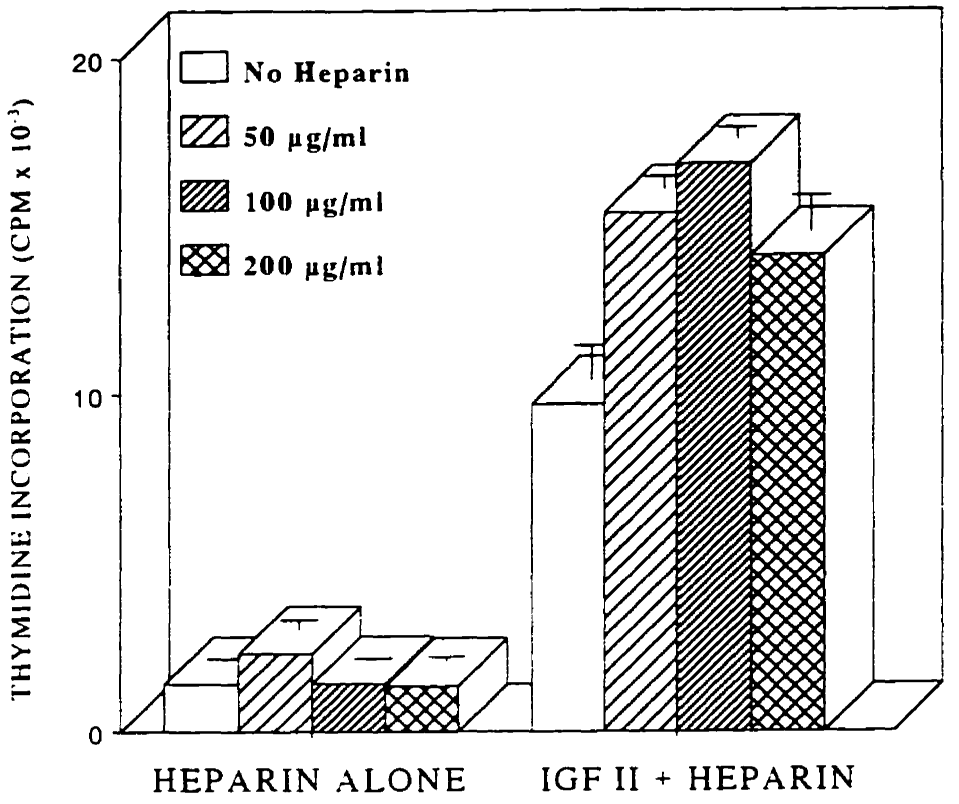


Figure 3.9. Effect of heparin on thymidine incorporation into fetal liver cells. Different concentrations of porcine heparin were incubated with fetal liver cells in the absence or presence of 8 nM of IGF II, and thymidine incorporated into the cells was determined as described. Mean ± SE of triplicate determinations.

3.3 ISOLATION OF A BOVINE APOLIPOPROTEIN H-LIKE PROTEIN AS AN ERYTHROID CELL INHIBITOR

During the process of purification of erythroid stimulating factors, it was noticed that, in addition to the erythroid stimulating activity, some fractions collected from HPLC purification procedures contained thymidine incorporation inhibiting activities (fig. 3.5). The inhibitory activity could be observed in the samples eluted immediately before the stimulating activity from reversed phase HPLC and was not evident before the step of HPLC purification. Fractions 21 and 22 of the sample from figure 3.5A. which had an apparent erythroid cell inhibitory activity, were further purified individually by Aquapore reversed phase HPLC. Aliquots of the HPLC fractions were tested for their effects on thymidine incorporation into bovine fetal liver erythroid cells. In some experiments, the batches of fetal liver cells used for thymidine incorporation did not respond well to the stimulating activity of fetal bovine serum, but were very sensitive to the inhibitory activities present in the preparations (fig. 3.10A). The fractions 41-42 and 43-44 of the sample from figure 3.10A were pooled together into two groups because it seemed that the compositions of fractions 41-42 were different from those of fractions 43-44. Fractions 41-42 from figure 3.10A were analyzed by gel permeation HPLC. There were apparently two different groups of proteins with inhibitory activities (fig. 3.10B). Most inhibitory activities fell into the second (smaller) peak. The fractions containing the inhibitory activity with retention times

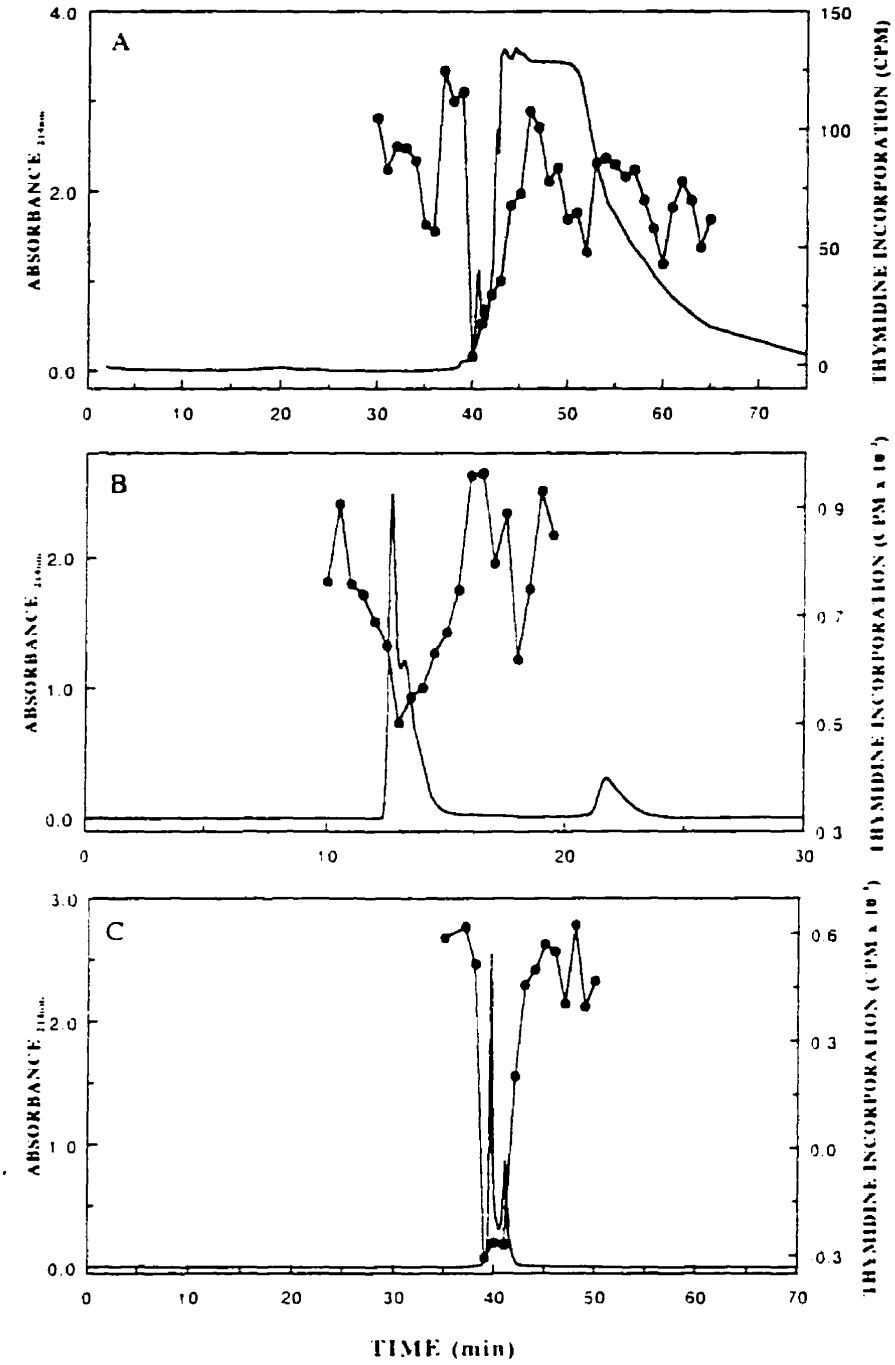

A
B
C
ABSORBANCE
THYMIDINE INCORPORATION (CPM)
TIME (min)

Figure 3.10. Purification of erythroid inhibitory activity by HPLC. Fractions 21 and 22 from the sample in figure 3.5A were pooled together and subjected to Aquapore reversed phase HPLC (A). The fractions which inhibited thymidine incorporation into fetal liver cells (fraction 40 and 41 from fig. 3.10A) were further purified by gel permeation HPLC (B) followed by another round of Aquapore HPLC (C) as detailed in chapter 2. (———), absorbance. (●), thymidine incorporated into the cells.

between 13-14.5 minutes from figure 3.10B were purified with Aquapore reversed phase HPLC (fig. 3.10C). There were two major fractions with absorbance at 214 nm, but it was not clear which one contained the inhibitory activity. Consequently, each fraction was further purified by gel permeation HPLC. The fractions 39 and 40 from figure 3.10C seemed to be identical on the gel permeation HPLC profile, and the inhibitory activity was concentrated in the second peak (fig. 3.11A). The fraction 41 from figure 3.10C did not have any inhibitory activity and was not further analyzed (results not shown). The final purification of the inhibitor (fractions 27-31 of fig. 3.11 A) was carried out by Aquapore reversed phase HPLC with a more shallow gradient of acetonitrile (fig. 3.11B). A single sharp symmetric eluent peak was obtained with inhibitory activity falling exactly into the fractions containing the protein peak. The molecular weight of this protein was 46 kD by gel permeation HPLC (fig. 3.11C) and 50 kD by SDS-PAGE of a single band under reducing conditions (result not shown). The N-terminal amino acid sequencing revealed 81% identity with the published sequence of human apolipoprotein H (apo H), also known as β_2-glycoprotein I (fig. 3.12).

The effects of the purified bovine apo H-like protein on thymidine incorporation were compared with commercially available human apo H (fig. 3.13). Thymidine incorporation into bovine fetal liver erythroid cells was inhibited by bovine apo H-like protein in a dose response manner with an ED_{50} of 38 nM. At high concentrations, thymidine incorporation was completely inhibited. Bovine apo

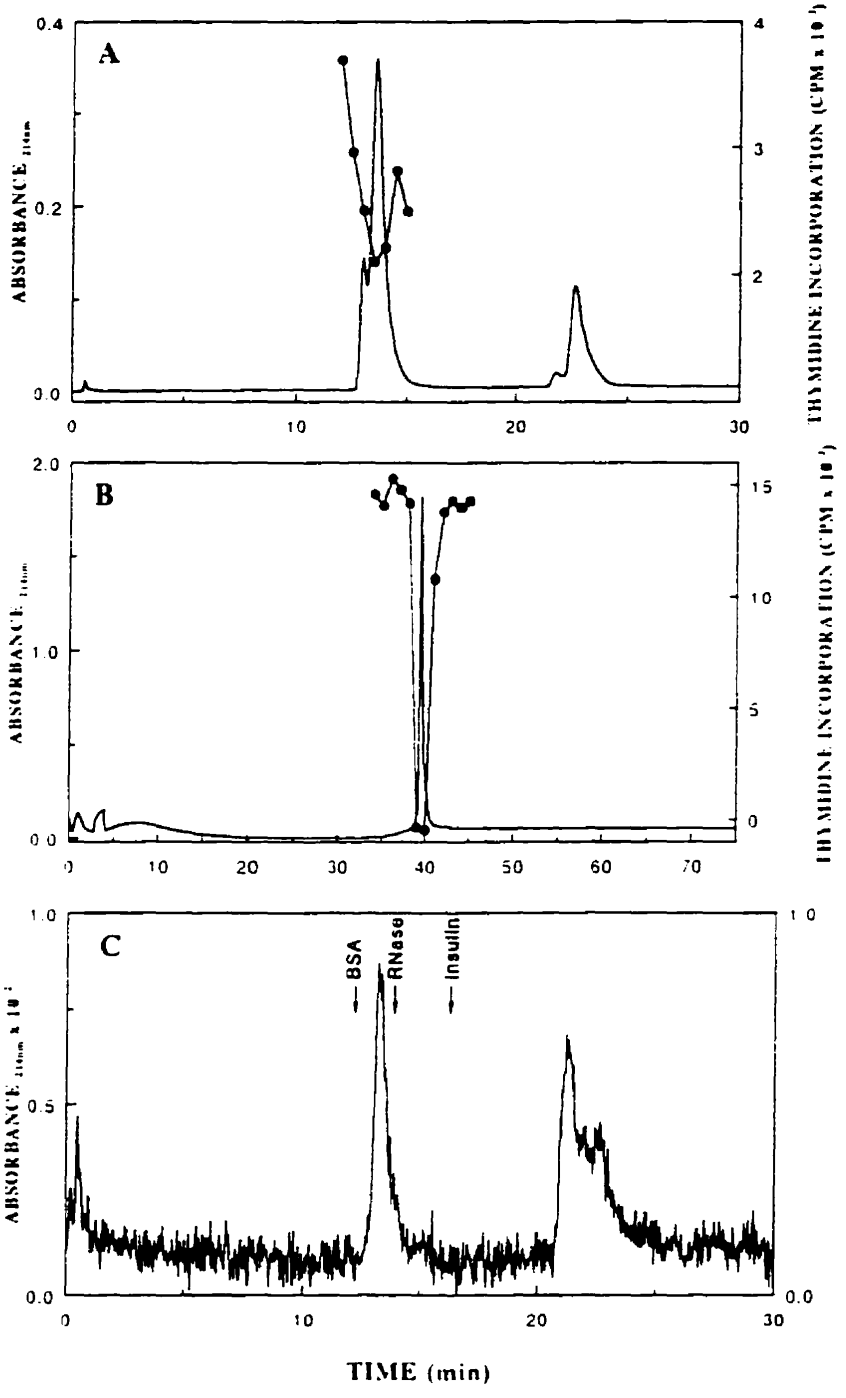

A
B
C
BSA
RNase
Insulin
TIME (min)

Figure 3.11. The final steps of purification of the erythroid inhibitory activity by HPLC. The sample containing erythroid inhibitory activity was purified by gel permeation HPLC (A), which gave rise to a single elution peak from Aquapore HPLC (B). The molecular weight of the purified protein was determined by gel permeation HPLC with BSA, RNase, and insulin as molecular weight markers (C). (———), absorbance. (●), thymidine incorporated into the cells.

