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INVOLVEMENT OF INSULIN-LIKE GROWTH FACTOR I AND ITS BINDING PROTEINS ON PROLIFERATION AND DIFFERENTIATION OF MURINE BONE MARROW MACROPHAGE PRECURSORS

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ABSTRACT

INVOLVEMENT OF INSULIN-LIKE GROWTH FACTOR-I AND ITS BINDING PROTEINS ON PROLIFERATION AND DIFFERENTIATION OF MURINE BONE MARROW MACROPHAGE PRECURSORS

The alteration of insulin-like growth factor I (IGF-I) and its binding proteins (IGFBP) and their effects on proliferation and differentiation of murine bone marrow-derived macrophage (BMM) precursors were investigated. Bone marrow cells exposed to 20% L929-fibroblast conditioned medium (LCM) were cultured in serum-free medium for 24 h before collecting the supernatant and the cell pellet. Western ligand blotting (WLB) analysis of the supernatant detected four bands in all samples. Both 41-kDa and 30-kDa bands were detected after 12 h and remained constant during BMM differentiation. The 28-kDa and 25-kDa proteins were almost undetectable until day 2, but accumulated significantly from day 3 to day 7. Immunoblotting analysis verified these two bands as IGFBP-4. Northern blotting analyses detected both IGFBP-4 and IGFBP-3 mRNA in the cells. The 41-kDa protein was postulated to be IGFBP-3 in a glycosylated form. However, the identity of the 30-kDa band is not known. Northern blotting analysis also showed that IGF-I mRNA level was increased in a time-dependent manner until day 3, and was decreased thereafter during BMM differentiation. The effect of IGF-I and its analogs (long R^3 IGF-I and des(1-3)IGF-I) on cell proliferation was studied at different time points by [^{3}H]

thymidine incorporation. IGF-I and its analogs enhanced cell proliferation of freshly isolated bone marrow cells. Both IGF-I and long R^3 IGF-I, but not des(1-3)IGF-I, continued to exert a stimulating effect on day 1, although to a lesser extent. The effect of IGF-I and its analogs on BMM differentiation was studied by checking the morphology, non-specific esterase-1 (NSE-1) activity, and mannose receptor expression. No significant differences in morphology and NSE-1 activity were observed among the treatment groups. There was no difference of mannose receptor expression on day 4 between the IGF-I group and the control cells, whereas long R^3 and des(1-3) IGF-I increased the receptor number by 260% and 228% respectively, with less increased K_d values. This suggests that IGF-I enhances BMM differentiation, but this stimulating effect could be blocked by IGFBP-4. These results support the notion that IGF-I and its binding proteins play important roles in proliferation and differentiation of BMM precursors.

RÉSUMÉ

RÔLES DU FACTEUR DE CROISSANCE INSULINE-LIKE I ET DE SES PROTÉINES FIXATRICES SUR LA PROLIFÉRATION ET LA DIFFÉRENCIATION DES PRÉCURSEURS DES MACROPHAGES MURINS DE LA MOELLE OSSEUSE

La modification du facteur de croissance insuline-like I (IGF-I) et de ses protéines fixatrices (IGFBP) ainsi que leurs effets sur la prolifération et la différenciation des précurseurs des macrophages murins de la moelle osseuse (BMM) ont été examinés. Les cellules de la moelle osseuse ont été exposées dans un premier temps à un milieu de culture contenant 20% de médium pour fibroblastes L929 puis ont été cultivées dans un milieu sans serum durant 24 h avant de récolter le surnageant et le culot cellulaire. L'analyse par le Western ligand blotting (WLB) du surnageant nous a permis de détecter 4 bandes dans l'ensemble des échantillons. Les bandes correspondant à 41 et 30 kDa ont été exprimées après 12 h et sont demeurées stables durant la différenciation des BMM. Par ailleurs, les protéines correspondant à 28 et 25 kDa étaient presque invisibles jusqu'au second jour, mais s'étaient accumulées de façon significative du troisième au septième jour. L'immunoblotting a démontré que ces deux protéines étaient des IGFBP-4. Le Northern blotting nous a aussi permis de détecter de l'ARNm codant pour l'IGFBP-3 et l'IGFBP-4. Nous avons présumé que la protéine de 41 kDa était la forme glycosylée de l'IGFBP-3. Toutefois, la bande de 30

kDa n'a pu être identifiée. Le Northern blotting a aussi démontré que le niveau de l'ARNm a augmenté jusqu'au troisième jour pour ensuite diminuer durant la différenciation des BMM. L'effet de l'IGF-I et ses analogues (long R^3 IGF-I et des(1-3)IGF-I) sur la prolifération cellulaire a été étudié à différentes périodes par incorporation de la thymidine tritiée. Au jour 0, l'IGF-I et ses analogues ont augmenté la prolifération cellulaire. Contrairement au des(1-3)IGF-I, l'IGF-I et le long R^3 IGF-I ont continué d'exercer un effet positif durant le jour 1, mais à un niveau moindre. L'effet d'IGF-I et de ses analogues sur la différentiation des BMM a été étudié en examinant la morphologie, l'activité non-spécifique de l'estérase-1 (NSE-1) ainsi que l'expression du recepteur mannose. Aucune différence significative n'a été observée pour la morphologie et l'activité NSE-1 des groupes étudiés. L'expression du récepteur mannose entre le groupe IGF-I et les cellules témoins n'a révélé aucune différence au jour 4. Par contre, le long R^3 IGF-I et le des(1-3)IGF-I ont augmenté le nombre de récepteurs de 260% et 228%, respectivement, ainsi que la valeur du K_d, mais à un niveau moindre. Ceci suggère que l'IGF-I accroît la différenciation des BMM, mais cet effet peut être bloqué par l'IGFBP-4. Les résultats obtenus confirment que l'IGF-I et ses protéines de fixation jouent un rôle important au niveau de la prolifération et de la différenciation des précurseurs des BMM.

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I. Introduction

Insulin-like growth factor I (IGF-I) has been found to exist in many tissues and has pleiotropic actions. On binding to cell surface receptors, it transduces signals inside the cell and induces cellular responses. IGF-I binds a variety of proteins which are homologous in structure, namely IGF binding proteins (IGFBP). At least 6 IGFBP have been characterized. IGFBP can either alter cellular responses through their interaction with IGF-I or directly influence cell functions independent of IGF-I. Accumulating evidence suggests that IGF-I and IGFBP are involved in the immune system. Arkins et al. (1995) found that IGF-I mRNA was up-regulated in growth and differentiation of murine bone marrow macrophage (BMM) precursors, but the effects of IGF-I in the process are still unclear. Moreover, whether the profile of endogenous IGFBP also changes and how it affects BMM development have not been tested up to now. Therefore, the objectives of this research are to study 1) the actions of IGF-I; 2) the alteration of IGFBP; 3) the effects of IGFBP during murine BMM development. These studies provide insight on the role of IGF-I and its binding proteins in proliferation and differentiation of BMM precursors.

II. Literature Review

1. Insulin-like growth factor I (IGF-I)

In the 1950s, IGF-I was first discovered to stimulate sulfation activity of cartilage (Salmon and Daughaday, 1957), and was therefore called "sulfation factor". Afterwards it was found that this factor mediated growth hormone (GH) functions in many systems and was renamed somatomedin (Daughaday et al., 1972). Meanwhile, some factors in serum were proved to have nonsuppressible insulin-like activity (NSILA) (Froesch et al., 1966). The above studies were based on crude extracts from tissues or serum. In the late 1970s, these factors were purified from different systems and it was shown that one of the NSILA factors has the identical sequence to that of somatomedin-C (Rinderknecht and Humbel, 1978a). This factor was named IGF-I. Another NSILA factor which was highly homologous to IGF-I was named IGF-II (Rinderknecht and Humbel, 1978b). Both IGF-I and IGF-II belong to the IGF family.

1.1. Protein structure of IGF-I

The primary structure of mature IGF-I was first characterized by Rinderknecht and Humbel (1978a). It is a monomer composed of 70 amino acids (AA). This polypeptide is divided into 4 regions, i.e., A, B, C and D domains. The B

domain starts from the amino terminus of the peptide, followed in sequence by C, A, and D domains. The A and B domains are about 40% and 60% homologous to those of proinsulin and IGF-II, respectively. Three disulfide bonds exist in IGF-I (Iwai et al., 1989).

Evidence proved that IGF-I is highly conserved in different species. In fact, the AA sequences of human (Rinderknecht and Humbel, 1978a), bovine (Honegger et al., 1986) and porcine (Tavakkol et al., 1988) IGF-I are completely identical. No more than 7 AA differences exist in rat (Tamura et al., 1989), mouse (Bell et al., 1986), ovine (Wong et al., 1989), and chicken (Dawe et al., 1988) IGF-I, in comparison with human IGF-I.

An additional peptide, E peptide, is present in the carboxyl terminus in the precursor of mature IGF-I. Two different cDNA sequences were characterized in rat, mouse and human, encoding two different E peptides (IGF-Ia and IGF-Ib) (Rotwein, 1986, Bell et al., 1986, Roberts et al., 1987a). Again, they showed high homology in different species, indicating high evolutionary conservation. The divergence of IGF-Ia and IGF-Ib may result from alternate processing of the original gene.

1.2. Gene structure of IGF-I

The IGF-I gene has been studied in several species, among which the rat IGF gene is best known. It is larger than 70 kb and contains at least 5 exons and 4 introns. The 5 exons are exon 1, 1A, 2, 3, and 4 (Shimatsu and Rotwein, 1987). Exons 1 and 1A are believed to encode the 5' untranslated region (UTR) and a part of the signal peptide. Exon 2 encodes the rest of the signal peptide and most of the B domain, while exon 3 encodes the rest of B domain, C, A and D domains, and some part of E peptide. Another part of E peptide is encoded by exon 4. Exon 5 encodes the left part of the E peptide and 3' UTR (Bucci et al., 1989).

1.3. IGF-I gene expression

Differential splicing occurs at the 5' end of IGF-I gene in many species, therefore distinct 5'-UTR sequences have been found in different IGF-I transcripts (Roberts et al., 1987b). Alternative splicing also exists in the regions which encode the E peptide, resulting in different E peptide expression. In human and mouse, two transcripts with different E peptide encoding sequences have been described (Rotwein, 1986, Bell et al., 1986). While IGF-Ia is encoded by exons 3 and 5, IGF-Ib is encoded by exons 3, 4 and 5. The length of the 3' UTR differs significantly in different transcripts due to the use

of alternate polyadenylation sites, which is the main reason for the size heterogeneity of IGF-I mRNA. Arkins et al. (1993) found that at least 3 IGF-I transcripts with different sizes were expressed in murine BMM. The physiological significance of this heterogeneity is still unclear.

Gene expression of IGF-I is regulated by GH (Isgaard et al., 1989, Closset et al., 1989), estrogen (Murphy et al., 1987), insulin (Fagin et al., 1989, Boni-Schnetzler et al., 1989), and nutritional status of the animal (Emler and Schalch, 1987, Lowe et al., 1989, Davenport et al., 1990). Differentiation of many cells also alters IGF-I expression. An increase of IGF-I mRNA level was observed during the differentiation of mouse C2 myoblasts (Tollefsen et al., 1989). Expression of IGF-I was first up-regulated and then down-regulated (Birnbaum et al., 1995) during *in vitro* murine osteoblast differentiation.