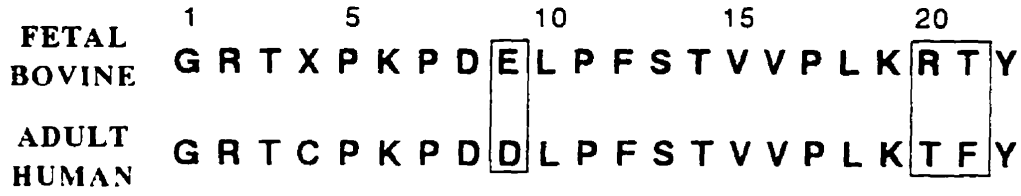


Figure 3.12. Comparison of the N-terminal sequence of the purified 48 kD erythroid inhibitor and the N-terminal sequence of human apo H ($ß_2$-glycoprotein I). The human apo H sequence was found using the program FASTP of the Protein Identification Resource of the National Biomedical Research Foundation, Georgetown University, WA, USA. Boxes indicate sequence differences.

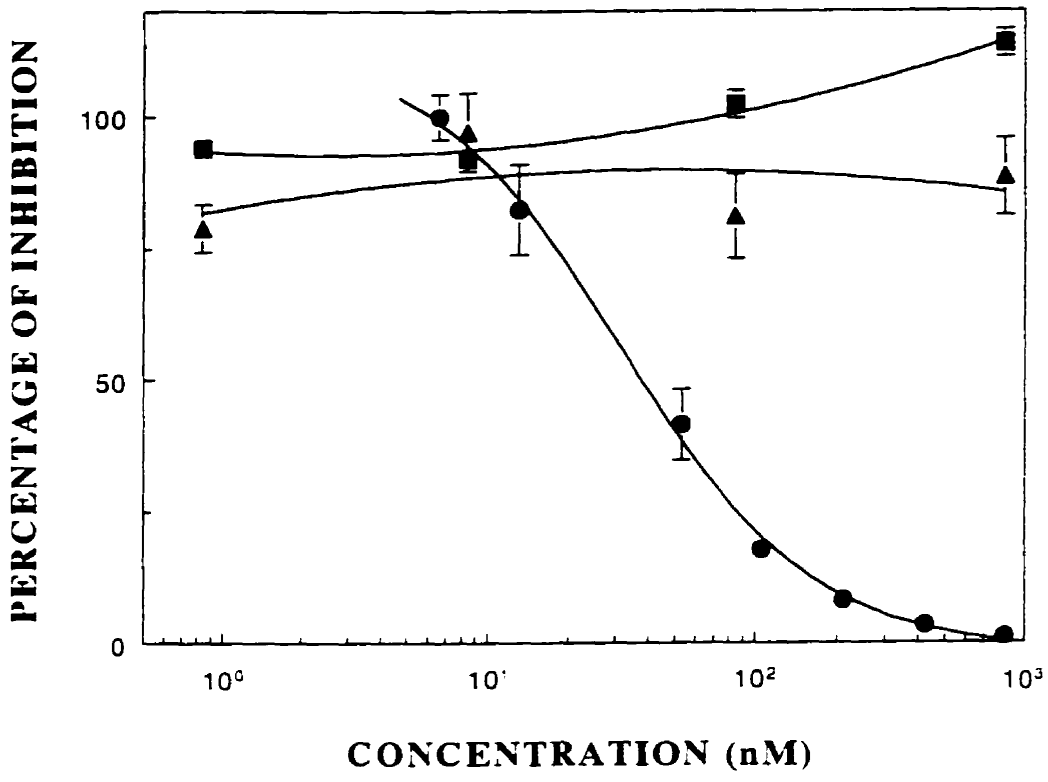


Figure 3.13. Effect of the purified bovine apo H-like protein on thymidine incorporation into fetal bovine liver cells. The purified protein in figure 3.11B at indicated concentrations was incubated with fetal bovine liver cells overnight; then thymidine incorporated into the cells was measured (●). NIH 3T3 cells were used as controls (■). Human apo H did not inhibit thymidine incorporation into bovine fetal liver cells (▲). Mean ± SE of triplicate determinations.

H-like protein did not have any effect on thymidine incorporation into NIH 3T3 cells at the highest concentration tested. Human apo H did not exert any inhibitory effect on bovine fetal liver cells. This lack of inhibitory activity of human apo H on bovine cells and that of bovine apo H-like protein on mouse cells may be due to the species specificity of the protein. The specificity of the erythroid regulators was also tested by comparing their effect on bovine and rat fetal liver erythroid cells during the purification process. Rat fetal liver cells were sensitive to the stimulatory activities but were not affected at all by inhibitory activities (data not shown).

The effects of bovine apo H-like protein on cell viability was also studied. At the highest concentration of bovine apo H-like protein tested (840 nM), [^{3}H]thymidine incorporated into fetal liver erythroid cells was completely inhibited even though 60.1% of the cells were still viable. This result indicated the bovine apo H-like protein inhibited fetal erythropoiesis mainly through a cytostatic, rather than a cytotoxic effect.

3.4 ISOLATION AND CHARACTERIZATION OF AN 11 KD ERYTHROID CELL INHIBITOR

The fractions 19 to 25 of the samples from anion exchange chromatography (fig. 3.4B) were pooled together and subject to semipreparative reversed phase HPLC, because the chromatographic profile implied the possible existence of a mixture of erythroid cell stimulating activities and inhibitory activities. It was a

surprise for us that this sample contained a very potent cytolytic activity (fractions 19-26), which was not species specific and destroyed both bovine fetal liver cells and human peripheral red blood cells (used here as carrier cells, fig. 3.14). The cytolytic nature of the inhibitory activity was indicated by the disappearance of red cells seen at the bottom of the microtubes after centrifugation, and the negative values of [^{3}H]thymidine incorporation comparing to the control values. These fractions were further purified with the combination of reversed phased HPLC and gel permeation HPLC as applied for the purification of the other factors (fig. 3.14 and fig. 3.15). After the last step of purification by gel permeation HPLC, a single peak with an apparent molecular weight of 11 kD was obtained. Nevertheless, N-terminal sequence analysis of the sample revealed that this peak contained a mixture of two peptides with equal ratio of 50:50. One of them had the N-terminal sequence of IGF II and the other one corresponded to the sequence of anaphylatoxin C4a, a fragment of complement 4 of the immune system. Since IGF II and its precursors are the major erythroid cell stimulating factors of fetal bovine serum, and all inhibitory activities coelute with IGF II, it is easy to understand that their presence in the preparations of the 11 kD inhibitor. The mixture was further purified with more shallow gradients of acetonitrile by Aquapore reversed phase HPLC. The dose response curves of the samples indicated the considerable enrichment of the inhibitory activity (fig. 3.16). At high concentrations [^{3}H]thymidine incorporation was below 0% (0% corresponds to [^{3}H]thymidine

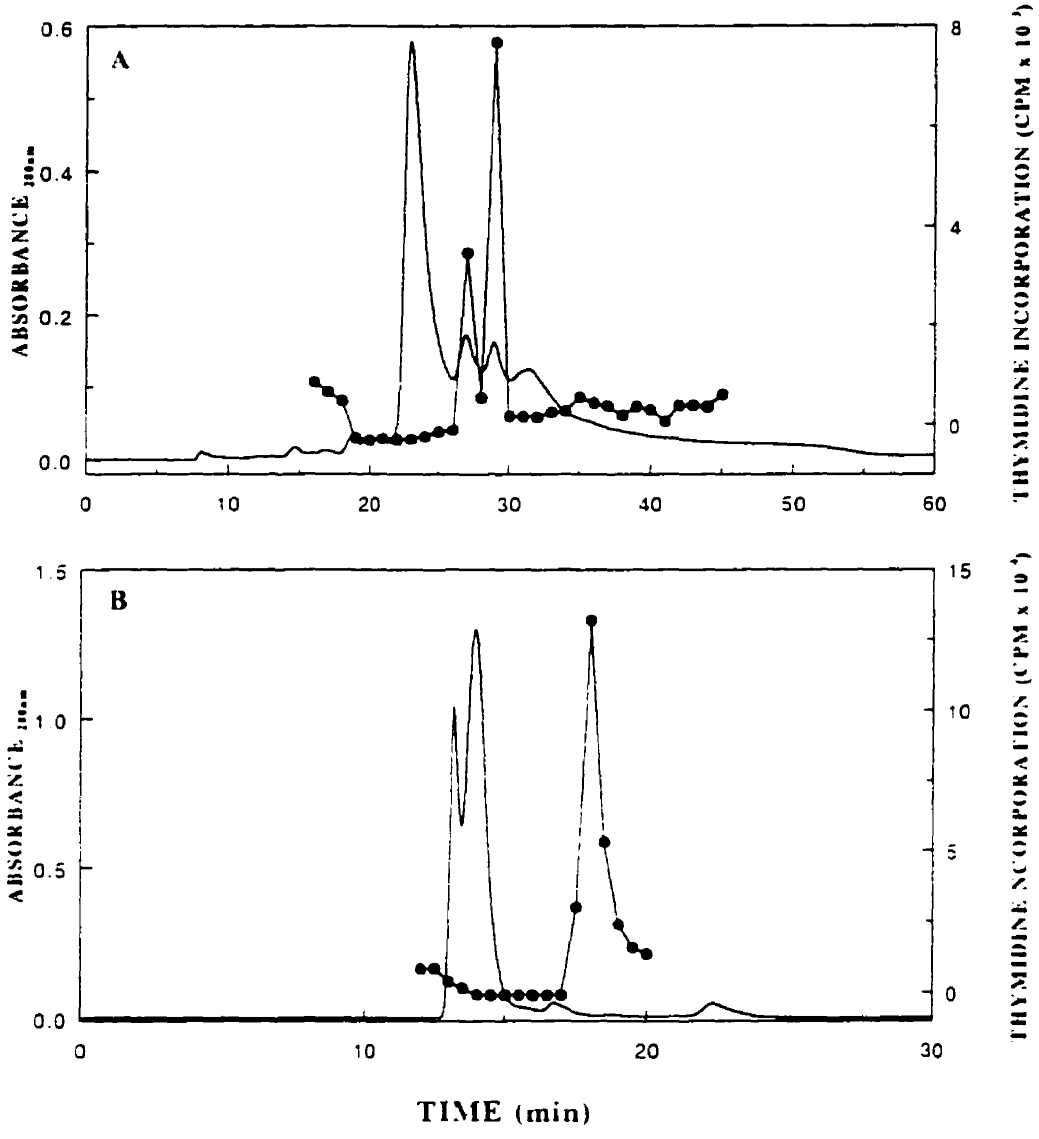

A
B
ABSORBANCE
THYMIDINE INCORPORATION (CPM x 10⁻³)
TIME (min)

Figure 3.14. Appearance of a cytolytic inhibitory activity after reversed phase HPLC. Fractions 19 to 25 were pooled together and purified by reversed phase HPLC with a semipreparative column (A). The inhibitory activity was then subjected to gel permeation HPLC (B). (———), absorbance. (●), thymidine incorporated into the cells.