1.4. Biological functions of IGF-I

It has been demonstrated that IGF-I is synthesized and secreted by many cells and tissues. It exerts multiple functions through endocrine, autocrine or paracrine mechanisms. Biological functions of IGF-I *in vivo* have been extensively reviewed by Sara and Hall (1990). Due to the nature of our research, the *in vitro* effects of IGF-I on

different cells will be briefly reviewed below. IGF-I is involved in cell cycle progression, cell proliferation, differentiation and other events. Furthermore, IGF-I exerts many other actions, including the regulation of glucose and AA uptake, glucose incorporation into glycogen, RNA and protein synthesis.

Using BALB/c 3T3 fibroblast cell lines, the role of IGF-I in cell cycle has been studied. Competence factors such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) induced quiescent cells to enter G1 from G0 phase, but G1 arrest occurred if IGF-I was not added into the culture medium. Only in the presence of IGF-I could the cells continue to enter S phase and subsequently complete the cell cycle. Thus, IGF-I was regarded as a progression factor in cell cycle. The synergistic effect of both competence and progression factors is a prerequisite for DNA synthesis and proliferation of BALB/c 3T3 cells (Stiles et al., 1979).

Growth-promoting activity is the major function of IGF-I in many cell types. A large number of studies have shown that IGF-I has stimulatory effects on cell proliferation. DNA synthesis measured by [^{3}H] thymidine incorporation in porcine and fetal chicken chondrocytes was increased by IGF-I (Taylor et al., 1988). Osteoblast proliferation in fetal rats was also enhanced by IGF-I (Hock et al., 1988). After stimulation by

IGF-I, cultured human lens epithelial cells were much more numerous than the control cells (Ibaraki et al., 1995). Besides, the effect of IGF-I on cell proliferation has been demonstrated on many other cell types, including fibroblasts, smooth muscle cells and endothelial cells, (Corps et al., 1988, Pfeifle et al., 1982, Bar et al., 1988). Apart from normal cells, the growth of some tumor cells can also be mediated by IGF-I. For example, IGF-I stimulated proliferation of neuroblastoma cell line SH-SY5Y and several non-small cell lung carcinoma (non-SCLC) cell lines (Meghani et al., 1993, Ankrapp and Bevan, 1993).

Differentiation of many types of cells involves IGF-I. Myogenesis of skeletal muscle cell line, L6E9, could be induced by IGF-I (Silverman et al., 1995). Murine 3T3-L1 preadipocytes did not differentiate into adipocytes when cultured in the medium depleted of IGF-I. Supplementation of IGF-I in the medium restored the differentiation (Smith et al., 1988). As reported by Schmid et al.(1984), IGF-I also enhanced rat osteoblast differentiation. In serum-free culture, IGF-I enhanced colony formation of erythroid progenitors, indicating its effect on erythropoiesis (Akahane et al., 1987).

2. IGF-I receptor

For IGF-I to exert its biological functions, it needs to bind with IGF-I receptor (IGF-IR) first. While the details are still sketchy, binding of IGF-I to IGF-IR does initiate a cascade of cellular events which consequently result in the final response, as described later.

2.1. Protein structure of IGF-IR

The precursor of IGF-IR is a single peptide (Jacobs et al., 1983). At its amino terminus, it starts from a 30-AA signal peptide containing many hydrophobic residues, followed by a 706-AA α-subunit, a 4-AA proteolytic region, and a 627-AA ß-subunit. The precursor undergoes post-translational modification after its synthesis. Upon removal of the signal peptide, glycosylation and proteolytic cleavage, the α- and ß-subunits are linked together by a disulfide bond, forming a half-receptor. Subsequently, two half-receptors are linked to each other by a disulfide bond between their α-subunits. Thus the mature receptor is a tetramer with two α- and two ß-subunits (reviewed by Jones and Clemmons, 1995).

Located extracellularly, the α-subunit contains a cysteine-rich region and 11 N-glycosylation sites. The cysteine-rich region of the α-subunit is important in specific

ligand binding, since IR3, an antibody directly against this region, abrogated IGF-I binding (Flier et al., 1986). The IGF-IR has a high binding affinity for IGF-I with the dissociation constant (K_d) value around 0.2-1 nM. While IGF-IR can also cross-bind with insulin and IGF-II, the binding affinity of IGF-IR is 100- to 1000-times lower for insulin, and 2- to 15-times lower for IGF-II (Steele Perkins et al., 1988, Frattali and Pessin, 1993, Germain Lee et al., 1992).

Unlike the α-subunit, the ß-subunit is a transmembrane peptide. Its cytoplasmic part contains a tyrosine kinase domain and several phosphorylation sites. Within the tyrosine kinase region, there is a ATP-binding site. The tyrosine kinase activity is suggested to be involved in the signalling functions of IGF-IR (Jacobs et al., 1983, Adamo et al., 1989).

2.2. Signal transduction

Phosphorylation of tyr or ser residues of the IGF-IR is induced by IGF-I binding (Steele Perkins et al., 1988, Kato et al., 1993). The tyrosine kinase of one ß-subunit can phosphorylate tyr residues on the other ß-subunit. (Frattali and Pessin, 1993, Frattali et al., 1992, Treadway et al., 1991). Phosphorylation of the receptor enhances its tyrosine kinase activity, which in turn phosphorylates its substrates in the cells.

An important substrate of IGF-IR in the cytoplasm is a 185-kDa protein, named insulin receptor substrate 1 (IRS-1). Being phosphorylated by IGF-IR, this protein can associate with two other proteins, Grb and Sos. The IRS-1-Grb2-Sos complex activates the Ras-mediated signal transduction pathways, resulting in mitogenic response (Blenis, 1993). Phosphorylation of IRS-1 also leads to activation of phosphatidylinositol-3 (PIP_3) kinase, which phosphorylates phosphatidylinositol-2 (PIP_2) to PIP_3. The latter is an important regulator involved in intracellular communications (Yamamoto et al., 1992).

2.3. Regulation of IGF-IR

In many situations, IGF-IR is regulated by its ligand, IGF-I. In IM-9 lymphoid cells, FRTL-5 thyroid cells, endothelial cells, and bovine articular chondrocytes, IGF-IR was down-regulated with increasing IGF-I concentrations (Poretsky et al., 1988). On the contrary, IGF-II up-regulated IGF-IR in IM-9 cells (Werther et al., 1989). Besides, IGF-IR expression is also mediated by various hormones, such as human chorionic gonadotropin (hCG) (Lin et al., 1987 and 1988) and follicle stimulating hormone (FSH) (Adashi et al., 1986).

Alteration of IGF-IR expression occurs during differentiation of certain types of cells. For instance, there

was an increase of IGF-IR mRNA level during rat adipose tissue differentiation (Teruel et al., 1995). During BMM differentiation induced by CSF-1, IGF-IR mRNA was reported to be down-regulated (Arkins et al., 1995). Overexpression of IGF-IR in myoblasts was suggested to have either positive or negative effects on myoblast differentiation depending on the ligand concentrations (Quinn et al., 1993).

3. IGF binding proteins (IGFBP)

In addition to IGF-IR, a family of proteins, termed IGFBP, can also bind to IGF-I with high affinities. At least 6 IGFBP have been characterized in various cells and tissues. They are highly homologous to one another at their amino and carboxyl termini, but divergent in their middle regions. Each IGFBP is quite conserved in different species. The IGFBP can either alter cellular responses by interacting with IGF-I or directly influence cellular functions independent of IGF-I.

3.1. Structures and binding affinities of IGFBP

The molecular structures and binding affinities of the IGFBP have been extensively reviewed by Rechler (1993). Molecular masses and special features of rat and human IGFBP-1 to IGFBP-6 are summarized in Table 1.

Table 1. Structural features of IGFBP*.

IGFBP	Species	Molecular mass(Da)**	Number of AA	Special features
IGFBP-1	Human	25,271	234	RGD, 18 Cys
IGFBP-1	Rat	26,919	247	RGD, 18 Cys
IGFBP-2	Human	31,355	289	RGD, 18 Cys
IGFBP-2	Rat	29,561	270	RGD, 18 Cys
IGFBP-3	Human	28,717	264	N-glycosylated, 18 Cys
IGFBP-3	Rat	28,856	265	N-glycosylated, 18 Cys
IGFBP-4	Human	25,957	237	N-glycosylated, 20 Cys
IGFBP-4	Rat	25,681	233	N-glycosylated, 20 Cys
IGFBP-5	Human	28,553	252	18 Cys
IGFBP-5	Rat	28,428	252	18 Cys
IGFBP-6	Human	22,847	216	16 Cys
IGFBP-6	Rat	21,461	201	14 Cys

* Modified from Rechler (1993).

** Molecular masses (Da) of mature IGFBP deduced from nucleotide sequences.

At the carboxyl terminus, IGFBP-1 and IGFBP-2 contain an arg-gly-asp (RGD) sequence, which is considered to be important in their binding with the cell surface integrin receptors. No RGD sequence exists in IGFBP-3 to -6.

Several N-glycosylation sites are present in IGFBP-3 in different species. One possible N-glycosylation site exists in IGFBP-4. No N-glycosylation has been found in IGFBP -1, -2, -5 and -6.

There are 18 cys residues in the amino and carboxyl termini of IGFBP-1 to -5. Two extra cys residues are also present in the exon 2-encoding region of IGFBP-4. While 18 cys exist in the amino and carboxyl termini in IGFBP-1 to 5, human and rat IGFBP-6 has only 16 and 14 cys, respectively.

All six IGFBP can bind with IGF-I and IGF-II. The binding affinity of IGFBP-1 to -5 for IGF-I and IGF-II is quite similar, but IGFBP-6 preferentially binds with IGF-II. In most situations, IGF-I binds to its binding proteins with a greater affinity than to its receptor.

3.2. Expression and regulation of IGFBP

The presence of IGFBP has been reported in plasma and other extracellular fluids. They have also been detected in many cells, such as hepatocytes, smooth muscle cells, myoblasts, osteoblasts, glial cells, thyroid cells, kidney cells, fibroblasts, endothelial cells and cancer cells, as reviewed by Rechler (1993).

Various factors regulate the expression of IGFBP. In insulin-dependent diabetes mellitus (IDDM) patients, insulin decreased the plasma level of IGFBP-1 (Brismar et al., 1988). Infusion of glucose and fructose also down-regulated IGFBP-1 in human plasma (Snyder and Clemmons, 1990). In human Hep G2 hepatocarcinoma cells, insulin inhibited IGFBP-1 transcription, and cAMP stimulated IGFBP-1 expression (Powell et al., 1991). In bovine mammary epithelial cells, IGF-I is an important regulator of IGFBP-2. It stimulated IGFBP-2 secretion by 50- to 75- fold into the serum-free medium (McGrath et al., 1991). Upon exposure to IGF-I, the secretion of IGFBP-3 was upregulated, whereas that of IGFBP-4 was downregulated in human fibroblasts. On the contrary, insulin treatment increased IGFBP-4 secretion in the same type of cells (Camacho-Hubner et al., 1992). Also, IGFBP-5 induction by IGF-I was observed in human fibroblasts by Clemmons and his colleagues (1990).

Alteration of IGFBP also occurs in differentiation of several cells. For instance, during differentiation of Caco-2, an intestinal epithelial cell line, IGFBP-2 and -3 secretion increased, but IGFBP-4 secretion decreased (Oguchi et al., 1994). Expression of IGFBP-2 mRNA and secretion of the protein both diminished with the differentiation of C_2C_{12} myoblasts (Ernst et al., 1992). Accumulation of IGFBP-4 and 6 was found during the myogenesis of rat L6E9 skeletal muscle cells (Silverman et al., 1995).

In addition to the quantitative change of IGFBP, the binding affinities of IGFBP for IGF-I may also be altered. For instance, IGFBP-3, when associated with human fibroblast cells, had a 10-fold lower affinity for IGF-I than that in the culture medium (McCusker et al., 1990). Phosphorylation of IGFBP-1 also affects its binding affinity. With the phosphorylation of a serine residue, the binding ability of IGFBP-1 decreased 4- to 6- fold compared to its dephosphorylated form (Jones et al., 1991).

3.3. Functions of IGFBP

The biological actions of different IGFBP are divergent. They can either modulate IGF-I actions, or directly influence the cell responses independent of IGF-I. Moreover, cell culture conditions affect the response of cells to IGFBP. The

functions of IGFBP are also affected by their localization and modification. It has been postulated that IGFBP perform the following functions in regulating IGF-I actions: (1) to act as transport proteins in plasma and to control the efflux of IGF-I from the vascular space; (2) to prolong the half-lives of IGF-I and regulate their metabolic clearance; (3) to provide a means of tissue- and cell type-specific localization; and (4) to directly modulate interaction of IGF-I with its receptor and thereby indirectly control biological actions (reviewed by Jones and Clemmons, 1995).

4. IGF-I analogs

Some peptides, either mutated from native IGF-I or synthesized artificially, are only slightly different from native IGF-I in their structures. However, their binding affinities for IGF-IR or IGFBP are quite distinct from those of IGF-I. Therefore, they have provided unique opportunities to study the biological functions of IGF-I, IGF-IR and IGFBP.

When tyr^{24} of IGF-I was replaced by leu or ser, its binding affinity for IGF-IR was reduced significantly, but that for IGFBP remained normal (Cascieri et al., 1988). Substitution of tyr^{24}, tyr^{31} or tyr^{60} with an ala or leu led to a 6-to 20-fold loss of affinity for IGF-IR (Bayne et al.,

1990). Thus, it is suggested that tyr residues at positions 24 and 31 are important in IGF-I binding to its receptor.

The first 16 AA of IGF-I at the amino terminus are considered to be critical in binding to IGFBP, since their replacement with the first 17 AA of the B-chain of insulin decreased the binding affinity for human serum IGFBP by more than 1,000 fold (Bayne et al., 1988).

A truncated form of IGF-I has been identified from both fetal and adult human brain (Carlsson-Skwirut et al., 1986). This isoform lacks the N-terminal tripeptide compared to normal human IGF-I, therefore called des(1-3)IGF-I. Recombinant des(1-3)IGF-I was more potent in stimulating DNA synthesis of fetal rat brain cells than intact IGF-I (Carlsson-Skwirut et al., 1989). In L6 myoblasts, des(1-3)IGF-I was also more effective in inducing ^{3}H-leu incorporation (Ballard et al., 1987). According to the radioreceptor assay, des(1-3)IGF-I had a similar binding affinity for IGF-I receptor to regular IGF-I (Ballard et al., 1988). However, its binding affinity for IGFBP was dramatically reduced in comparison with regular IGF-I, and its activity on cells could not be blocked by IGFBP (Szabo et al., 1988, Carlsson-Skwirut et al., 1989). These data suggest that the increased potency of this truncated IGF-I results from the loss of its binding ability to endogenous IGFBP.

Another analog, Long R^3 IGF-I, which was synthesized for the purpose of changing IGF-I potency, contains 83 AA. It replaces the glu^3 of human IGF-I with an arg, and has 13 more AA at the N-terminus. It has a slightly lower binding affinity for IGF-IR on L6 myoblasts than regular IGF-I, but the binding ability to bovine IGFBP-2 is much lower. Like des(1-3)IGF-I, it was also more potent in stimulating protein synthesis in L6 myoblasts when compared to normal IGF-I (Francis et al., 1992). Thus, long R^3 IGF-I, as well as des(1-3)IGF-I, is a good candidate for studying the functions of IGFBP.

5. IGF-I and the immune system

While the above review has focused on cells other than immune cells, some immunocompetent cells, such as leukocytes (Baxter et al., 1991), and an immortalized T cell line(Geffner et al., 1990), expressed and secreted IGF-I. In addition, IGF-IR has been characterized in many types of immune cells, including human monocytes, lymphocytes, bovine neutrophils and mononuclear cells (Stuart et al., 1991, Kooijman et al., 1992a, Zhao et al., 1992). Finally, several types of immune cells were also found to express IGFBP. It has been shown that Jurkat helper T cells expressed IGFBP-2 mRNA (Binkert et al., 1989), and IGFBP-5 was expressed in U937 human monocyte cell line (Keifer et al., 1991).

Evidence showed that IGF-I had a similar function to that of IL-2 in increasing human lymphocyte proliferation. Moreover, it had an additional stimulatory effect when used in combination with saturable doses of IL-2 (Schillaci et al., 1995). Kooijman et al. (1992b) reported that recombinant IGF-I enhanced proliferation of human peripheral blood mononuclear cells and purified T lymphocytes induced by lectins. When a series of human lymphoblastoid B cell lines were used to test the IGF-I actions on DNA synthesis and immunoglobulin (Ig) production, augmenting effects were observed in each cell line (Kimata and Yoshida, 1994). The growth of a transformed T cell line was also stimulated by IGF-I (Geffner et al, 1992).

In addition to the growth-stimulatory effects, IGF-I can also influence the differentiation of several immune cells. It has been demonstrated that IGF-I played an important role in B cell lymphopoiesis (Jardieu et al., 1994), granulopoiesis (Merchav et al., 1988 and 1993) and erythropoiesis (Kurtz et al., 1988). Furthermore, IGF-I can directly regulate the immune functions of some cells. Natural killer (NK) cell activity was potentiated by IGF-I in vitro (Kooijman et al., 1992a). Hydrogen peroxide release by bovine neutrophils was enhanced by IGF-I (Zhao et al., 1993).

Even though the effect of IGF-I on BMM has only received limited attention, accumulating evidence suggests the

potential involvement of IGF-I in proliferation and differentiation of BMM. Colony-stimulating factor-1 (CSF-1) could induce murine BMM differentiation, accompanied by IGF-I mRNA induction. Moreover, the development of other myeloid lineages from primary bone marrow cultures induced by various CSFs was also associated with the enhancement of IGF-I mRNA (Arkins et al., 1995).

6. BMM differentiation

Macrophage plays an important role in the immune system. It has numerous functions including phagocytosis and destruction of the invading microorganisms, presentation of antigen to T cells, secretion of enzymes to kill pathogens and tumor cells (Auger and Ross, 1992). In the bone marrow, macrophage precursors develop into monoblasts, promonocytes, and finally divide into monocytes, which enter the peripheral blood. Monocytes circulate in the blood, and then enter different tissues, where they differentiate into mature macrophages (Auger and Ross, 1992). In some situations, the macrophage may also originate from local proliferation (North, 1969). Due to stimulation by different factors, macrophages display functional, morphological and phenotypical heterogeneity in different tissues, or in different subpopulations within the same localization (Dougherty and McBride, 1984).

Boltz-Nitulescu et al. (1987) proved that freshly-isolated murine BMM precursors could first proliferate and finally differentiate into typical mature macrophages in the presence of mouse L929-conditioned medium (LCM), which contained colony-stimulating factor-1 (CSF-1). Hence, this method has been adopted by many researchers to study the events occurring during macrophage differentiation.

7. Characteristics of macrophages

Characterization of a macrophage is based on several criteria, mainly morphology, cytochemistry, functions, proliferative capacities and cell surface markers.

7.1. Morphology

Macrophages adhere to and elongate on glass surfaces. They are large compared to other mononuclear phagocytes, with a diameter of about 25 to 50 μm. Usually, the cell surface is ruffled, and two to four pseudopodia can be observed on each cell. The nuclear-to-cytoplasmic ratio is less than 1. The cytoplasm contains many vacuoles and granules (Goud et al., 1975).

7.2. Cytochemistry

Cytochemical studies were performed on human bone-marrow hematopoietic cells by Willcox et al (1976). Macrophages react positively in non-specific esterase-1 (NSE-1), methyl green pyronin, periodic acid-Schiff, and acid phosphatase staining. However, they are peroxidase and AS-D chloroacetate esterase negative. NSE-1 staining is a commonly used method in identifying macrophages. Murine BMM are also NSE-1 positive. Besides, these macrophages can be stained for adenosintriphosphatase intensely (Boltz-Nitulescu et al., 1987).

7.3. Functions

During BMM differentiation induced by CSF-1, lysozyme production and secretion increased. Meanwhile, the differentiated cells exerted higher cytotoxicity (Keller and Keist, 1982). With the differentiation from monoblasts, promonocytes, monocytes to macrophages, the ability of pinocytosis of dextran sulfate and phagocytosis of bacteria or latex particles by the cells increased gradually (Goud et al., 1975).

7.4. Proliferative capacity

When [^{3}H] thymidine incorporation was used as a parameter, mature BMM showed very low proliferative ability, whereas the immature mononuclear phagocytes, such as promonocytes and monoblasts, displayed much higher proliferative capacity (Goud et al, 1975).

7.5. Cell surface markers

A large variety of cell surface markers are expressed on well-differentiated macrophages, but with heterogeneity in different tissues and subpopulations. Cell surface receptors for the Fc region of IgG and IgE, and for complement C_3b are expressed on BMM (Boltz-Nitulescu et al., 1987 and 1988, Cline et al., 1972). These receptors are probably involved in the effects of phagocytosis and antigen presentation by macrophages. A series of cytokine receptors, such as the receptors for interleukin-1, tumor necrosis factor α, interleukin-3, interleukin-4, multi-CSF, granulocyte-macrophage CSF, and interferons, are also anchored on macrophage surface (Auger and Ross, 1992).

Some lectin-like proteins also exist on the surface of macrophages, binding specifically with oligosaccharides. Mannose receptor is one of them. It is important in mediating

endocytosis of mannose-terminal glycoproteins independent of Ig molecules and complements. The uptake of these proteins has been proved to be macrophage-specific and does not occur in polymorphonuclear leukocytes (Stahl et al., 1980)

III. Hypotheses and Objectives

Macrophages play an important role in the immune system. They are derived from bone marrow stem cells, which undergo several steps of differentiation and finally develop into mature macrophages. Up to now, the mechanism of macrophage development is still not very clear, although the involvement of a variety of factors has been suggested. Arkins et al. (1995) found that IGF-I mRNA was altered during BMM differentiation and that IGF-I was involved in bone marrow cell proliferation. Moveover, Li et al. (1996) showed that macrophages synthesized and secreted IGFBP-4. Since IGF-I and IGFBP are involved in the differentiation of many cells, it is plausible to hypothesize that IGF-I and its binding proteins may be important regulators in macrophage proliferation and differentiation. Specifically, IGF-I and IGFBP might affect CSF-1-induced BMM development. Furthermore, IGF-I and IGFBP expression could be altered during BMM differentiation induced by CSF-1. Therefore, the objectives for this study are: (1) To test whether and how IGF-I and IGFBP mRNA expression alters in BMM differentiation induced by CSF-1; (2) To examine

whether and how IGFBP secretion changes during BMM differentiation induced by CSF-1; and (3) To compare the effects of IGF-I and its analogs on proliferation and differentiation of BMM precursors.

IV. Materials and Methods

1. Materials

L929 fibroblasts and ß-actin cDNA probe were kindly provided by Dr. Kris Chadee (Institute of Parasitology, McGill University, Montreal, Quebec). Rat IGF-I and IGFBP cDNA probes, and rabbit anti-rat IGFBP antibodies were obtained from Dr. H. T. Huynh (Lady Davis Research Institute, Montreal, Quebec). Except for those specified elsewhere, all reagents were purchased from Sigma (St. Louis, MO).