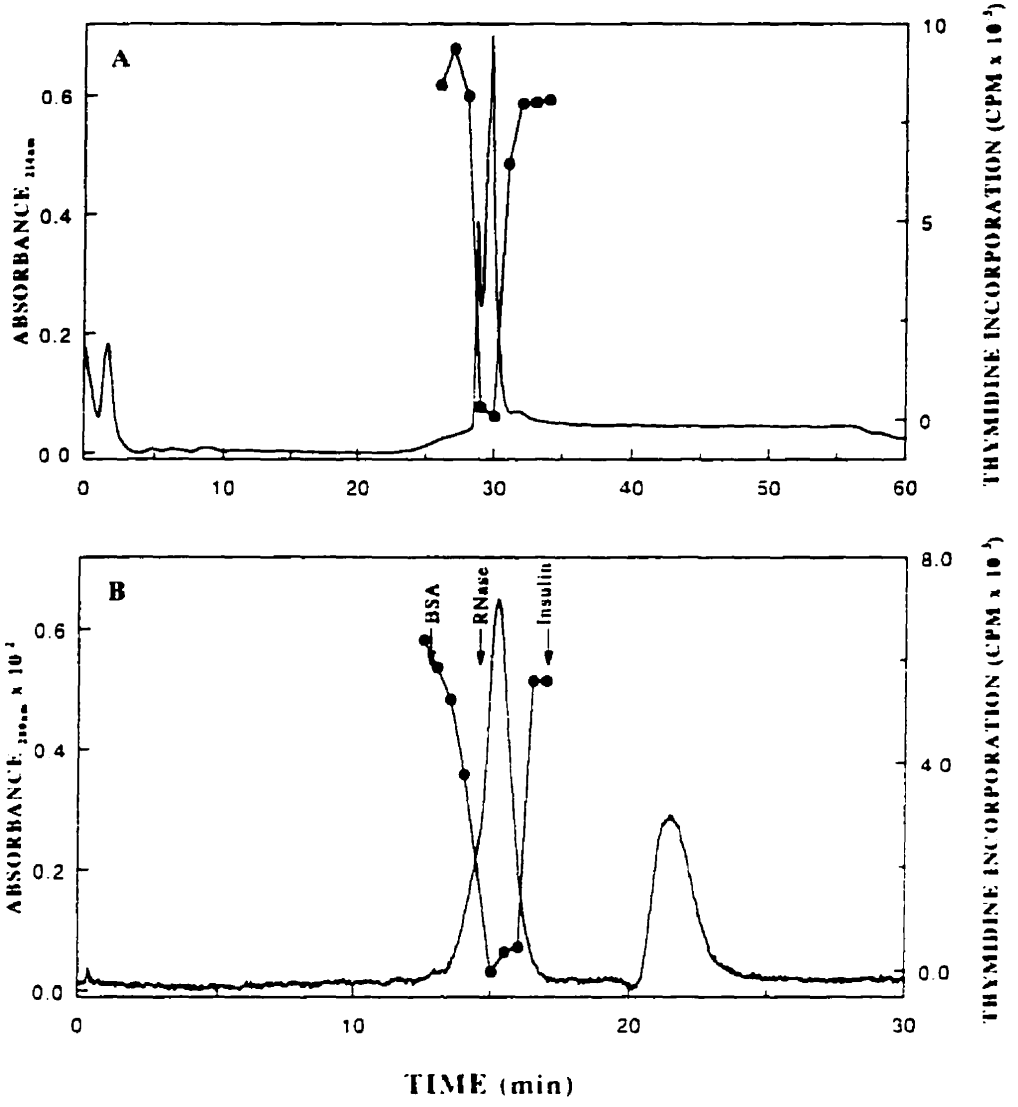

A
B
BSA
RNase
Insulin
ABSORBANCE 280nm
ABSORBANCE 280nm x 10⁻²
THYMIDINE INCORPORATION (CPM x 10⁻³)
TIME (min)

Figure 3.15. Further purification of the cytolytic inhibitory activity. Each fraction of the sample from fig. 3.14B was subject to Aquapore reversed phase HPLC individually (a representative profile of the cytolytic inhibitory activity after Aquapore reversed phase HPLC (A) is shown). The molecular weight of the peptide was determined by gel permeation HPLC just before the peptide was sequenced (B). (———), absorbance. (●), thymidine incorporated into the cells.

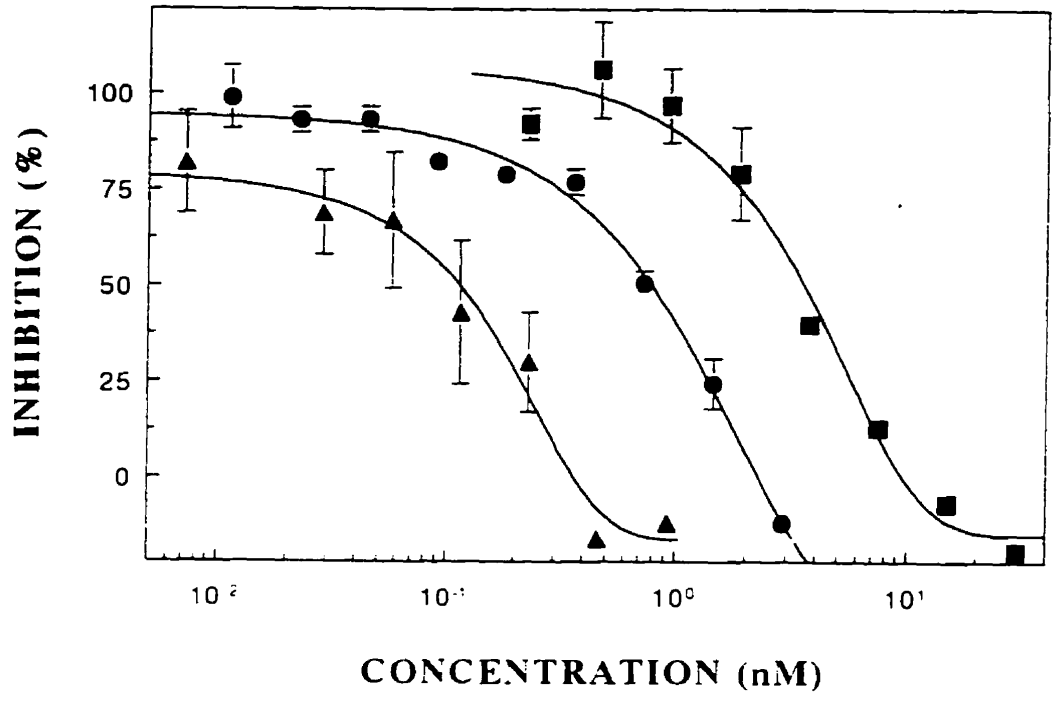


Figure 3.16. Effect of the 11 kD inhibitor at three different stages of purification on fetal liver erythroid cells. (■), Partially purified preparation from fig 3.15B containing a mixture of peptides similar to IGF II and anaphylatoxin C4a. (●) and (▲), preparations used after one and two furhter purification steps, respectively. 0% indicates thymidine incorporation equal to that at time 0 hr. 100% indicates incorporation after a 1-hr incubation of control cell cultures with [^{3}H]-thymidine. Values lower than 0% are due to destruction of the cells. Mean ± SE of triplicate determinations.

incorporation at time 0 in control cell cultures) and most of the fetal liver cells and mature human red blood cells were destroyed (fig. 3.16). The effective inhibitory dose (ED_{50}) was shifted from about 36 ng/ml to 1.1 ng/ml, which was about 0.1 nM of the peptide. Unfortunately, there was not enough peptide for an N-terminal sequence analysis with the preparation after the final purification.

3.5 COMPARISON OF THE EFFECT OF APO H-LIKE PROTEIN WITH THAT OF C4A-LIKE PEPTIDE

The C4a-like peptide is about 1000 times more potent than the apo H-like protein. Therefore, it is possible that the inhibitory activity of bovine apo H-like protein might be due to the contamination with the C4a-like peptide. Figure 3.17A shows that the bovine apo H-like protein could inhibit [^{3}H]thymidine incorporation completely at high doses, reaching a plateau at a concentration of 420 nM. 60% of the erythroid cells were still alive after overnight incubation with a protein concentration of 840 nM. Furthermore, the effects of the bovine apo H-like protein seemed to be species specific as indicated above (fig 3.12 and 3.17B). On the other hand, the C4a-like peptide was strongly cytolytic, destroying both bovine and human erythroid cells in minutes. [^{3}H]Thymidine incorporation was below 0% at a peptide concentration of 0.5 nM and lower, which was another indication that erythroid cells in the microtubes were destroyed. Another major difference between them was their different elution time in gel permeation HPLC. All these data demonstrate that there are at least two erythroid cell inhibitors in fetal bovine serum, with different molecular weight and different mechanisms of action.

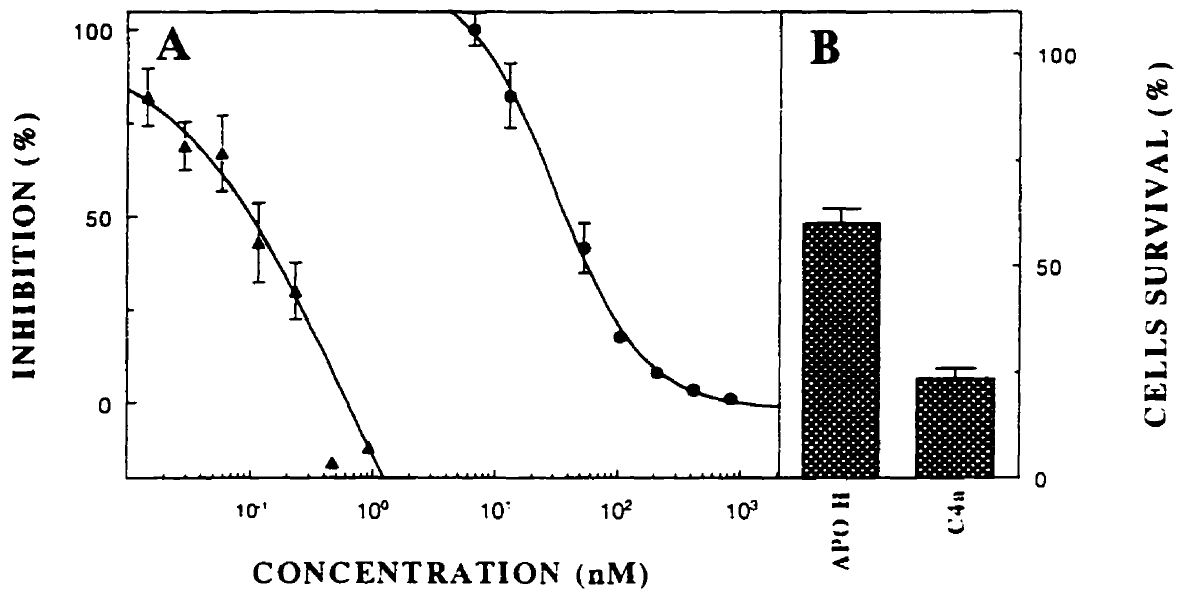


Figure 3.17. Comparison of the apo H-like inhibitor and the C4a-like inhibitor. Various concentrations of the apo H-like inhibitor (●) and C4a-like inhibitor (▲) were studied for their ability to inhibit thymidine incorporation into fetal liver erythroid cells (A), and their ability to lyse the cells (B). The concentrations of apo H-like inhibitor and C4a-like inhibitor in (B) were 840 nM and 0.9 nM, respectively. Mean ± SE of triplicate determinations.

CHAPTER 4

FACTORS REGULATING FETAL ERYTHROID COLONY FORMATION

Although the isolation and characterization of some erythroid cell regulators present in fetal bovine serum have been described, it is still not clear how these factors regulate erythroid cell proliferation and differentiation. Regulation of thymidine incorporation into fetal bovine liver cells does not provide information on how these factors act on erythropoiesis, because fetal bovine liver cells isolated contained a mixed population of cells at different stages of maturation. In this section, an in vitro hematopoietic clonal assay was developed to investigate if IGF II could stimulate the proliferation of erythroid cells, and on what differentiation stage of erythropoiesis it might act.

4.1 DEVELOPMENT OF A BOVINE FETAL ERYTHROID CELL CLONAL ASSAY

4.1.1. Establishment of an FBS containing colony formation assay system

Because there was not an erythroid clonal assay system available for bovine

hematopoiesis, a general method of clonal assay in vitro for erythroid progenitors of other species was adopted to study bovine fetal liver erythropoiesis (277). Bovine fetal liver mononuclear cells separated by centrifugation on Ficoll-Paque (1×10^5 cells/ml) were cultured in 35 mm tissue culture dishes in the presence of 30% FBS. There were 772 ± 46 (mean ± SE) CFU-Es formed after three days of incubation and 74 ± 9 (mean ± SE) BFU-Es formed after nine days of culture in a representative experiment. However, the number of erythroid colonies formed changed significantly depending on the batches of fetal liver cells used. Therefore, the effect of some components of the culture medium on bovine fetal erythroid colony formation was tested in order to optimize the culture conditions (Table 1). The concentrations of methylcellulose in the range from 0.7% (w/v) to 1.2% did not significantly alter BFU-E formation, but at higher concentrations it reduced the number of CFU-Es formed. Subsequently, 0.8% methylcellulose was used in all other experiments. FBS is one of the most important components in the culture system. Variation of FBS concentrations significantly altered hematopoietic colony formation. In the presence of Epo (1 U/ml), erythroid colonies increased in dose-dependent manner with increased concentrations of FBS. CFU-Es formed became saturated at a concentration of about 15% FBC, but maximal BFU-E formation required a FBS concentration of 30% (data not shown). It was also observed that FBS promoted CFU-E formation in the absence of Epo (Table 1). One third of CFU-E formed in the presence of Epo could be achieved by adding 30% FBS.