2. Endotoxin assay

Endotoxin levels for all the medium and reagents used in the study were measured by the Limulus amoebocyte lysate assay, following the protocol provided by Sigma.

3. Preparation of L929-conditioned medium (LCM)

L929 fibroblasts were cultured in T75 flasks (Nunc, Naperville, Ill) with 50 ml of the RPMI complete medium (RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 1 mg/ml of gentamicin (GIBCO/BRL, Grand Island, NY) until confluence. The LCM was collected by passing the supernatant through a 0.22 μm sterile filter (Costar, Cambridge, CA) and used as a source of CSF-1 (Boltz-Nitulescu, 1987).

4. Isolation of bone marrow cells

Bone marrow cells were obtained from 4 to 6-week-old female BALB/c mice (Charles River, Montreal, Quebec). Animals were sacrificed by cervical dislocation and cells were flushed from the femurs and tibia with RPMI 1640 medium. Cell suspension was then centrifuged (1000 x g, 8 min, 4°C) and red blood cells (RBC) were lysed in a lysing solution (130 mM NH_4Cl, 5 mM KCl, 0.84 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 5.6 mM glucose, 1 mM $MgCl_2$, 0.3 mM $MgSO_4$, 1.5 mM $CaCl_2$ and 13.5 mM $NaHCO_3$). For mRNA and protein analyses, the remaining cells were cultured in 100 mm^2 non-adherent culture plates (Baxter, Mississauga, Ontario) at a density of 10^7 cells/plate in RPMI complete medium containing 20% LCM. For the other assays, cells were put in adherent petri dishes (Nunc) with the same medium as above. After overnight incubation, fibroblastic cells and

mature macrophages were attached to the bottom of the plates, and the non-adherent cells (almost pure macrophage precursors) were transferred to 24-well plates (Becton Dickinson, Lincoln Park, NJ) at a density of 10^6 cells/ml/well for further use. These cells were further cultured in the same medium.

5. Northern, Western and Immunoblotting analyses

5.1. Sample collection

At different time points, culture medium was replaced with serum-free RPMI 1640 for 24 h incubation. Subsequently, cells were scraped and centrifuged (1000 x g, 8 min, 4°C). The supernatant was collected for the Western ligand blotting (WLB) and immunoblotting analyses, while the pellet was lysed in TRIzol reagent (GIBCO/BRL) for RNA extraction and Northern blotting analysis. Samples were stored at -70°C until use.

5.2. Western ligand blotting (WLB) analysis

Samples were concentrated ten times by 10-kDa cut-off filters (Costar) and electrophoresed on a non-reducing SDS-polyacrylamide (Bio-Rad) gel (4% stacking gel and 12% separating gel). Size-separated proteins were electrotransferred to a nitrocellulose membrane (Bio-Rad) at 25 V overnight with a transblot unit (Bio-Rad). The membrane

was air-dried, and immersed into Tris-buffered saline (TBS, 0.15 M NaCl, 0.01 M Tris base, 0.05% NaN_3, pH 7.4) with 3% Nonidet P-40 for 30 min at 4°C. Afterwards, the membrane was blocked in TBS containing 1% BSA for 2-3 h, and then hybridized overnight with [^{125}I]IGF-I (Amersham) in TBS containing 0.1% Tween-20 and 1% BSA. The following day, free [^{125}I]IGF-I was removed by two washes with TBS containing 0.1% Tween-20 and three washes with TBS. All the above-mentioned blocking, hybridization and washing procedures were performed at 4°C. The membrane was then exposed to Kodak XAR-5 film (Inter Sciences) on an intensifying screen for 24-48 h at -70°C. The developed film was scanned and the intensity of the bands was densitometrically analyzed using NIH image 1.60 program.

5.3. Immunoblotting analysis

Samples were concentrated, electrophoresed and transferred as described in section 5.2, and immunoblotting analysis was performed using the Enhanced Chemiluminescence Method (ECL) following the instruction provided by Amersham. Briefly, the membrane was soaked in the blocking buffer (TBS with 3% BSA and 0.15% Tween-20) for 1 h, washed with TBS containing 0.15% Tween-20 (TBS-T) once for 15 min and twice for 5 min, and then incubated with the first antibody (rabbit anti-rat IGFBP antibody) (1:5000 dilution) in TBS-T containing

1% BSA for 1h, followed by one wash for 15 min and two washes for 5 min in TBS-T. Subsequently, the membrane was incubated with the second antibody (goat anti-rabbit IgG peroxidase conjugate) (1:5000 dilution) in TBS-T for 1 h, and washed with TBS-T once for 15 min and at least 4 times for 5 min. The washed membrane was covered with the ECL detection solution (Amersham) for 1 min, and exposed to Kodak XAR-5 film (Inter Sciences) on an intensifying screen for 5 sec. The developed film was scanned and the intensity of the bands was analyzed using NIH image 1.60 program.

5.4. Extraction of RNA from cell pellet

Extraction of RNA was performed using TRIzol reagent, based on the guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Briefly, 1 x 10^7 cells were lysed in 1 ml of TRIzol by pipetting up and down thoroughly, and left at room temperature for 5 min. Subsequently, 0.2 ml of chloroform was added, followed by vigorous agitation for 15 sec. After sitting at room temperature for 2-3 min, samples were centrifuged at 12,000 x g for 15 min at 4°C. The aqueous phase was mixed with 0.5 ml of isopropanol at room temperature for 10 min and centrifuged at 12,000 x g for 10 min at 4°C. The precipitated RNA was washed with 75% ethanol once and then dissolved in 0.1% Diethyl pyrocarbonate (DEPC)-water. RNA concentration was measured by spectrophotometry (1 x OD_{260} = 40

μg/ml of RNA). Purity of RNA was tested by measuring the ratio of OD_{260} to OD_{280}.

5.5. Northern blotting analysis

Northern blotting analysis was performed as described by Sambrook et al. (1989) with minor modifications. Briefly, RNA (15 μg per sample) was denatured by glyoxal (Sigma, St. Louis) for 1 h at 50°C, separated by 1% agarose (Bio-Rad, Mississauga, Ontario) gel electrophoresis in 10 mM sodium phosphate buffer (pH 7.0), and then blotted onto Hybond-N nylon membrane (Amersham, Oakville, Ontario) by capillary transfer overnight. The membrane was baked at 80°C for 2 h and cross-linked under U.V. light for 10 min to fix the RNA. Prior to hybridization, the membrane was prehybridized in a solution including 5 x Denhardt (0.1% Ficoll, 0.1% polyvinylpyrodilone, 0.1% Bovine serum albumin (BSA)), 5 x SSPE (0.75 M NaCl, 0.05 M NaH_2PO_4, 0.05 M EDTA), 50% formamide, 0.25 mg/ml of salmon sperm DNA and 0.1% SDS (Bio-Rad) at 42°C for at least 3 h. The cDNA probes were labelled by ^{32}P incorporation using a nick-translation kit (Amersham) and purified by spinning in a Sephadex G-50 (Pharmacia, Uppsala, Sweden) column. The labelled rat IGF-I and IGFBP cDNA probes were added into the above-mentioned solution and incubated with the membrane for 12-18 h at 42°C. Afterwards, the membrane was washed once with 0.5 x SSC (0.075 M NaCl and 7.5 mM sodium citrate) for 30 min

at room temperature, twice with 0.5 x SSC containing 0.1% SDS for 30 min at 55°C, and once or twice with 0.1 x SSC containing 0.1% SDS for 30 min at 65°C, and then exposed to Kodak XAR-5 film (Inter Sciences, Markham, Ontario) on an intensifying screen for 24 to 48 h at -70°C. The developed film was scanned and the intensity of the bands was analyzed using NIH image 1.60 program. ß-actin was used as a reference for the relative quantitation of mRNA.

6. Effect of IGF-I on bone marrow cell proliferation

To investigate the effect of IGF-I on BMM proliferation, cells were stimulated with IGF-I at different time points, and then [^{3}H] thymidine incorporation was used as an index of cell proliferation. Since the cells might secrete some IGFBP to interfere with the effect of IGF-I, two analogs, long R^3 IGF-I and des(1-3)IGF-I, which have low affinities for IGFBP, were also used as stimulants.

6.1. Cell stimulation

Bone marrow cells were isolated from female BALB/c mice, and then cultured in RPMI complete medium, as described in section 4. At different time points, the culture medium was replaced with serum-free RPMI 1640, and 50 ng/ml of different stimulants (recombinant hIGF-I, long R^3 IGF-I, and des(1-

3)IGF-I, purchased from Gropep, Adelaide, Australia) were added for 24 h incubation, followed by different measurements, as described below. A similar dose of IGF-I has been used to test its effect on bone marrow cell proliferation by Arkins et al. (1995).

6.2. Cell proliferation

Cell proliferation was determined by [^{3}H] thymidine incorporation into DNA as previously described (Arkins et al., 1995) with slight modifications. One μCi of [^{3}H] methyl thymidine (ICN-biomedical, Montreal, Quebec) was added into each well and cells were incubated for 6 h. The medium was removed and the cells were washed three times with prewarmed phosphate-buffered saline (PBS). Then, 0.25 ml of 1 N NaOH was added into each well to lyse the cells, and 0.2 ml of the liquid was mixed with 4 ml of scintillation cocktail (ICN-biomedical), and radioactivity was counted in a scintillation counter.

7. Effect of IGF-I on BMM differentiation

It was hypothesized that IGF-I might have some effect on BMM differentiation. Therefore, cells were stimulated by IGF-I at different time points, and then differentiation was measured using several parameters. First, the effect of IGF-I

on cell morphology and NSE-1 activity was tested. Further, mannose receptor expression, a more sensitive and quantitative marker, was used to investigate the effect of IGF-I on BMM differentiation. As mentioned in section 6, long R^3 IGF-I and des(1-3)IGF-I were also used as stimulants to see whether IGFBP could influence the effect of IGF-I.

7.1. Cytochemistry and morphology

Cells were stained for NSE-1 activity using α-naphthyl acetate esterase as substrate. The assay was performed using a commercial Sigma α-naphthyl acetate esterase kit (91-A), following the procedure provided by the company. Briefly, adequate amount of deionized water was prewarmed at 37°C. One millilitre of the Sodium Nitrite Solution and 1 ml of the Fast Blue BB Base Solution were mixed together and allowed to stand for at least 2 min until the color changed from dirty brown to deep yellow. The mixed solution was then diluted into 40 ml of prewarmed deionized water. Five millilitres of TRIZMAL™ 7.6 Buffer Concentrate was added, followed by the addition of 1 ml of the α-Naphthyl Acetate Solution. The resultant staining solution was mixed until it turned homogeneously greenish.

After removal of the culture medium, cells were fixed with the Citrate-Acetone-Formaldehyde Solution for 30 sec and washed for 45-60 sec. The staining solution was incubated with

cells for 30 min at 37°C in the absence of light. Afterwards, cells were washed again for 6 times and stained with the Hematoxylin Solution for 2 min. Finally, cells were washed thoroughly and the plates were air-dried. The presence of NSE-1 was visualized by light microscopy (400 times amplified). Also under microscope, the morphology of the cells was checked and the number of macrophages was counted.

7.2. Mannosylated-BSA iodination

One hundred micrograms of Mannosylated-BSA (EY Laboratories, San Mateo, CA) and 300 μg of chloramine T were added into 80 μl of 0.1 M sodium phosphate buffer with 1 mCi of $Na^{125}I$ (Amersham) for 10 min on ice. Then, 190 μl of sodium metabisulfate (2.4 mg/ml) and 190μl of potassium iodide (10 mg/ml) were added to stop the reaction. Sephadex G-50 column chromatography (1 x 20 cm, buffered in Tris. HCl, pH 7.5) was used to remove free iodine. Finally, liquid from the column was collected at 0.5 ml per tube and the labelled protein fractions were identified by γ-counting (Clohisy et al., 1987).