TABLE 1. THE EFFECTS OF METHYLCELLULOSE AND FBS ON ERYTHROID COLONY FORMATION IN VITRO

COMPONENTS OF CULTURE		CFU-Es Formed (MEAN ± SE)	BFU-Es Formed (MEAN ± SE)
Methylcellulose (in the presence of 30% FBS and 1 U/ml Epo)	0.7%	1670 ± 134	47 ± 1
	0.8%	1695 ± 25	46.5 ± 3.5
	0.9%	1413 ± 45	48 ± 3
	1.0%	1006 ± 19	57.5 ± 7.5
	1.1%	n.d.	49 ± 1
	1.2%	n.d.	64.5 ± 10.5
FBS (in the absence of Epo)	5%	0	
	15%	77 ± 3	
	30%	426 ± 23	
	30% + Epo (1 U/ml)	1302 ± 30	

There was not BFU-E formation without the addition of Epo, suggesting the existence of some factor(s) in FBS, which could regulate erythropoiesis at late differential stages. In addition to erythroid colonies, there were also some monocyte/macrophage colonies formed, which were variable with the changes of FBS concentrations in culture but not Epo.

4.1.2 Enrichment of Erythroid progenitor Cells

Frozen bovine fetal liver cells were used for most experiments of clonal assays, but in early experiments freshly prepared cells were also used. It was observed that there were still some hemoglobinized erythroid cells in the mononuclear cells collected at the interface after Ficoll-Paque separation when the cells were stained with benzidine followed by Giemsa stain (data not shown). The presence of mature cells may contribute to the variations of colony formation among different batches of the fetal liver cells. In subsequent experiments, fetal liver cells were treated with ammonium chloride solution to selectively destroy differentiated erythroid cells prior to Ficoll-Paque separation (276). The erythroid progenitors separated from the fetal liver cells after this modification were enriched about 10 times more than those without ammonium chloride treatment. As a result, the concentration of fetal liver mononuclear cells used to produce similar numbers of erythroid colonies in vitro could be reduced from 10^5 cells/ml to 10^4 cells/ml. Moreover, it seemed that the use of NH_4 treated cells provide more

consistent results between different batches of the cells (data not shown).

4.1.3. A Serum Free System Failed to Support Bovine Fetal Erythropoiesis

It was our intention to use an erythroid colony formation system in which FBS could be replaced by chemically defined supplements, because the factors planned to be studied were isolated from FBS. Efforts have been made to find a proper serum free system for fetal bovine erythroid clonal assay according to several published references (180, 280, 281, 282). However, a method of colony formation in vitro for bovine fetal erythropoiesis could not be established by simple adoption of published methods for other species, even after many modifications of these published methods. It seemed that, in general, some component(s) in FBS necessary for fetal bovine erythroid colony formation was missing in the serum-free methods for other species. Subsequently, a different approach was tried, i.e., to reduce the concentration of FBS to 2% in erythroid colony formation assays.

4.2. EFFECTS OF FETAL STROMAL CELLS ON ERYTHROPOIESIS IN VITRO

Because the inability of fetal bovine erythroid cell colony formation in serum-free system seemed to be due to the lack of certain factor(s) which were critical for fetal bovine erythropoiesis, we decided to study whether fetal liver stromal cells

could provide the element(s) required for erythroid cell colony formation in serum free conditions or in reduced FBS concentrations. This approach allowed us to observe some interesting aspects of fetal liver stromal cells on erythropoiesis.

4.2.1. Establishment of a Bovine Fetal Liver Stromal Cell Line

During the culture of freshly prepared fetal bovine liver cells in liquid culture medium supplemented with 30% FBS, it was observed that gross clusters of cells were floating on the surface of the culture medium after two weeks of incubation. After transferring these cells to a new culture flask, they attached to the surface of the flask. These cells continued to grow in fresh medium. During subsequent cultures, floating cells formed again. After two weeks, the floating cells were transferred to a new flask. This procedure was repeated three times, and after the third repeat of this procedure, a monolayer of stromal cells were formed and very few floating cells formed after subsequent cultures. These stromal cells were maintained in IMDM with 10% FBS. The monocyte/macrophage colonies were not observed anymore in subcultures of the stromal cells after trypsinization. Under the phase contrast microscope these BFLS cells appeared as large cells which had a prominent round or oval nucleus or, in some cases two or more nuclei, with some perinuclear vesicles (fig. 4.1A). In the paranuclear area of the cytoplasm, there were phase-dark fine granules. The periphery of the cells was characterized by a spreadout cytoplasm membrane, which formed a transparent sheet-like

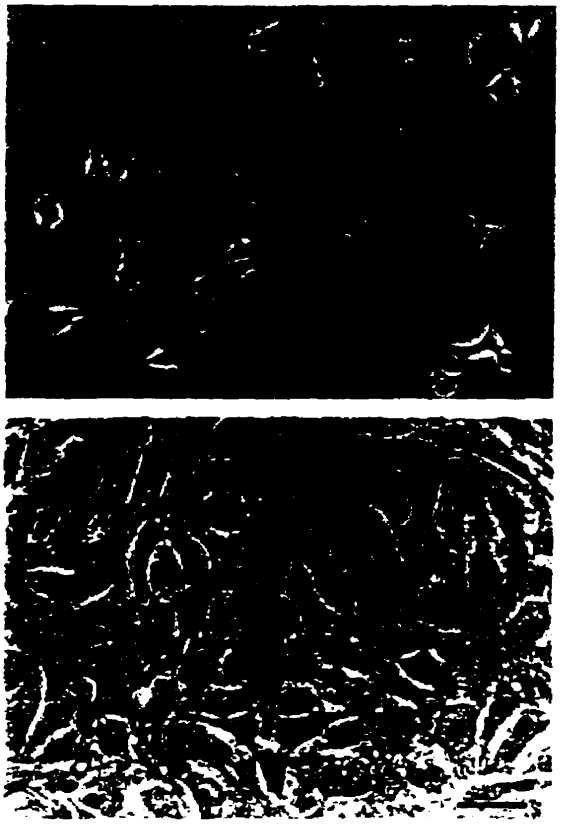


Figure 4.1. Morphological appearance of the bovine liver stromal cells. Stromal cells were cultured in IMDM with 10% FBS for two days (A) or at confluence (B) in 35 mm cell culture dishes (phase contrast microscopy). Bar = 50 μm.

structure. After 3 days in culture, these cells became elongated and had one or more long dendritic cytoplasmic processes which reached out to form contacts with surrounding cells. At confluence, BFLS cells showed cobblestone-like appearance (Fig. 4. 1B). In overgrown cultures, these cells became squeezed and their cytoplasm overlapped with each other, giving rise to a bipolar look.

4.2.2 Cytohistological Characteristics of BFLS Cells

Because both epithelial and endothelial cells give rise to cobblestone morphology, monoclonal antibodies against cytokeratins and vimentin were used to identify the origin of the cells (Fig. 4.2). Immunocytochemistry revealed these cells to be vimentin-positive and cytokeratin-negative, indicating that they were of mesenchymal origin. This result excludes the BFLS cells from being hepatocytes, which have an epithelial origin. These cells did not express acid or alkaline phosphatase activity in routine cytochemical staining and were negative for PAS stain and oil red O stain (results not shown).

4.2.3 Phagocytic Activity of the BFLS Cells

Because the immunocytochemistry results indicated that BFLS cells could be from endothelial origin, and endothelial cells have phagocytic activity (283), the ability of the BFLS cells to take up carbon particles was assessed by culturing BFLS cells in the medium containing 1:1000 India Ink. CHO cells (epithelial) and

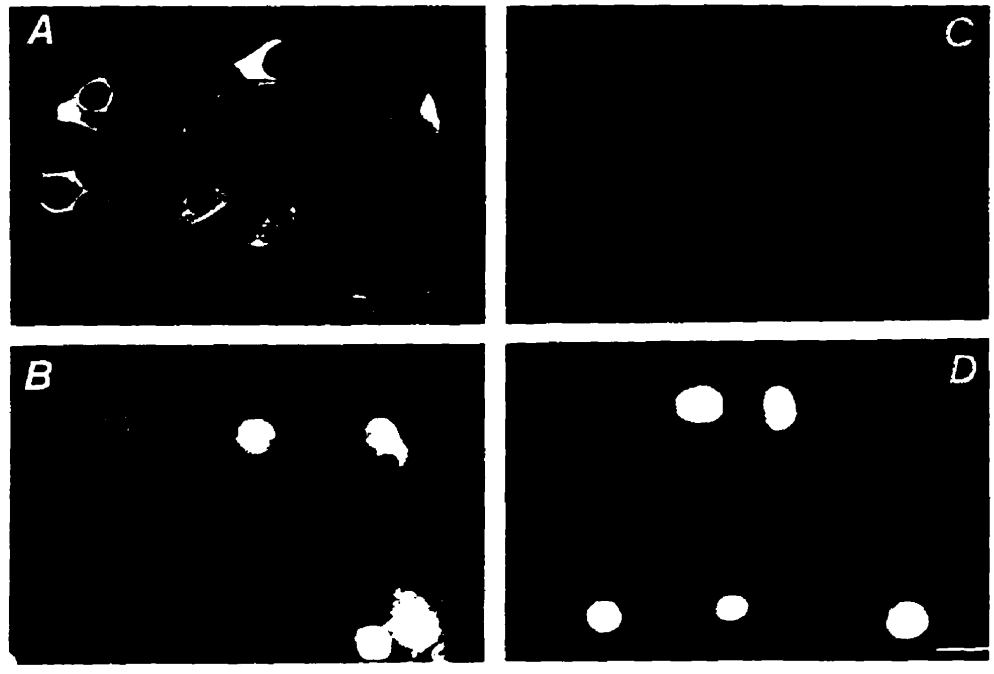


Figure 4.2. Immunofluorescent Staining of BFLS Cells. BFLS cells were cultured on coverslips for 3-5 days, fixed and treated with anti-vimentin (A) or anti-cytokeratin (C) antibodies. Nuclear dye DAPI was used to reveal the locations of the cells in respective fields (B and D). Bar = 25 μm.

NIH-3T3 cells (embryonic fibroblastoid cells) were cultured under the same conditions as control cell cultures. After one day in culture, phagocytosis of carbon particles into the cytoplasm around nuclear area of the BFLS cells could be observed under a reversed bright field microscope. This accumulation became more prominent after two days in culture (Fig. 4.3). CHO cells and NIH-3T3 cells did not show this phagocytic activity at all. The accumulation of carbon particles into the cytoplasm of BFLS cells did not interfere with cell growth because they continued to proliferate until confluence and all cells contained carbon particles in their cytoplasm.

4.2.4. BFLS cells Support Erythropoiesis

To test if BFLS cells could support erythropoiesis, fetal liver mononuclear cells were co-cultured with the stromal cells (Fig. 4.4). BFLS cells (2 x 10^4/well) were cultured according to the method described in Chapter 2 (BFLS cells at confluence were not very efficient in supporting erythroid colony formation). Figure 4.4A shows the number of colonies formed with 0.1 U/ml of Epo in the presence of 2 and 10% FBS but in the absence of stromal cells. The concentration of FBS was lowered to 2% to reduce the interference of unknown factors from FBS. Without BFLS cells, at this concentration of FBS, there were rarely erythroid colonies formed, even in the presence of high concentrations of Epo (Fig. 4.4A). Nevertheless, in the presence of BFLS cells, the number of CFU-Es formed

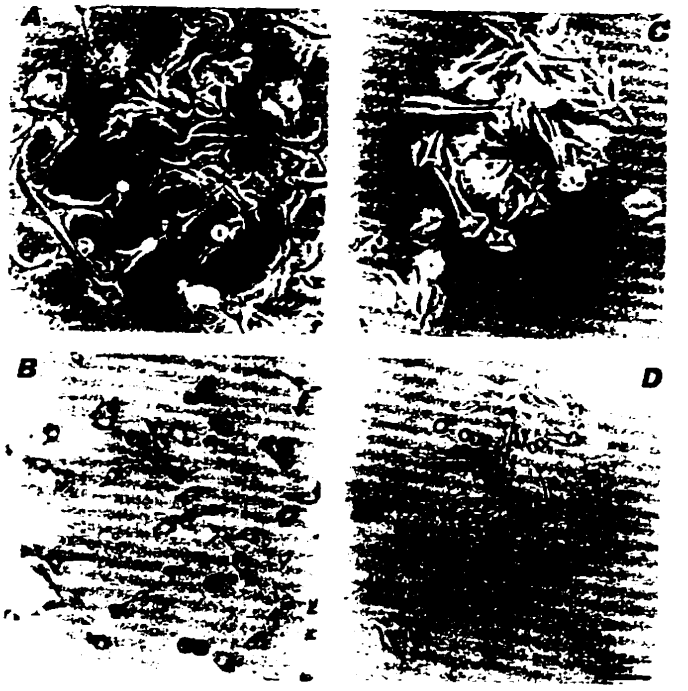


Figure 4.3. Phagocytic activity of BFLS cells. BFLS cells were cultured with 1:1000 India Ink in IMDM with 10% FBS for 2 days. Accumulation of black carbon particles in the cytoplasm of the cells was observed under the microscope (A, B). All BFLS cells phagocytosed carbon particles, which were visible under phase contrast and bright field microscope. CHO cells cultured in 1:1000 India Ink in F 12 medium with 10% FBS (C and D). A and C, phase contrast. B and D, bright field. Bar = 50 μm.

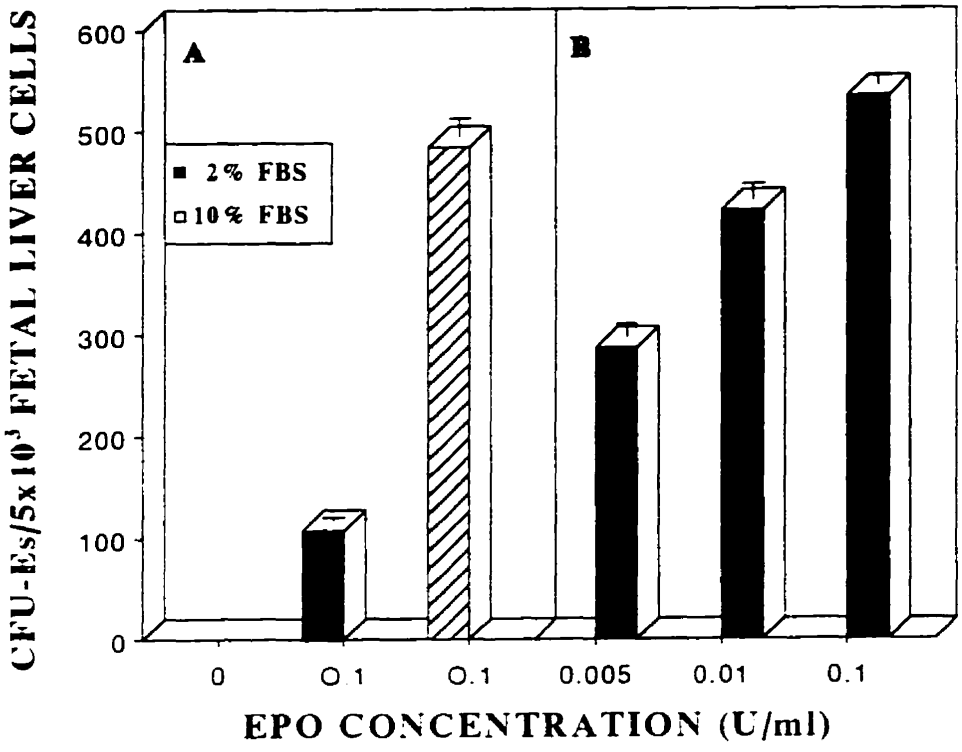


Figure 4.4. Effects of fetal stromal cells on erythropoiesis. CFU-Es formed in the absence of stromal cells (A) and in the presence of BFLS cells (B) with different concentrations of Epo supplemented with either 2% or 10% FBS, as indicated. Mean ± SE of triplicate determinations.

increased dramatically, and showed Epo dependent growth (Fig. 4.4B). The number of erythroid colonies formed in the presence of BFLS cells with 5 mU/ml of Epo was higher than those formed with 100 mU/ml Epo in the absence of BFLS cells, indicating that other factors different from Epo were supplied by BFLS cells and were required for fetal erythropoiesis. In subsequent experiments, 2% FBS was used in colony formation assay.

4.3 IGFs ENHANCES ERYTHROPOIESIS IN VITRO

We and others have previously found that IGFs stimulated erythropoiesis in the presence or absence of Epo (109, 177, 178, 179, 180, 181, 189, 284, 285). One of the purposes of developing the fetal bovine erythroid cell clonal assay system was to study the effects of IGF II on erythropoiesis in this system. Figure 4.5 showed the effects of IGF II on erythropoiesis. In the absence of BFLS cells, IGF II alone did not stimulate erythroid colony formation. But more CFU-Es were observed in the presence of Epo. In the presence of BFLS cells, IGF II alone slightly stimulated CFU-E formation. In the presence of 2 mU/ml Epo, IGF II increased CFU-E formation by more than 80% over control cultures (Fig 4.5 and 4.6A). Furthermore, the erythroid colonies survived longer in culture with IGF II than those without IGF II addition. Addition of IGF II at two different concentrations of Epo increased CFU-E formation (Fig. 4.6A), but the effects of IGF II at low Epo concentration (2 mU/ml) were greater than those observed at high Epo

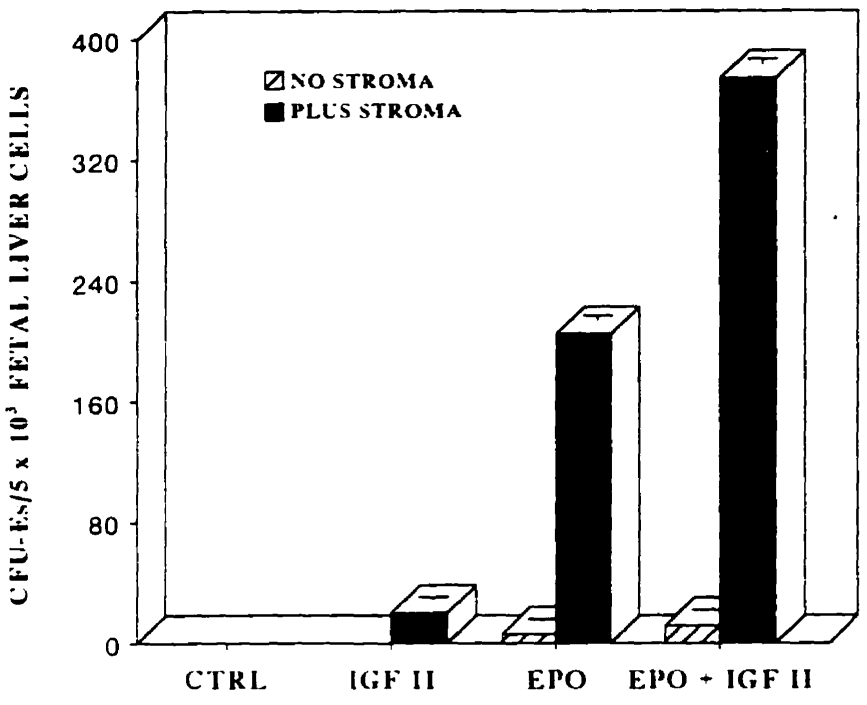
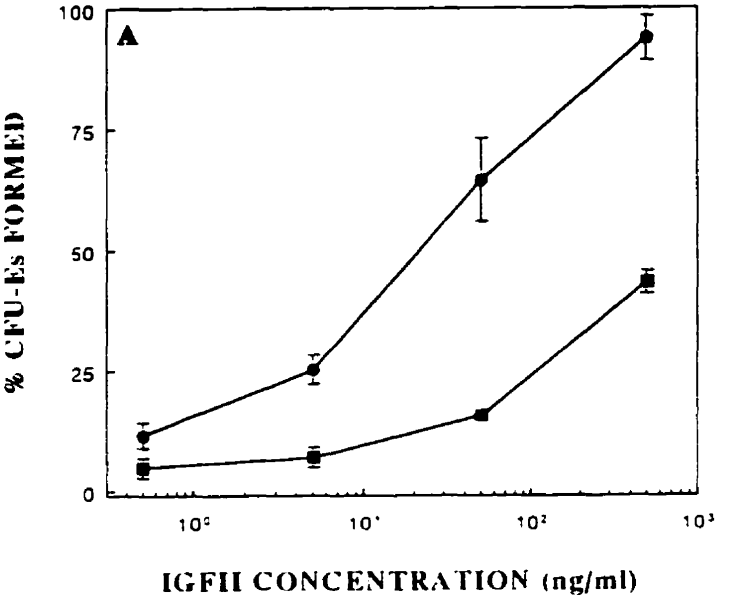


Figure 4.5. Enhancement of erythropoiesis by IGF II in the presence of Epo. Various combinations of Epo (2 mU/ml) and IGF II (0.5 μg/ml) were added to the cultures in the absence or presence of BFLS cells. The cultures were supplemented with 2% FBS. Mean ± SE of triplicate determinations.


A
100
75
50
25
0
% CFU-Es FORMED
10^0
10^1
10^2
10^3
IGFII CONCENTRATION (ng/ml)

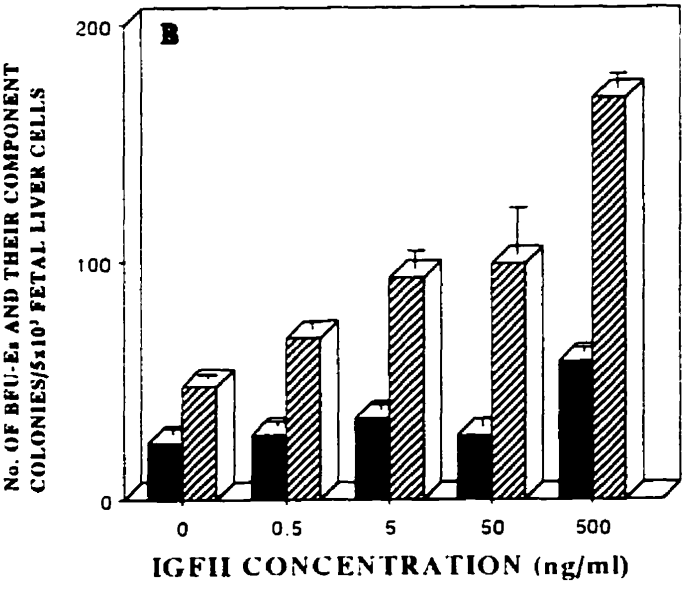

B
200
100
0
No. OF BFU-Es AND THEIR COMPONENT COLONIES/5x10⁵ FETAL LIVER CELLS
0
0.5
5
50
500
IGFII CONCENTRATION (ng/ml)

Figure 4.6. Response of erythroid progenitor cells to IGF II. (A) Percentage of CFU-Es formed with increasing IGF II concentrations in the presence of 2 mU/ml (●) or 50 mU/ml of Epo (▪). The percentage increase was calculated as:

$$\frac{\text{CFU-Es formed - CFU-Es without IGF II addition}}{\text{CFU-Es without IGF II addition}} \times 100$$

The numbers of colonies in the control cultures were 303 ± 10 at 2 mU/ml Epo and 621 ± 24 at 50 mU/ml Epo, respectively. (B) Effect of increased concentrations of IGF II on BFU-E formation (solid bars), and total number of colonies of the bursts (hatched bars) in the presence of 50 mU/ml of Epo. Mean ± SE of triplicate determinations.

concentration (50 mU/ml).

IGF II also increased the burst sizes of BFU-Es. Even though BFU-Es increased only at the highest concentration of IGF II tested, the number of the component colonies within BFU-Es increased in a dose responsive pattern to IGF II (Fig. 4.6B). The size of each individual colony of BFU-Es also appeared larger in the presence of IGF II than in control cell cultures.