7.3. Mannosylated-BSA receptor binding assay

Mannose receptors are a cell surface marker expressed during BMM differentiation (Clohisy et al., 1987). [^{125}I]-

Mannosylated-BSA ([^{125}I]-Man-BSA) was used as a ligand in determining this receptor expression. Cells were incubated and stimulated following the procedure described in section 6.1, washed with HHBG (Hank's balanced salt solution (HBSS) (GIBCO/BRL), 10 mM Hepes, 10 mM Tris, 0.1% glucose, 10 mg/ml of BSA, pH 7.1) for 3 times and then incubated with [^{125}I]-Man-BSA at different concentrations in 0.4 ml of HHBG with or without 2 mg/ml of mannan for 48 h at 4°C. At the end of incubation, the buffer was removed and cells were washed six times with HBSS. Then, 0.5 ml of 1 N NaOH was added into each well to lyse the cells, and 0.4 ml of the liquid was taken out for γ-counting. Data were analyzed by the Scatchard analysis (Scatchard, 1949).

8. Statistical analysis

All data were obtained from at least 3 independent experiments, except the receptor binding assay, which was only performed twice. Statistical analysis was performed by analysis of variance (ANOVA) and multiple comparison test. For the receptor binding assay, Scatchard analysis was performed using EBDA (version 2.0, 1985) and Ligand (1980) programs (McPherson, 1985).

V. Results

1. Detection of endotoxin in the medium and reagents

In order to check the presence of endotoxin in the medium and reagents, which might influence the accuracy of this study, the Limulus amebocyte lysate assay was performed. The results showed that all culture medium and relevant reagents contained less than 0.1 ng of LPS per ml.

2. Alteration of endogenous IGFBP during BMM differentiation

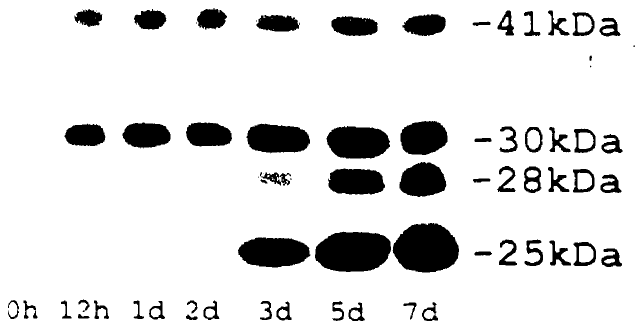
To determine if IGFBP secretion is altered during BMM differentiation, WLB analysis was performed. Freshly isolated bone marrow cells were cultured in RPMI complete medium containing 20% LCM for 12 h, 24 h, 2 d, 3 d, 5 d and 7 d, and then the medium was replaced with serum-free RPMI for an additional 24 h incubation. One group of freshly isolated bone marrow cells was directly cultured in serum-free medium for 24 h (referred as 0 h later). The cells were scraped and pooled into the medium. After centrifugation, the supernatant was collected and concentrated ten times for analysis of IGFBP profiles. No IGFBP was observed at 0 h by WLB analysis. However, two bands, with approximate molecular weights of 41 kDa and 30 KDa, were detected in the samples collected from 12 h to 7 d. Secretion of the 41-kDa protein was quite stable in

the whole process of BMM development, while the intensity of the 30-kDa band increased in a time-dependent manner until day 5 and decreased slightly on day 7. Starting from day 3, two additional bands with molecular weights of approximately 28-kDa and a 25-kDa were also detected, which increased during differentiation in a time-dependent manner. These two bands could also be observed at 12 h, 24 h and on day 2, but could not be measured using NIH image 1.60 program due to the low intensity (Fig. 1).

Posttranslational modification makes it difficult to identify the IGFBP profiles based on their molecular weights. For instance, different glycosylation and phosphorylation could make the same protein to have quite divergent molecular masses. To verify the IGFBP detected in WLB analysis, immunoblotting analysis was performed. Anti-IGFBP-4 antibody recognized two bands in the samples. The 25-kDa band could be detected in all samples, although it was faint in the samples collected at 0 h and 12 h. The expression of this band increased in a time-dependent manner. The other band reacting with anti-IGFBP-4 antibody had a molecular mass of 28 kDa, and it was detected from day 2. Compared to the intensity of the 25-kDa band, this band was much weaker, but it still increased in a time-dependent manner (Fig. 2). These data indicate that both the 28-kDa and the 25-kDa bands detected in the samples are IGFBP-4.

Fig. 1. Western ligand blotting (WLB) analysis of IGFBP secretion during BMM differentiation induced by CSF-1. Freshly isolated bone marrow cells were cultured in the complete medium with 20% LCM for different time periods (indicated in the figure), and then incubated in serum-free medium for 24 h. Conditioned medium was collected and WLB analysis was performed as described in Materials and Methods. Panel A shows the autoradiograph of WLB analysis. Panel B displays the densitometric units of the binding proteins analyzed by NIH image 1.60 program. This is a representative from three independent experiments.

A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-30kDa
-28kDa
-25kDa
0h 12h 1d 2d 3d 5d 7d

B

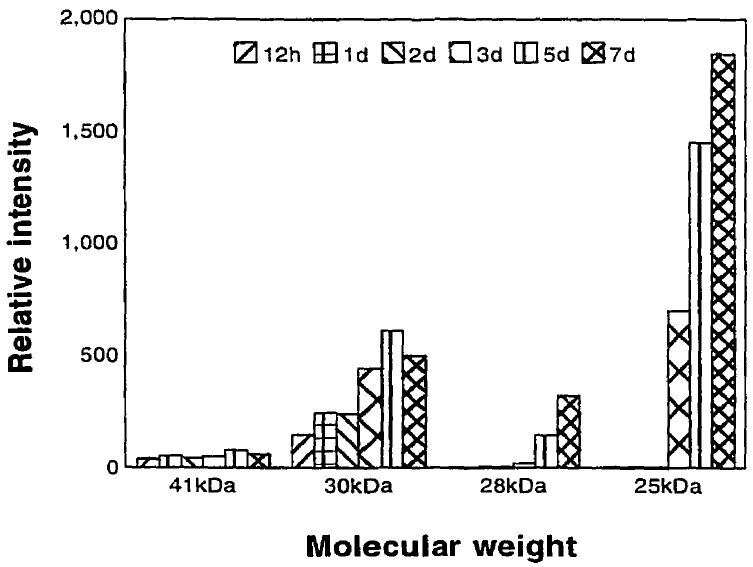
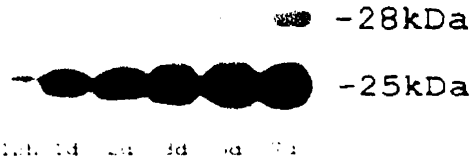
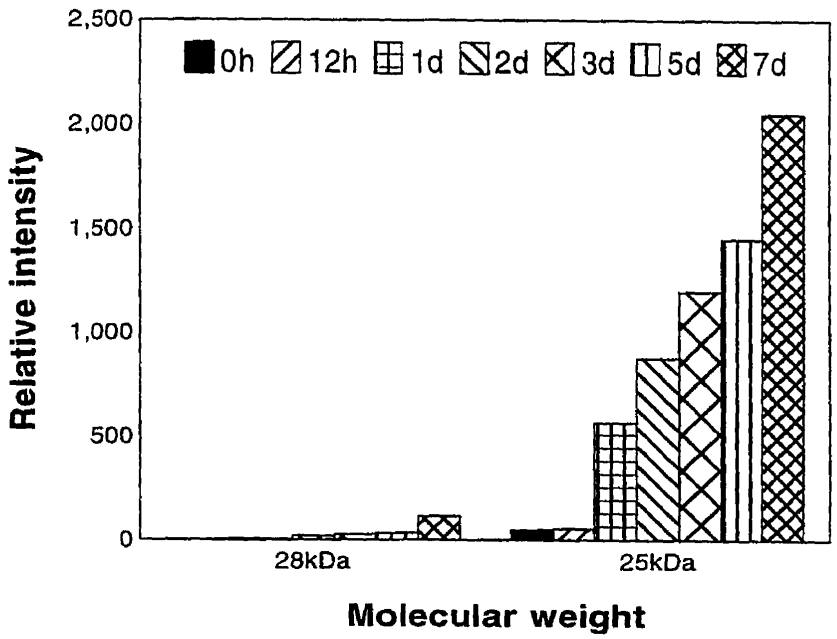

2,000
1,500
1,000
500
0
12h 1d 2d 3d 5d 7d
Relative intensity
41kDa
30kDa
28kDa
25kDa
Molecular weight

Fig. 2. Immunoblotting analysis of IGFBP secretion during BMM differentiation induced by CSF-1. Freshly isolated bone marrow cells were cultured in complete medium with 20% LCM for different time periods (indicated in the figure), and then incubated with serum-free medium for 24 h. Conditioned medium was collected and immunoblotting analysis was performed using anti-IGFBP-4 antibody. Panel A shows the flurograph of immunoblotting analysis. Panel B displays the densitometric units of the binding proteins analyzed by NIH image 1.60 program. This is a representative from three independent experiments.

A


-28kDa
-25kDa

B


2,500
2,000
1,500
1,000
500
0
0h
12h
1d
2d
3d
5d
7d
Relative intensity
28kDa
25kDa
Molecular weight

When Anti-IGFBP-5, anti-IGFBP-2 and anti-IGFBP-1 antibodies were used in the immunoblotting analysis, they did not react with any protein in any of the samples (data not shown).

3. Alteration of IGF-I and IGFBP mRNA expression during BMM differentiation

While the protein levels were determined by the Western analysis, the mRNA levels were measured by the Northern blotting analysis. Samples were collected as described in Materials and Methods, RNA was extracted from the cell pellets and separated by agarose gel electrophoresis. Stained with ethidium bromide, 18s and 28s rRNA could be clearly visualized under U.V. light (data not shown), indicating that RNA was extracted without degradation. The mRNA level was tested by Northern blotting analysis using cDNA fragments as probes.

IGF-I mRNA level was undetectable in freshly isolated bone marrow cells, but its expression increased with the culture time until day 3, and decreased gradually on day 5 and 7. In the early stages of culture (12 h and 24 h), only two IGF-I transcripts (0.8-1.2 kb and 1.8 kb) were detected, and in the day 2 sample, another transcript of 7.4 kb was observed. Four transcripts with different sizes (0.8-1.2 kb,

1.8 kb, 4.4 kb and 7.4 kb, respectively) existed in the samples collected after day 3 (Fig. 3).

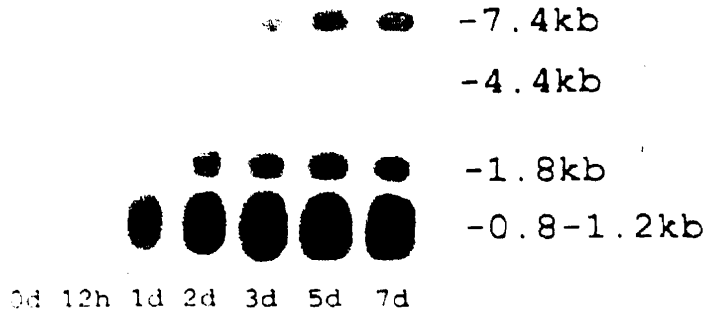
The IGFBP-3 transcripts were detected in all the samples. IGFBP-3 mRNA level appeared to decrease during the first 3 days, and then increase gradually until day 7 (Fig. 4). Like IGFBP-3, IGFBP-4 mRNA (2.4 kb) was also detected in all the samples, but its level was relatively constant (Fig. 5). IGFBP-2 and IGFBP-5 mRNAs were undetectable by the Northern blotting analysis (data not shown). Due to the unavailability of the IGFBP-1 cDNA probe, IGFBP-1 mRNA expression was not investigated.

4. Effects of IGF-I and its analogs on cell proliferation

To test the effect of IGF-I on bone marrow cell proliferation, [^{3}H] thymidine incorporation was compared between control cells and cells stimulated with recombinant human IGF-I (rhIGF-I). To determine if IGFBP could interfere with IGF-I, two IGF-I analogs, long R^3 IGF-I and des(1-3)IGF-I, which have much lower affinities for IGFBP, were also used as stimulants. At different time points (day 0, day 1, day 3 and day 5), cells were incubated with IGF-I or its analogs in serum-free medium for 24 h, cell proliferation being measured by [^{3}H] thymidine incorporation, as described in Materials and Methods. All stimulants displayed a stimulatory effect on

Fig. 3. Expression of IGF-I mRNA during BMM differentiation induced by CSF-1. Freshly isolated bone marrow cells were cultured in complete medium with 20% LCM for different time periods (indicated in the figure), and then incubated with serum-free medium for 24 h. Total RNA was extracted from the cells and IGF-I mRNA level was checked by Northern blotting analysis using rat IGF-I cDNA probe, as described in Materials and Methods. Panel A is the autoradiograph of IGF-I expression. Panel B shows the autoradiograph of ß-actin. Panel C displays the results of densitometric analysis of IGF-I expression corrected for ß-actin (data shown as the percentages of ß-actin expression). This is a representative from three independent experiments.

A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-4.4kb
-1.8kb
-0.8-1.2kb
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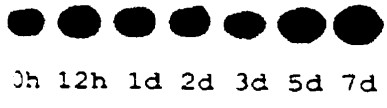
B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C

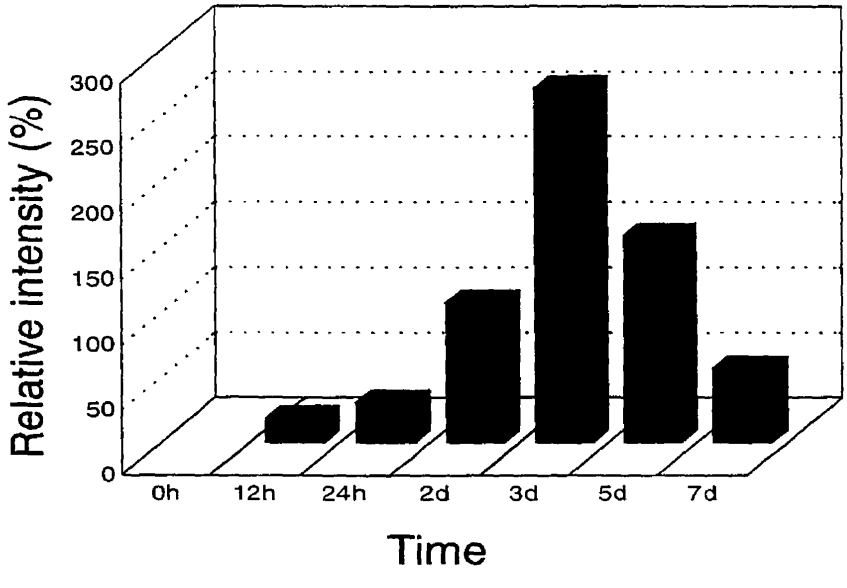
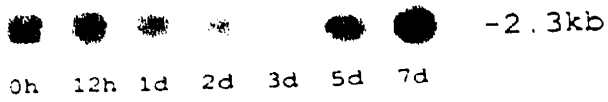

Relative intensity (%)
300
250
200
150
100
50
0
0h
12h
24h
2d
3d
5d
7d
Time

Fig. 4. Expression of IGFBP-3 mRNA during BMM differentiation induced by CSF-1. Freshly isolated bone marrow cells were cultured in complete medium with 20% LCM for different time periods (indicated on the X-axis), and then incubated with serum-free medium for 24 h. Total RNA was extracted from the cells and IGFBP-3 mRNA level was determined by Northern blotting analysis using rat IGFBP-3 cDNA probe, as described in Materials and Methods. Panel A is the autoradiograph of IGFBP-3 expression. Panel B shows the autoradiograph of ß-actin. Panel C displays the results of densitometric analysis of IGFBP-3 expression corrected for ß-actin (data shown as the percentages of ß-actin expression). This is a representative from three independent experiments.

A


-2.3kb
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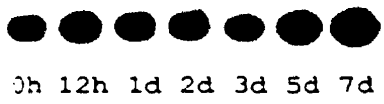
B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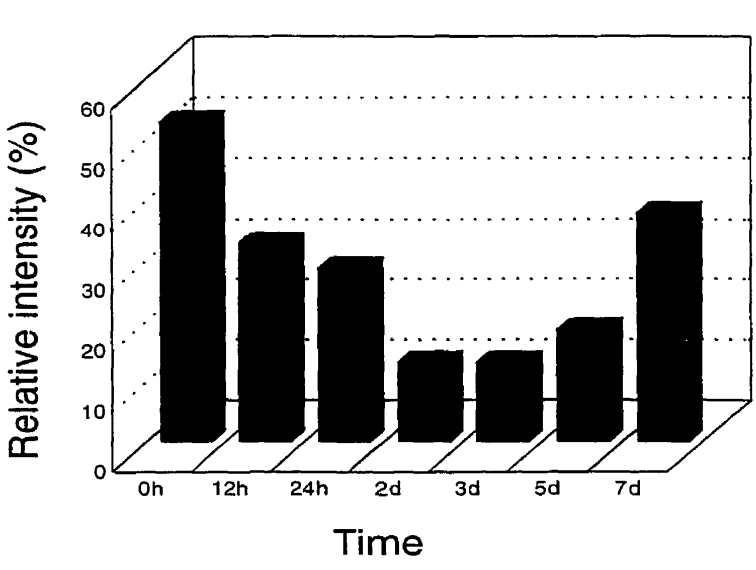
C


Relative intensity (%)
60
50
40
30
20
10
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0h
12h
24h
2d
3d
5d
7d
Time

Fig. 5. Expression of IGFBP-4 mRNA during BMM differentiation induced by CSF-1. Freshly isolated bone marrow cells were cultured in complete medium with 20% LCM for different time periods (indicated on the X-axis), and then incubated with serum-free medium for 24 h. Total RNA was extracted from the cells and IGFBP-4 mRNA level was checked by Northern blotting analysis using rat IGFBP-4 cDNA probe, as described in Materials and Methods. Panel A is the autoradiograph of IGFBP-4 gene expression. Panel B shows the autoradiograph of ß-actin. Panel C displays the results of densitometric analysis of IGFBP-4 expression corrected for ß-actin (data shown as the percentages of ß-actin expression). This is a representative from three independent experiments.

A

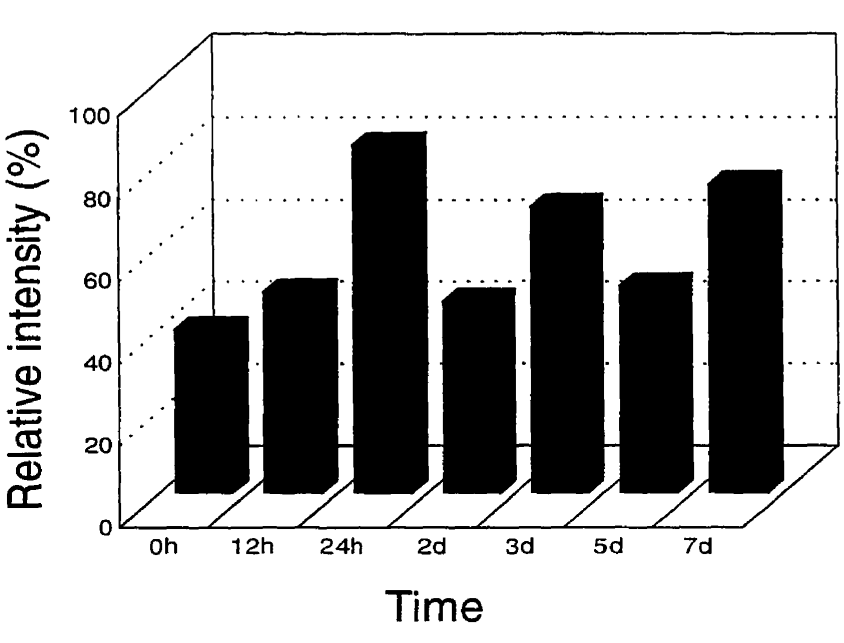
-2.4kb

0h 12h 1d 2d 3d 5d 7d

B

0h 12h 1d 2d 3d 5d 7d

C

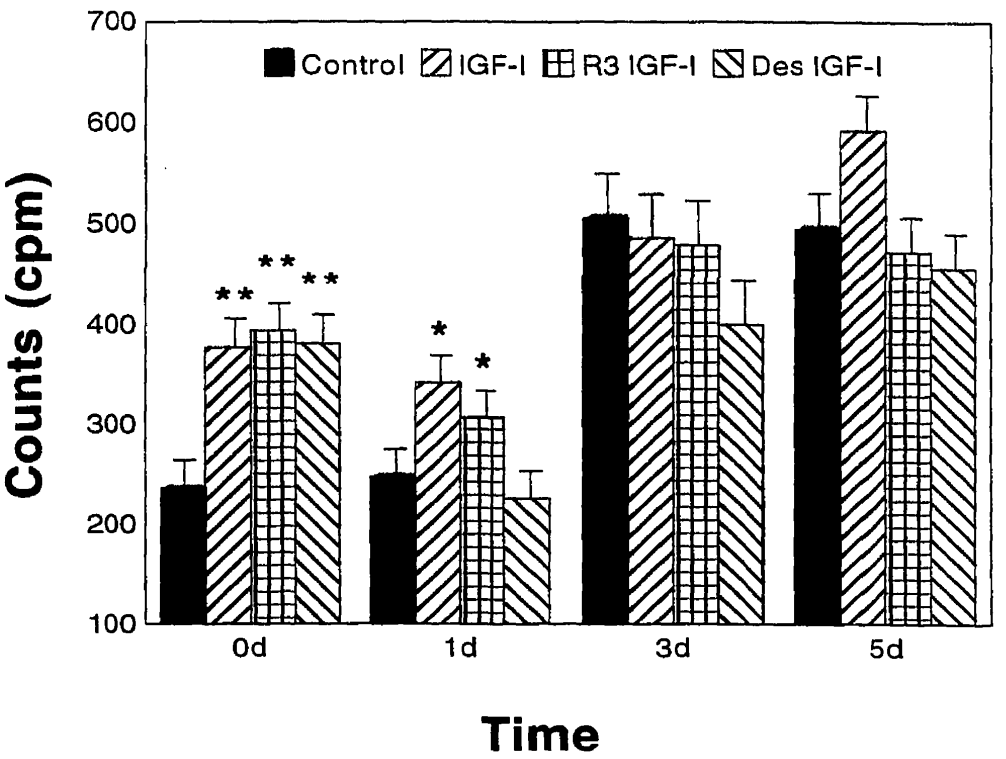


freshly isolated bone marrow cell proliferation after 24 h incubation ($P<0.01$). Compared to that of control, [^{3}H] thymidine incorporation increased 59.7%, 66.7%, and 61.1% upon incubation with rhIGF-I, long R^3 IGF-I, and des(1-3)IGF-I, respectively. On day 1, rhIGF-I and long R^3 IGF-I continued to enhance cell proliferation, albeit to a lesser extent ($P<0.05$). The former increased DNA synthesis by 37.9% and the latter increased by 23.8% compared to that of control. However, des(1-3)IGF-I was ineffective. On days 3 and 5, the stimulants did not have any effect on cell proliferation (Fig. 6).