4.4 BOTH DIRECT CELL TO CELL CONTACT AND SOLUBLE FACTOR(S) PLAY A ROLE IN ERYTHROPOIESIS

To study whether cell to cell contact or soluble factor(s) were responsible for the effects of BFLS cells on erythropoiesis, a 0.3% agar layer was inserted between BFLS cells and mononuclear progenitor cells. High concentrations of insulin (10 μg/ml) were added as a substitute for IGF II to optimize the erythropoietic response. The physical separation of these two cell types decreased but did not abolished the number of CFU-Es formed (Fig. 4.7). This result suggested that both cell to cell contact and soluble factor(s) were involved in erythropoiesis. It is possible that erythroid colony formation is stimulated by a factor with a membrane-bound and soluble forms, such as the KL (82). Therefore medium conditioned for five days by BFLS cells was tested in its capacity to stimulate CFU-E formation. Addition of 20% CM into the culture improved CFU-E formation (fig. 4.8), and IGFs increased even further the number of colonies. We

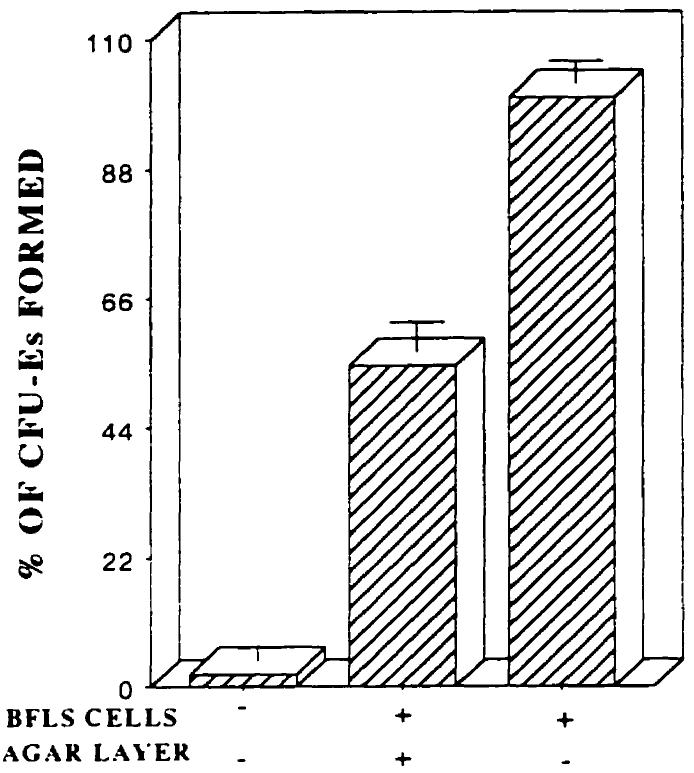


Figure 4.7. Both soluble factor(s) and cell-cell contact played a role for erythropoiesis. A 0.3% agar layer was laid between BFLS cells and mononuclear cells. The culture contained 50 mU/ml Epo and 10 μg/ml insulin. High concentrations of insulin were used as a substitute for IGF II. Number of CFU-Es formed with BFLS cells and without agar layer was used as 100% (430 ± 10). Mean ± SE of triplicate determinations.

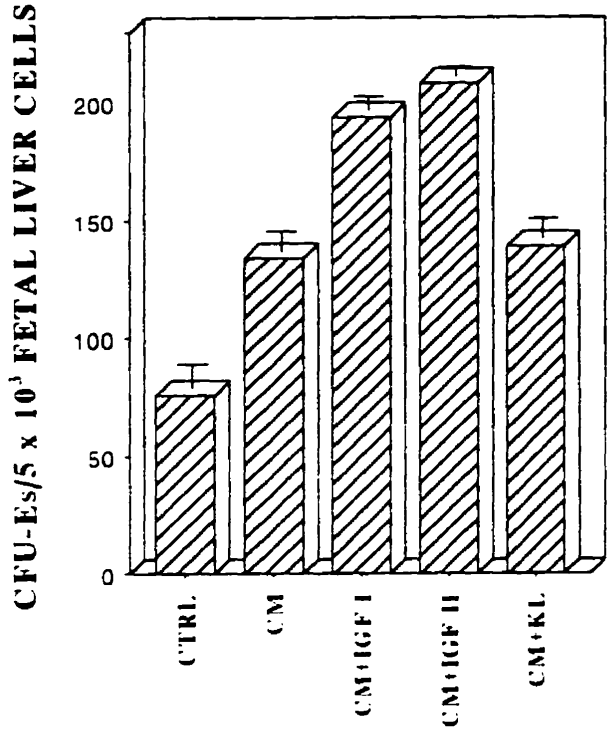


Figure 4.8. BFLS cell conditioned medium promoted CFU-E formation. The assay was carried out as described in chapter 2 in the presence of 50 mU/ml Epo, but no BFLS cells were added. The fetal liver mononuclear cells were cultured with Epo alone (CTRL), with Epo plus 20% CM (CM), with Epo plus 20% CM and 500 ng/ml IGF I (CM + IGF I)/IGF II (CM + IGF II), or with Epo plus 20% CM and 100 ng/ml KL (CM + KL). Mean ± SE of triplicate determinations.

were not able to see any significant change in CFU-E formation with human KL (fig. 4.8). Furthermore, human KL did not stimulate erythroid colony formation in the presence of BFLS cells.

4.5 DETERMINATION OF THE MOLECULAR WEIGHT OF THE SOLUBLE ERYTHROID-STIMULATING FACTORS IN THE CM

Centricon™ microconcentrators with molecular weight cut-offs of 100, 10 and 3 kD were used to determine the molecular weight range of the erythroid stimulating factor(s) in the CM. As shown in figure 4.9, the erythroid stimulating activity in the CM was not retained by any of the microconcentrators, implying its molecular weight was less than 3 kD.

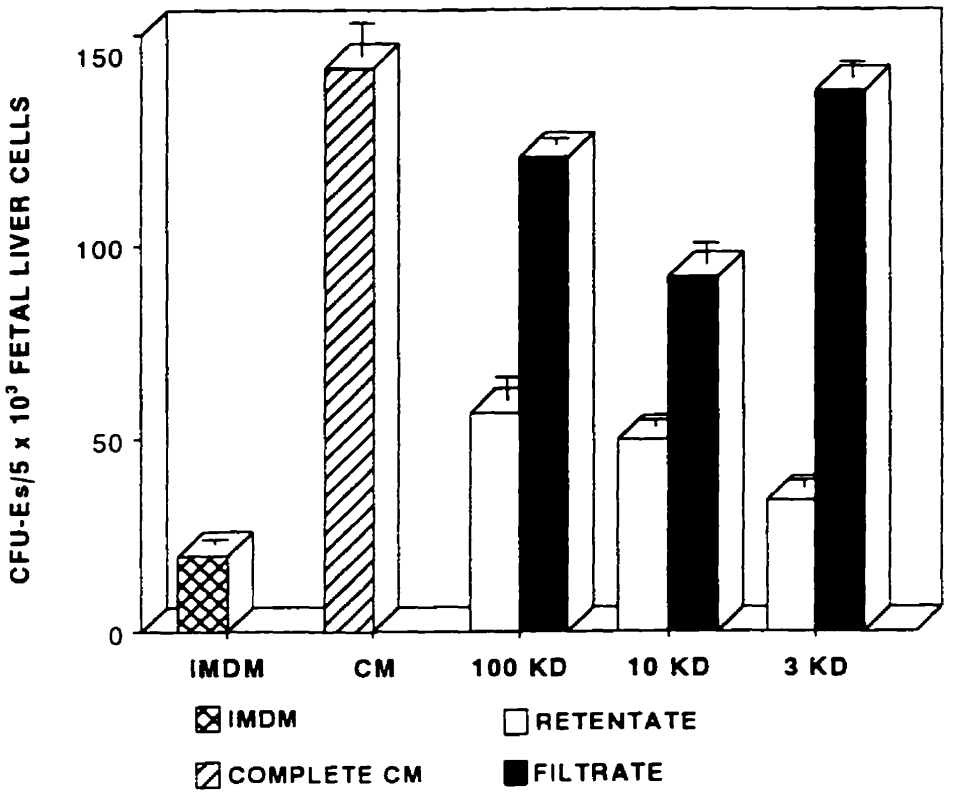


Figure 4.9. Determination of the molecular weight of the soluble factor(s). The retentates and filtrates separated by the Centricon™ microconcentrators with indicated molecular weight cutoff were tested for their erythroid stimulating activity. The IMDM and complete CM (CM) were used as controls. Mean ± SE of triplicate determinations.

CHAPTER 5

DISCUSSION

During ontogeny, hematopoietic cells are produced in the yolk sac in early embryonic development, then in the liver at mid-gestation, and finally in the bone marrow at late gestation and adulthood. Erythropoiesis also undergoes sequential changes. In many mammalian species including bovine and human red blood cells, the hemoglobin produced by erythroid cells switches from an embryonic type to a fetal type during development. Eventually, the production of adult hemoglobin becomes predominant and is maintained in adult life. The factors regulating the shift of the sites of hematopoietic cell production and the hemoglobin switching remain unknown. Nonetheless, there is clear evidence that fetal bovine serum contains growth factors which stimulate fetal erythropoiesis (274, 284). We found that heparin affinity chromatography could be used to partially purify certain erythropoietic growth factors of FBS. The major objective of our studies was the identification of these erythroid cell regulating factors and their role on fetal red blood cell formation.

5.1 ESTABLISHMENT OF THE ISOLATION PROCEDURES

A combination of heparin affinity chromatography, anionic ion exchange chromatography and reversed phase HPLC has been applied for the purification of the heparin-binding erythropoietic regulators. The use of heparin affinity columns eliminates a great number of proteins in the serum and makes it possible the subsequent application of other chromatographic techniques of protein purification, because the capacities of the columns for HPLC are limited. However, there are many proteins in FBS which can bind to heparin, and non-specific binding can also increase the heterogeneity of the proteins retained by heparin affinity columns. Therefore, QAE anion exchange chromatography further reduces the amount of the contaminating proteins isolated by heparin affinity chromatography and contributes to the purification of the erythroid-regulating factors. The final purification is accomplished by HPLC.

In the process of the protein purification, the biological activity of large number of samples must be tested. This requires a rapid and reliable bioassay method to trace the heparin-binding growth factors. In order to attain the final purification of these factors, a serum-free thymidine incorporation bioassay method has been developed to screen the biological activities of the samples (275). This method is very sensitive, highly reproducible, and requires only very small aliquots of the samples to be analyzed. Other advantage of this system is that hundreds of the chromatographic fractions can be tested at one time. The application of this

bioassay method has tremendously facilitated the purification process. This method can be used to study thymidine incorporation into both anchorage-independent and anchorage-dependent cells.

It was found during the purification process that the activity of the samples was lost when the factors were purified near homogeneity. BSA (150 μg/ml) was added as carrier to fractions of the highly purified samples for the bioassay in order to prevent the loss of the bioactivity in the process. Commercial BSA preparations are known to contain erythroid-cell-stimulating factors (178, 286), and can therefore increase the base-line levels of thymidine incorporation. This property of BSA was very useful for the purification of thymidine incorporation-inhibiting activities because increased base-line levels of thymidine incorporation make the inhibitory activities easy to trace (fig. 3.11).

5.2 IGF II as a heparin-binding erythroid stimulator

We have found that some of the erythroid cell-stimulating activities could be separated from FBS by heparin affinity chromatography (fig. 3.4A). One of the heparin-binding erythroid cell-stimulating activities has been purified to homogeneity by using a combination of several chromatographic techniques. The peptide has an apparent molecular weight of about 8 kD and stimulates thymidine incorporation into bovine fetal liver erythroid cells in a dose-dependent manner (fig. 3.7). The N-terminal sequence of this peptide was identical to that of bovine IGF

II (178, 287, 288). This result was surprising because, to the best of our knowledge, there have not been any reports on the heparin-binding properties of IGFs. The only information available at the time about the possible interaction between IGFs and heparin was that heparin might release IGFs from IGF-binding proteins (289). However, it has been reported that the sequence of an IGF-binding protein contained the tripeptide RGD (290). This tripeptide is found in proteins such as fibronectin and represents the binding site for membrane receptors called integrins, typical of many cells interacting with extracellular matrices (291). These studies raise the question whether IGFs can bind heparin directly, or indirectly through their binding-proteins. By using heparin-Shodex affinity chromatography, we demonstrated that both IGF I and IGF II bind to heparin (fig. 3.8). The affinity of IGFs and insulin to heparin is in the order of IGF II > IGF I > insulin, where insulin does not bind heparin at all. By analyzing this result it is easily to predict that the amino acids involved in the binding may be located in the C-loop of IGFs because the structure of mature insulin does not contain this loop. Moreover, it also indicates that the local arrangement of the basic amino acids of the molecules may play a more important role than the overall electrical charges of the molecules for the heparin-binding property because the isoelectrical point of IGF I is higher than that of IGF II (164). In previous studies, consensus sequences have been formulated for heparin-binding (292). One of the predicted sequences is BBXB, where B represents any of the basic amino acids and X is a non-basic amino

acids, often hydrophobic. An analysis of the reported sequence of IGF II indicates that the arginine-rich C-peptide region may be responsible for the heparin-binding property because the amino acids at positions 37 to 40 constitute one of the heparin-binding consensus sequence (fig. 5.1). Nevertheless, the three-dimensional structure proposed by Blundell et al (158) indicates that the loop formed by the C-peptide region, which is on the surface of the IGF II molecule. may have additional binding sites with other arginines such as the ones at positions 30 and 34.