5. Time course study of NSE-1 staining and morphology of the cells stimulated by IGF-I and its analogs

While the macrophage precursors transit to its mature forms, their morphology and NSE-1 enzyme activity are expected to be altered. Therefore, using NSE-1 staining and morphology of the cells as markers, the effect of IGF-I on BMM differentiation was studied. Bone marrow cells were cultured in complete medium with 20% LCM. On days 1, 2, 4 and 6, the culture medium was replaced with serum-free RPMI, and rhIGF-I, long R^3 IGF-I or des(1-3)IGF-I was added for 24 h incubation. Subsequently, the medium was removed, cells were fixed and stained. Morphology and NSE-1 activity were visualized microscopically in 10 fields (400 times amplified) for each

Fig. 6. Effects of rhIGF-I and its analogs on [^{3}H] thymidine incorporation during BMM differentiation. Freshly isolated bone marrow cells were cultured in complete medium with 20% LCM for different time periods (indicated in the figure), and then incubated with rhIGF-I, long R^3 IGF-I or des(1-3)IGF-I in serum-free medium for 24 h. [^{3}H] thymidine incorporation was measured as described in Materials and Methods. This is a representative from three independent experiments.



*: different from control within the same day (P<0.05).

**: different from control within the same day (P<0.01).

sample. The cytoplasm of almost all the adherent cells were positive for NSE-1 staining. Although cells at later stages of development appeared to be stained more intensively in the cytoplasm, it is difficult to distinguish the extent of differentiation by this assay. Morphologically, mature macrophages were identified as 1) being comparatively large; 2) with several pseudopodia; 3) having a large part of cytoplasm (cytoplasm: nucleus >1); and 4) containing many vacuoles and lysosomes in the cytoplasm. There was no significant difference in the percentage of mature macrophages among all four groups within the same day. Then all data from different groups in the same day were pooled in order to analyze the culture time effect on macrophage differentiation. The percentage of macrophages increased significantly from day 1 to day 2 ($P<0.05$), and from day 2 to day 4 ($P<0.05$). However, no significant increase was found from day 4 to day 6 ($P>0.05$) (Table 2). Representatives of cells collected at different days with different stimulants are shown in Fig. 7. In the control group, from day 1 to day 6, the cytoplasm area and the vacuoles and granules increased gradually. However, within the same day (day 4), no difference could be observed among different treatment groups. Cells collected on the other days did not display any difference in morphology and NSE-1 activity among all the four groups, either (data not shown).

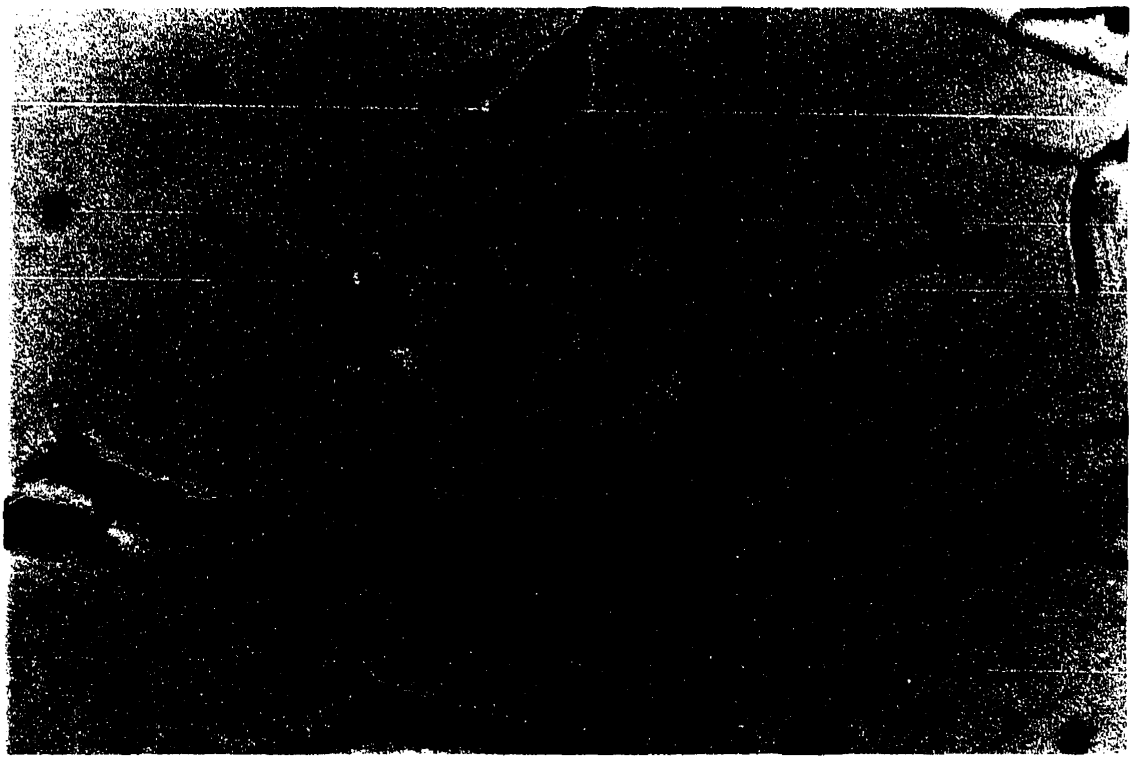
Table 2. Effects of rhIGF-I and its analogs on percentages of macrophages based on cell morphology*.

	Control	rhIGF-I	Long R^3 IGF-I	Des (1-3) IGF-I
1 d	52.4±3.9%	62.7±3.9%	62.7±3.9%	61.9±3.9%
2 d	72.4±3.2%	66.8±3.2%	67.8±3.2%	71.5±3.2%
4 d	76.0±2.8%	77.5±2.8%	75.6±2.8%	78.5±2.8%
6 d	80.4±1.8%	79.0±1.8%	80.8±1.8%	82.5±1.8%

* Cells were incubated for different time periods, and stimulants (rhIGF-I, long R^3 IGF-I or des(1-3)IGF-I) were added, as described in Materials and Methods. Cells were counted from ten randomly selected microscopic fields. Percentages of macrophages were calculated.

Fig. 7. NSE-1 staining of the cells. Freshly isolated bone marrow cells were cultured in complete medium with 20% LCM for different time periods, and then incubated with one of the stimulants (rhIGF-I, long (R^3) IGF-I and des (1-3) IGF-I) in serum-free medium for 24 h. Subsequently the medium was removed and cells were fixed and stained as described in Materials and Methods. Each figure is a representative of cells cultured for a certain time period with a certain stimulant. Panel A: 1 d control; Panel B: 2 d control; Panel C: 4 d control; Panel D: 6 d control; Panel E: 4 d rhIGF-I; Panel F: 4 d long R^3 IGF-I; Panel G: 4 d des(1-3)IGF-I. The time stands for the culture days excluding the 24 h period in serum-free medium.