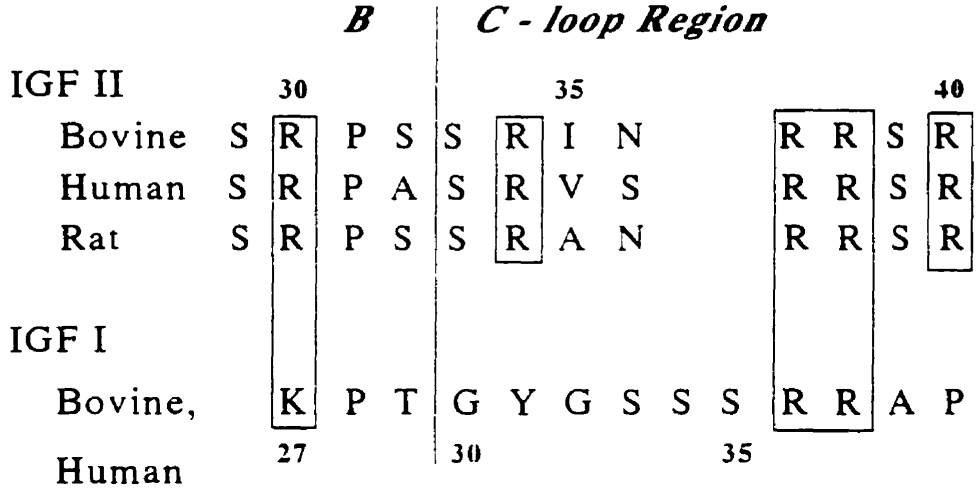


Figure 5.1. Comparison of the C-loop region of human, rat and bovine IGF I and II using the proposed B-C peptide assignment of Blundell et al (158).

A comparison of the same sequence of IGF I suggests that this molecule probably has a much lower affinity towards heparin than IGF II (fig. 5.1), which is in agreement with our data. The lysine 27 of the B-loop of IGF I (corresponding with arginine 30 of IGF II in the B-peptide region) is not far from the pair of arginines of the C-loop and could be important in heparin binding.

The results here detail a previously undescribed interaction of IGFs with components of the extracellular matrix (EM), and imply that EM may influence the biological activity of IGFs to stimulate cell growth as described for other heparin-binding growth factors (232, 293). Indeed, addition of heparin in the bovine fetal liver cell cultures potentiate the effect of IGF II on thymidine incorporation into these cells in a dose-dependent fashion, but is ineffective when added alone (fig. 3.9). Recent studies have provided additional evidence of possible involvement of EM components in regulation of IGF biological activities (294, 295, 296). One of the IGF binding proteins (IGFBP-5) binds heparin and is believed to retain IGFs in the extracellular matrix (296a, 296b). Based on the observations of heparin binding of IGF II indicated above, a new IGF II produced by site directed mutagenesis and containing additional putative heparin binding sites at the C-peptide region and the N-terminal region has been shown to have increased heparin-binding activity (296c). However, the exact role of EM in the regulation of IGF activities in vivo needs to be further studied.

5.3 IDENTIFICATION OF A 46 KD ERYTHROID INHIBITOR AS AN APO H-LIKE PROTEIN

During the initial purification steps for heparin-binding growth factors for liver erythroid cells, it was not at all evident that inhibitory factors were present, because the very high thymidine-incorporation-stimulating activity effectively masked their identification (fig. 3.3 and 3.4). Only the use of reversed phase HPLC allowed us to separate and purify these inhibitory factors (fig. 3.10). Further analysis of the nature of the fractions with thymidine incorporation-stimulating or -inhibiting activities indicated the presence of several factors. The major heparin-binding erythroid cell-stimulating factor of fetal bovine serum has been found to be similar, if not identical, to erythrotropin/insulin like growth factor II as described early (chapter 3.2) (297). After a series of purification steps, one of the major inhibiting factors with an apparent molecular weight of 46 kD has been purified to homogeneity. The N-terminal sequence of this factor is similar to that of human apo H (also called β_2-glycoprotein I, fig. 3.12) (298).

Apo H is a serum glycoprotein with poorly defined physiological functions. While a portion (30%) of apo H is associated with the lipoprotein fraction, the majority (70%) is found free in serum (299). Because it binds to a number of negatively charged substances, including phospholipids, heparin DNA, and platelets, several functions of apo H have been postulated (299,300, 301, 302). They includes inhibition of the blood coagulation pathway (303, 304), inhibition of

adenylate cyclase activities (302), and inhibition of the platelet prothrombinase (305). Apo H, when added together with lipoprotein C-II, stimulates the catalytic activity of lipoprotein lipase (306). It may also be involved in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus (307).

The sequence of human apo H was initially determined directly from a purified protein and contains 326 amino acids (298). The protein and nucleic acid sequences of apo H from other species is known. Among them, the bovine sequence shows the same N-terminal amino acids as the one we obtained here (308, 309, 310, 311, 312). Sequence analyses indicate apo H is a highly conserved protein with 5 contiguous complement control protein (CCP) repeat modules (also called short consensus repeats, or sushi domains because of their shape) (313, 314). The CCP repeats have been found in many members of the complement proteins, and a growing number of non-complement proteins such as the IL-2 receptor and members of the selectin family (315). Since most of these proteins bind to other proteins, and are usually assembled as multiple CCP structures or in combination with other segments such as growth factor domains, it is suggested that CCP structures may function as a protein-binding module (313).

Apo H, to our best knowledge, has not been associated with cytostatic or cytotoxic events. Nevertheless, other heparin-binding apolipoproteins such as apolipoprotein B100 are inhibitors of lymphocytes, and the synthetic peptides

based on two heparin-binding domains of apolipoproteins B and E have been shown to inhibit lymphocyte proliferation (316). Furthermore, human apo H also has a consensus sequence shared with other heparin-binding proteins such as antistasin which is involved in the inhibition of blood coagulation and metastasis of tumors (317). The inhibitory action of the bovine apo H-like protein seems to be restricted to certain cell types, because it was not active in cultures of 3T3 fibroblasts. Furthermore, the inhibition is evident at concentrations which are about 100 times lower than the concentrations reported for human apo H in adult plasma (318). Although the N-terminal sequences of the bovine and human proteins show a high degree of similarity, human apo H did not inhibit thymidine incorporation into fetal bovine liver cells at the same range of concentrations at which 100% inhibition can be observed with the bovine protein. This could be due to a species-specific effect, or to differences in the sequences of other sections of the molecule. Another possibility which cannot be excluded is that the bovine apo H-like protein is carrying a potent low molecular weight inhibitor. Nevertheless, it is unlikely that a bound inhibitor would survive the very severe conditions of high acetonitrile concentrations and 0.1% trifluoroacetic acid used during the purification procedure. Finally a minor post-translational modification of the apo H-like protein could confer special properties not found in a native apolipoprotein.

The inhibition of thymidine incorporation elicited by the apo H-like protein is far more striking than the decrease in cell numbers under identical culture

conditions (fig. 3.13B). These results suggest that the action of this protein is mainly cytostatic rather than cytolytic. It is not known if the bovine apo H plays a role during fetal development of the liver. It has been recently reported that apo H, like many other plasma proteins, is mainly synthesized in hepatocytes (309, 319). Therefore, the development of hepatic functions during fetal life may result in an increase of the synthesis of apo H, which could be useful in reducing erythroid cell development in the liver in favour of a higher rate of erythropoiesis in the developing bone marrow at the end of gestation. Recent studies demonstrate that the expression of apo H mRNA could be down-regulated by IL-1ß and IL-6 (320). Studies on the synthesis of this protein during fetal development could shed some light on its possible role in hepatic red cell formation. It is also possible that this protein may have cytostatic effects on other embryonic or fetal cells. It could be one of the factors present in fetal bovine serum which inhibit mouse embryonic cell proliferation in vitro (321).

5.4 THE POSSIBLE ROLE OF A C4a-LIKE PEPTIDE AS A CYTOLYTIC FACTOR OF ERYTHROID CELLS

The second major inhibitor of thymidine incorporation obtained from FBS has been found to have a molecular weight of 11 kD. However, its structure remains to be elucidated. The N-terminal sequence analysis of a partially purified fraction revealed it was a mixture of IGF II and anaphylatoxin C4a. The

contamination of IGF II with the 11 kD inhibitor is not quite a surprise because we consistently found some erythroid stimulating activity coeluting with the inhibitor. Since it has been identified as an erythroid cell stimulator, IGF II in the sample should not induce the inhibitory effect of thymidine incorporation into the cells (fig. 3.14 and 3.15). So, the cell-lysing activity seen here must come from the other component of the sample, which was identified as anaphylatoxin C4a-like peptide.

C4a is a peptide composed of 77 amino acids and is released from the fourth component of complement (C4) by C1s protease during activation of the complement classical pathway (322). Because C4a shares structural characteristics with those of C3a and C5a, it has been classified as an anaphylatoxin. However, although human C4a promotes contraction of isolated guinea pig ileum and enhances vascular permeability in human and animal skins, these activities are 100- to 1,000-fold less potent than those of either C3a or C5a on a molar basis (323). Whereas C5a elicits chemotactic response to polymorphonuclear leukocytes (PMNs) and monocytes at nanomolar ranges, C4a does not have this activity at all (323, 324). Thus, the biological role of C4a remains unclear. However, recent investigations indicate that C4a may, at very low concentrations, elicit the release of monocyte chemotaxis inhibitory factors from monocytes (325).

The 11 kD C4a-like peptide is a very powerful non-specific cytolytic factor. It not only inhibits thymidine incorporation into bovine fetal liver cells, but also

destroys human adult red blood cells. The dose-response curves in figure 3.16 go below 0% (here 0% corresponds to [^{3}H]thymidine incorporation at time 0 in control cells, representing the non-specific binding of the cells). This occurs because this peptide is a very powerful lysing factor and, at high concentrations, effectively destroys most of the cells present in the cultures (fig. 3.17B). The human red blood cells added in cultures as carriers have been completely destroyed in less than five minutes, suggesting the non-specific cytolytic nature of the action of this peptide.

5.5 BOVINE ERYTHROID CELL COLONY FORMATION ASSAY

The clonal culture of hematopoietic cells in semi-solid medium has been used to characterize hematopoietic precursor cells, and to identify regulators of hematopoietic cells. Although the general methods for culturing cells of different species are similar, the specific details of how the cultures are prepared differ quite significantly according to the source of the hematopoietic precursors (326).

Until now, we identified and characterized several factors regulating fetal erythropoiesis solely in their capacity to stimulate or inhibit thymidine incorporation. Because of the heterogeneity of the erythroid cells used for this bioassay, it is difficult to identify the stage of erythropoiesis in which these factors are acting. It is not known whether other types of cells may be involved in the process. The

application of the method for erythroid colony formation assay is helpful to understand the mechanisms of action of these factors.

However, before starting to study the target cells of the identified factors, the influence of the components in the culture medium has to be defined. Methylcellulose is on of the most commonly used components in semi-solid culture systems. We found that methylcellulose at concentrations higher than 0.8% somehow reduced CFU-Es formed, while it did not seem to interfere with BFU-E formation (Table 4.1). Therefore, 0.8% methylcellulose was used in all of the subsequent experiments. Another important factor influencing the outcome of the experiment is the number and purity of the mononuclear cells used. It is well-known that accessory cells and cell break-down products may alter the type of hematopoietic colonies formed (34). The bovine fetal mononuclear cells separated by Ficoll-Paque still contained some differentiated erythroid cells, which might contribute to the variable colonies formed from different batches of bovine fetal liver cells. After removing the differentiated erythroid cells by NH_4Cl treatment, more consistent results were obtained from different batches of bovine fetal liver cells. Moreover, the mononuclear cells were substantially enriched after NH_4 treatment and the number of cells seeded could be decreased 10 times as compared with the non-treated mononuclear cells. The treatment with NH_4Cl not only enriched erythroid precursor cells, but also reduced the possible interference by accessory cells. Addition of FBS at a concentration of 30% (v/v), in the presence of 1 U/ml

of Epo, greatly improved erythroid colony formation of both types. In the absence of Epo, FBS could still stimulate CFU-E formation in a dose-dependent manner, even though BFU-Es could not grow anymore (Table 4.1). This is not surprising because it has been clearly demonstrated that FBS contains erythroid-stimulating activities (274, 284, 285, 297).

FBS contains many factors, which usually influence the outcome of the cultures. It is advantageous to replace FBS with chemically defined elements. Some FBS-free hematopoietic clonal culture systems have been successfully developed. Unfortunately, removal or reduction of FBS resulted in a complete elimination of bovine fetal erythroid cell colony formation. Replacement of FBS with a combination of different elements, such as BSA, lipids, lipoproteins, hemin, nucleotides, and rare inorganic elements at different concentrations, which have been found to improve hematopoietic cell colony formation in different species (181, 192, 280, 281, 282, 327), did not induce the development of any hematopoietic colony of bovine fetal liver cells. However, we found that in the presence of bovine fetal stromal cells, reduction of FBS to 2% could still stimulate the formation of significant numbers of erythroid colonies, as will be explained in Section 5.6.

5.6 EFFECT OF BOVINE FETAL STROMAL CELLS ON ERYTHROPOIESIS

Although dozens of hematopoietic regulators have been identified, there are still many aspects of the control of hematopoiesis which require further

investigation. There is also evidence that fetal and adult hematopoiesis are regulated differently. For example, fetal hematopoiesis undergoes constant expansion throughout fetal life and the hematopoietic progenitor cells differentiate in vivo almost exclusively along the erythroid pathway (260), though the Epo levels in fetal sera are extremely low and do not change with gestational stage (328). Fetal liver is a major organ for erythropoiesis and hepatocytes have been thought to be important for erythroid growth because they synthesize Epo and may regulate hematopoietic progenitor cell proliferation and differentiation locally in a paracrine fashion. It is less clear if fetal liver stromal cells have any impact in erythropoiesis.

Our data show that fetal liver stromal cells are absolutely required for bovine erythroid cell proliferation and differentiation in vitro (fig. 4.4). BFLS cells are of mesenchymal origin because they were cytokeratin-negative and vimentin-positive (fig. 4.2). They had an endothelial-like morphology and at least one typical characteristic of endothelial cells: a strong phagocytic activity (fig. 4.3). Nevertheless, they could not be unequivocally classified as endothelial, as attempts to identify specific cell markers were inconclusive. It is not clear if von Willebrand's factor (vWF), an endothelial cell marker, is present in these cells. An anti-serum against human vWF did not cross-react with an established bovine endothelial cell line and BFLS cells. Northern blots of BFLS cell RNA for vWF and ribonuclease protection assays with an anti-sense RNA probe of human vWF were not

conclusive (results not shown). These results may be due to loss of endothelial cell markers during in vitro culture as described by Doron et al. (329). Alternatively, a lack of affinity of the human vWF antibody or the human vWF-probe to bovine vWF is also possible.

BFLS cells potentiate the effects of Epo, lowering the concentrations of Epo required for erythropoiesis in vitro to physiological levels. In the absence of BFLS cells, there were hardly any CFU-Es in culture, even with a high concentration of Epo. By coculturing BFLS cells with fetal liver mononuclear cells, the number of erythroid colonies formed in the presence of Epo increased dramatically (Fig. 4.4 and 4.5). The numbers of colonies formed with 2 to 5 mU/ml of Epo in the presence of BFLS cells were much higher than the numbers of CFU-Es observed with 100 mU/ml to 1 U/ml Epo in the absence of BFLS cells. BFLS cells also supported BFU-E formation at a physiological concentration of Epo in our culture system. Therefore, bovine fetal liver erythroid cells show a similar dependence on accessory cells for proliferation under serum-free conditions as previously described for BFU-E formation in primary cultures of human bone marrow (189, 330). These data indicate that some other factor(s) different from Epo are also required for erythroid cell growth. Recent studies from other groups also support the existence of erythroid cell stimulating factors different from Epo. Ohneda et al. (276) and Yanai et al. (331) reported that several stromal cell lines from mouse spleen or fetal liver, when metabolically active, could selectively support erythroid

colony formation. Correa and Axelrad (189) found that accessory cells were absolutely required for BFU-E formation in a serum free system. Aye et al. (332) also found that some cells derived from human bone marrow cultures contained factor(s) selectively promoting erythroid colony formation.

To study how BFLS cells support fetal erythroid cell growth, i.e., if cell-cell contact or secretion of soluble factors is required, an agar layer was inserted between BFLS cells and fetal liver mononuclear cells (Fig. 4.7). Physical separation of erythroid progenitor cells from BFLS cells by the agar layer, which allowed soluble factors to pass through, only reduced CFU-E formation by about 50%, suggesting that both cell-cell contact and secretion of soluble factor(s) were involved. Alternatively, in extracellular vesicles, membrane-bound growth factors with erythroid-stimulating activity may be shed into the culture medium (333, 334). The *c-kit* ligand (KL), which has both a membrane bound form and a soluble form, also stimulates erythropoiesis in the presence of Epo (82) and may account for both effects of BFLS cells. However, medium conditioned by BFLS cells improved CFU-E formation in the absence of BFLS cells, but this effect could not be mimicked by human KL. In addition, KL did not have any effect on erythropoiesis in the presence of CM (Fig. 4.8). This might be due to the lack of effect of human KL on bovine cells. However, the molecular weight of the factor(s) present in the BFLS cell conditioned medium estimated with Centricon™ microconcentrators (Fig. 4.9) was quite different from that reported for KL of different species, excluding the

possibility that KL might play a role here. The molecular weight of the factor(s), with a nominal molecular weight lower than 3 kd, did not correspond to the molecular weight of known colony-stimulating factors, interleukins, IGFs or other cytokines known to influence erythroid colony formation. It should be pointed out that there was some CFU-E growth with retentates of the centricon™ concentrators of all molecular weight ranges (fig 4.9). This might be due to the presence of a small amount of the stimulating factor(s) in the CM left behind with retentates. It may also be explained by the presence of additional factors of high molecular weight or factors associated with membrane vesicles in the case of the 100 kD cut-off filters. These factors could be either less active than the <3 kD factor(s) or concentrated together with inhibitory factors.

5.7 EFFECT OF IGFs ON ERYTHROID COLONY FORMATION

We have studied the effect of IGF II on erythropoiesis in this culture system, because we and other investigators previously showed that IGFs had erythropoietic activities in the presence and absence of Epo (109, 177, 178, 179, 180, 181, 188, 189, 192, 284, 285, 335, 336). In the absence of BFLS cells, addition of IGF II did not stimulate erythroid colony formation. However, it did enhance the effect of Epo, even though the absolute numbers of the colonies formed in the absence of BFLS cells were quite low (fig. 4.5). In the presence of BFLS cells, IGF II alone slightly stimulated erythroid colony formation. The effect of IGF II on erythropoiesis

was evident in combination with low concentrations of Epo in the presence of BFLS cells (Fig. 4.6A). The size of CFU-E colonies was larger and the colonies formed lasted longer in culture in the presence of IGF II than in control cell cultures. IGF II also increased the size of bursts of individual BFU-Es, but only increased the number of BFU-Es at the highest concentration tested (Fig. 4.6B). This suggests that IGF II may act on late erythroid progenitor cells and synergize with other erythroid cytokines to regulate normal erythropoiesis. The effects of IGF II on erythropoiesis could still be observed in the absence of stromal cells (Fig. 4.8), suggesting that IGF II acted directly on erythroid cells. Nevertheless, it is possible that IGF II could also influence erythropoiesis indirectly through actions on accessary or stromal cells. Our results suggest that during fetal development, IGF II may play a crucial role in erythropoiesis, because Epo levels are very low and are kept constant during ontogeny while concentrations of IGFs increase with gestation and are particular high at late gestation (164).

Using human erythroid cells, it has been shown the proliferation and maturation of erythroid cells are regulated by the interaction of multiple factors (194). IGF I and KL are required for erythroid maturation and proliferation, while Epo prevents apoptosis and maintains erythroid cell viability and development. The effects of both IGF I and IGF II are believed to be conducted through their interaction with IGF I receptors. Our results (fig. 4.8) and those of others have shown that IGF I and IGF II have similar effect on erythropoiesis (188). However,

a recent report demonstrated that although both IGF I and IGF II can stimulate erythropoiesis, they may play a distinct role at different developmental stages (192).

SUMMARY

We have developed a serum free bioassay system, which can be used for studies of thymidine incorporation into both anchorage-dependent and anchorage-independent cells in culture. By using this method, in combination with heparin affinity chromatography, ion exchange chromatography, and HPLC, several heparin-binding growth factors have been identified. One of them is an 8 kd peptide which is similar, if not identical, to bovine IGF II. This peptide stimulates thymidine incorporation into erythroid cells in serum free culture, and this effect can be enhanced by heparin.

During the process of isolation and purification of the heparin-binding erythroid stimulating factors, we found that they were almost invariably contaminated with substances that inhibited thymidine incorporation into erythroid cells of fetal bovine liver. We have isolated and partially sequenced two of these inhibitory factors. The first one was a 46 kd heparin-binding protein from FBS. It has a N-terminal amino acid sequence identical to bovine Apo H. It can completely inhibit thymidine incorporation into erythroid cells with an ED_{50} of 36 nM, although human Apo H had no inhibitory activity on the same cells.

The second inhibitor isolated from FBS was clearly cytotoxic at a concentration of 1 nM. This 11 kd peptide seems to be structurally related to the anaphylatoxin C4a. It not only inhibited thymidine incorporation into bovine fetal erythroid cells, but also destroyed human red blood cells.

We have also developed an erythroid clonal assay method in order to further characterize the heparin-binding factors. This culture system requires the presence of a monolayer of bovine fetal liver stromal cells and a medium supplemented with 2% FBS. The fetal liver stromal cell lines developed by us have some properties of endothelial cells and support erythropoiesis in the presence of Epo. Although the supportive effect could be best appreciated in cocultures of stromal cells and erythropoietic precursors, medium conditioned with the stromal cells was also able to stimulate CFU-E formation. The erythroid colony formation could be further enhanced by IGF II. This suggests that fetal liver stromal cells play an important role in fetal hematopoiesis.

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IMAGE EVALUATION
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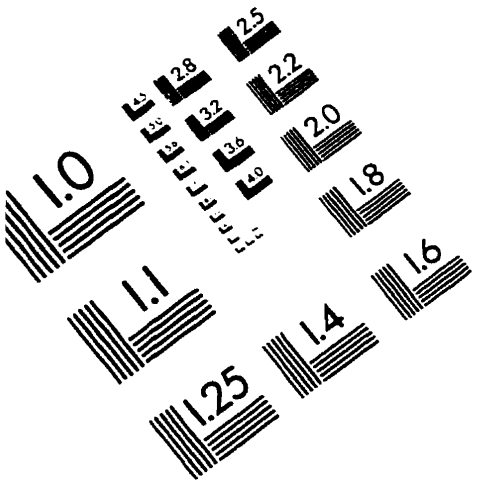

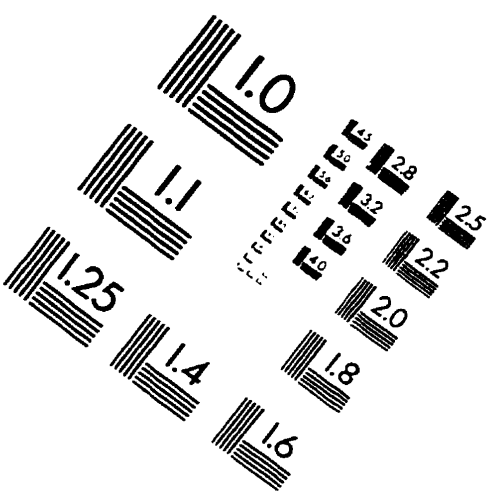

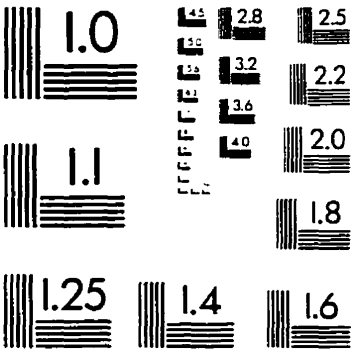

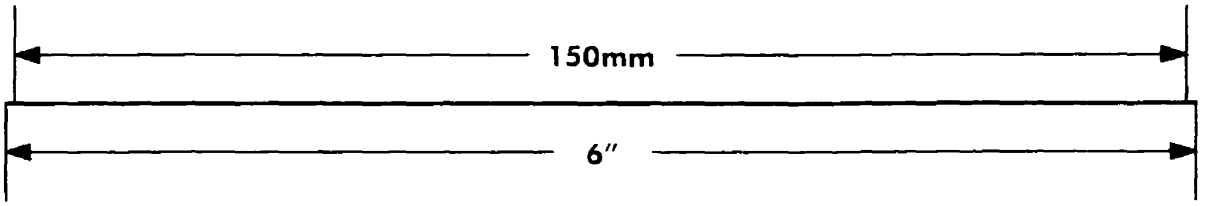

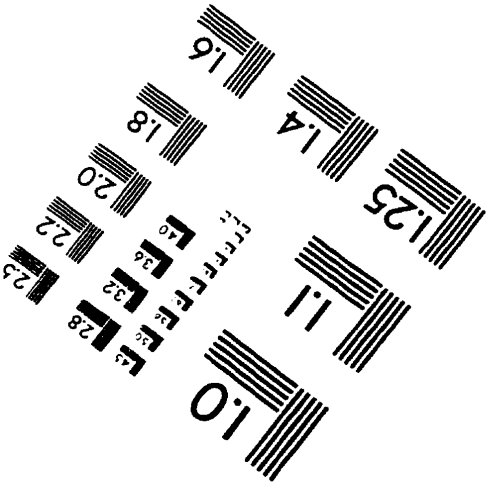

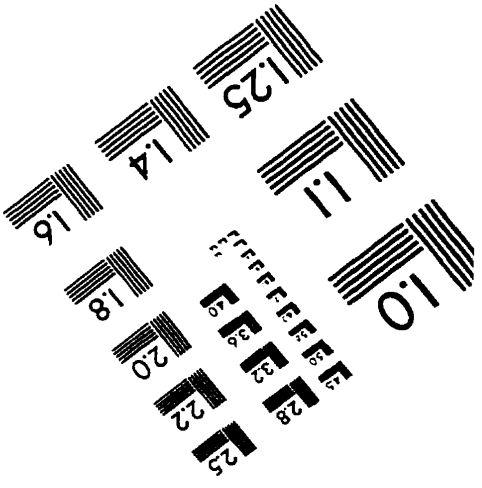

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989