A

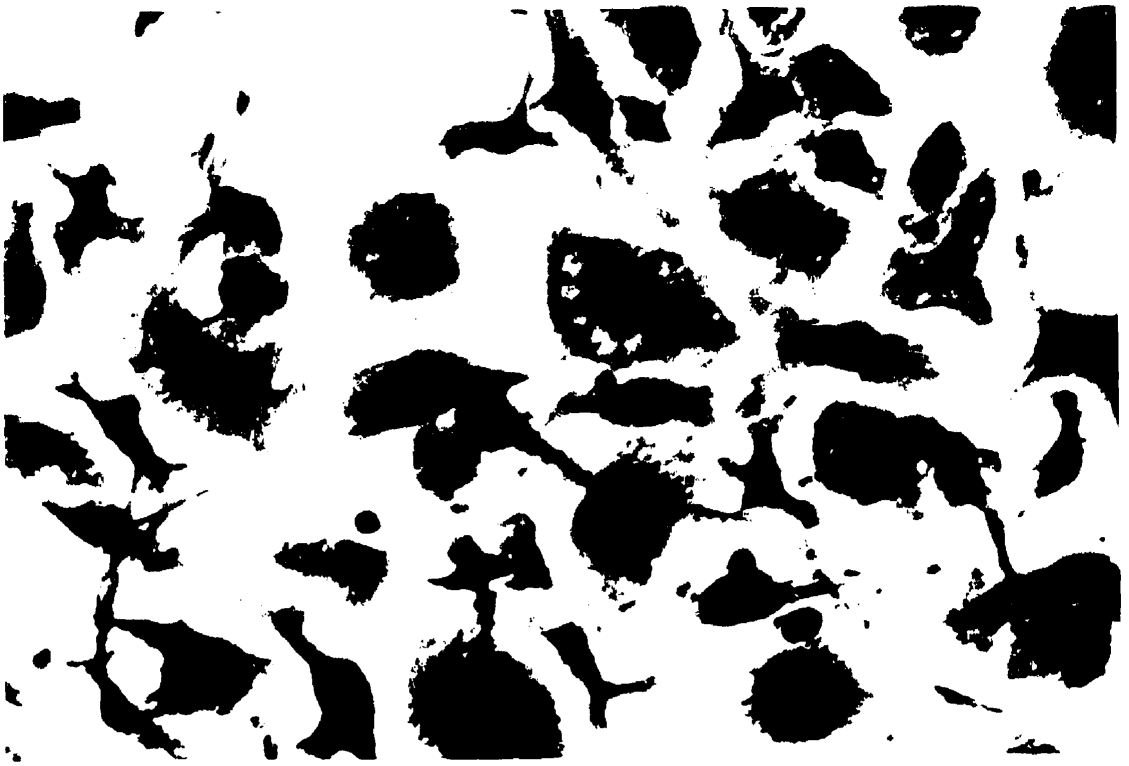


B

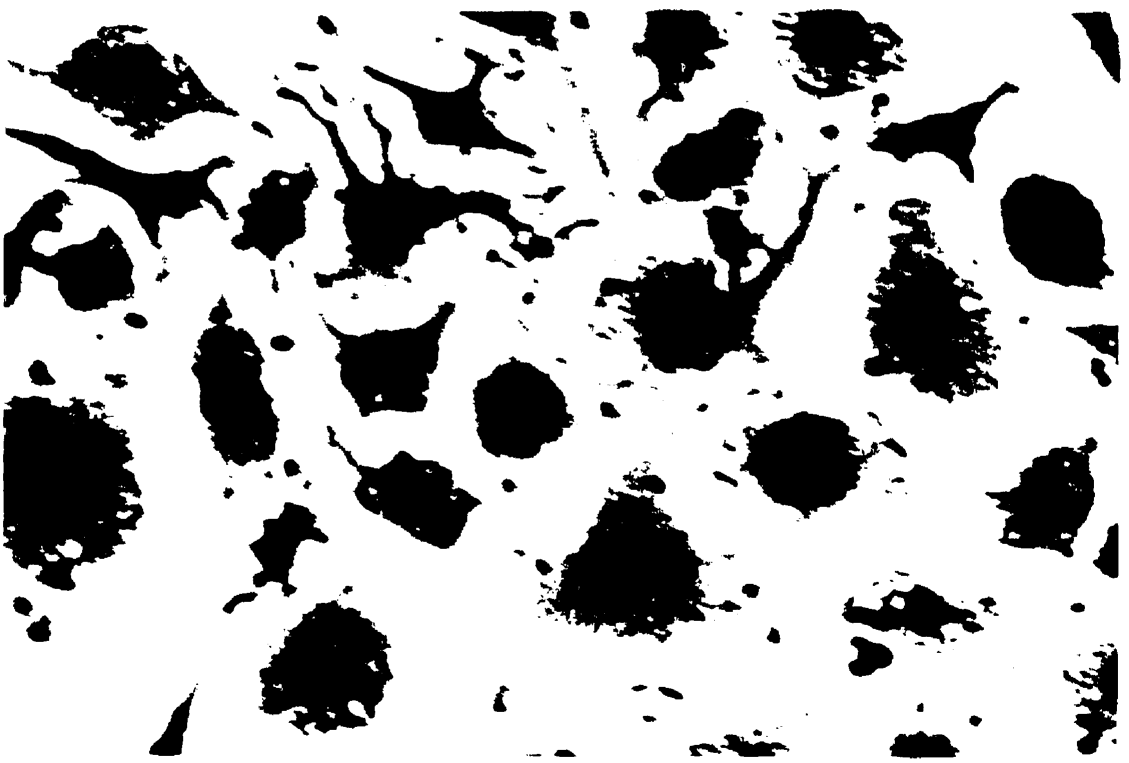
C

D

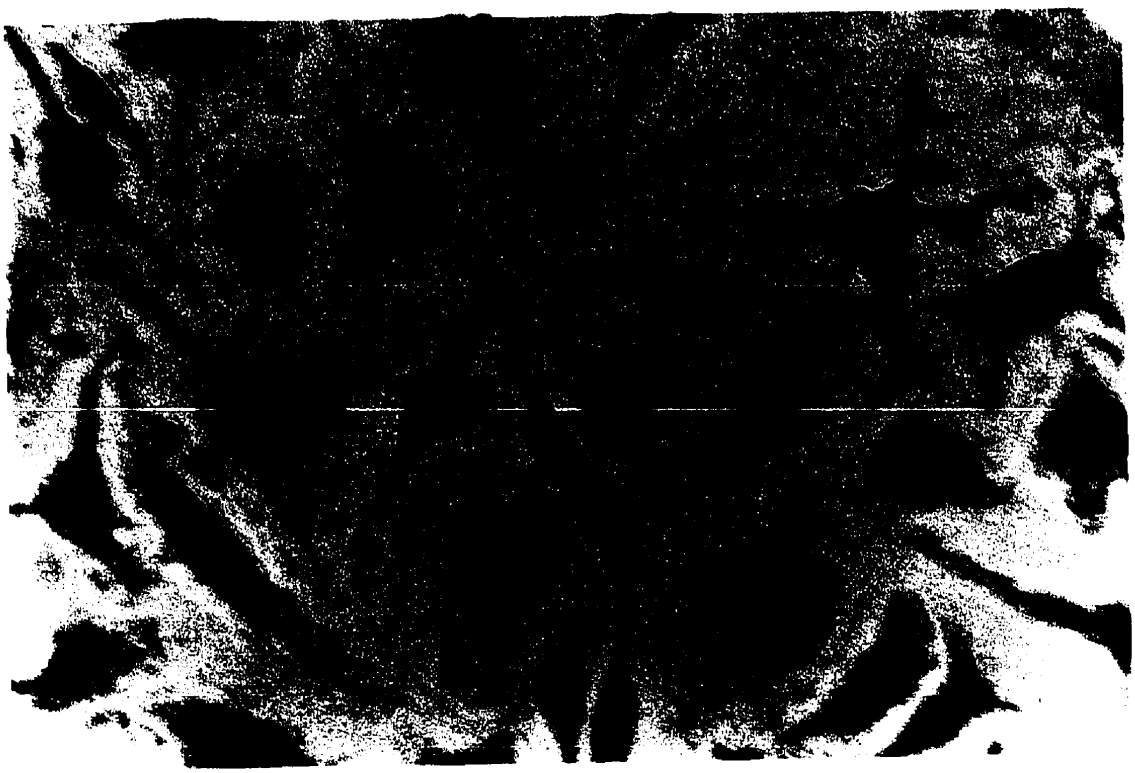
E



F



G



6. Effects of IGF-I and its analogs on mannose receptor binding

Expression of the mannose receptors, a specific and quantitative marker for macrophage differentiation, was also examined for the effect of IGF-I and its analogues on BMM differentiation with [^{125}I]-Man-BSA as the ligand. Freshly isolated bone marrow cells were cultured in complete medium with 20% LCM for 2 or 4 days, and then treated with different stimulants (rhIGF-I, long R^3 IGF-I or des(1-3)IGF-I) in serum-free medium for 24 h. Afterwards, cells were incubated with different concentrations of [^{125}I]-Man-BSA HHBG for 48 h at 4°C, in the presence or absence of unlabelled mannan (2 mg/ml). Cells were washed and lysed by NaOH, the radioactivity being measured in a γ-counter. Scatchard analysis was performed by using EBDA and Ligand program. For day 2 samples, data could not be used for analysis, presumably due to insufficient receptors on the cells. For day 4 samples, the rhIGF-I group did not show any significant difference in both K_d and B_{max} value in comparison with the control group. However, the maximum binding sites (B_{max} values) for the Long R^3 IGF-I and des(1-3)IGF-I groups was increased by 260% and 228%, respectively. At the same time, K_d values for these two groups increased to a lesser extent (Table 3 and Fig. 8).

Table 3. Effects of rhIGF-I and its analogs on Mannosylated-BSA binding to the cell receptors*.

	Control	rhIGF-I	Long R^3 IGF-I	Des (1-3) IGF-I
K_d (nM)**	47.8±16.9	61.3±44.4	73.5±14.1	89.5±8.63
B_{max}(pM)***	74.1±14.7	121.0±68.2	267.2±24.4	243.1±9.94

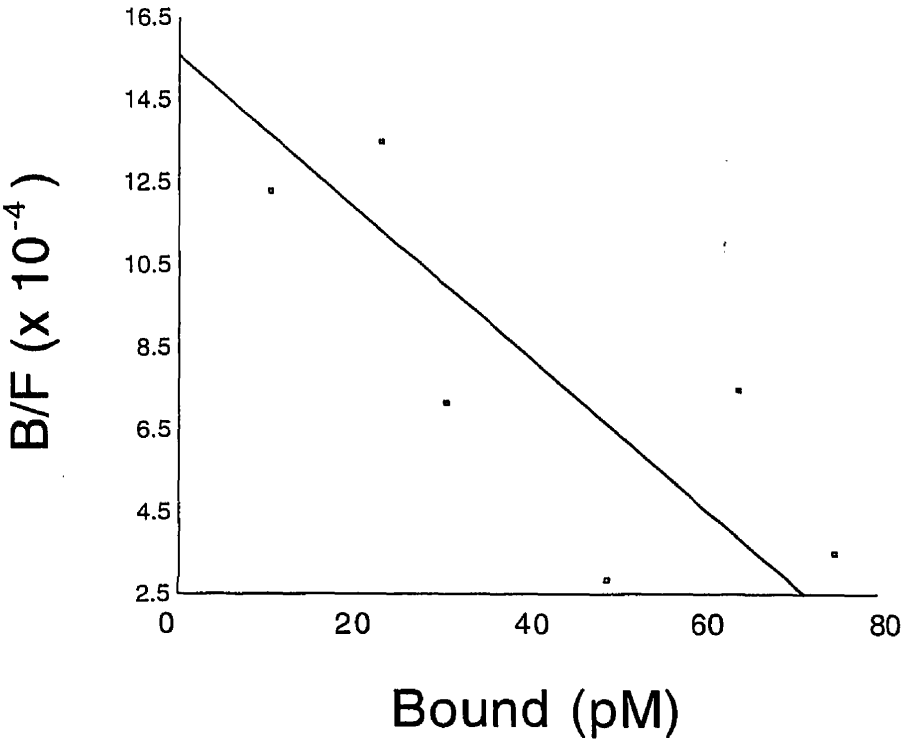
* Cells were cultured in complete medium with 20% LCM for 4 days, kept in serum-free medium with or without one of the three stimulants (rhIGF-I, long R^3 IGF-I or des(1-3)IGF-I) for 24 h, and then incubated with [^{125}I]Mannosylated-BSA in HHBG for 48 h in the absence or presence of unlabelled mannan, as described in Materials and Methods. Binding was measured by γ-counter, and data were analyzed by Scatchard method using Ligand program. This is a representative from two independent experiments.

** K_d stands for the equilibrium binding constant of dissociation.

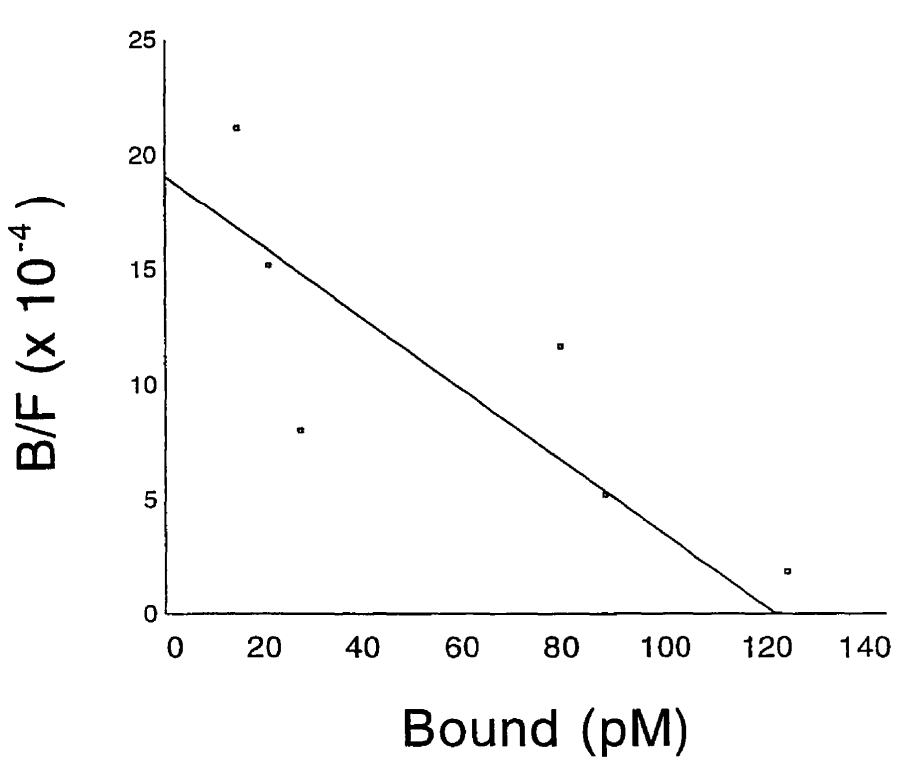
*** B_{max} stands for receptor site concentration.

Fig. 8. Scatchard analysis of [^{125}I]-Man-BSA binding to the cells. Freshly isolated bone marrow-derived cells were cultured in complete medium with 20% LCM for 4 days, then in serum-free medium with one of the three stimulants (rhIGF-I, long R^3 IGF-I or des(1-3)IGF-I) for 24 h, and finally incubated with [^{125}I]-Man-BSA in HHBG for 48h in the absence or presence of unlabelled mannan, as described in Materials and Methods. Binding was measured by γ-counter, and the data was analyzed by Scatchard method using Ligand program. Panel A: control; Panel B: rhIGF-I; Panel C: long (R^3) IGF-I; Panel D: des (1-3) IGF-I.

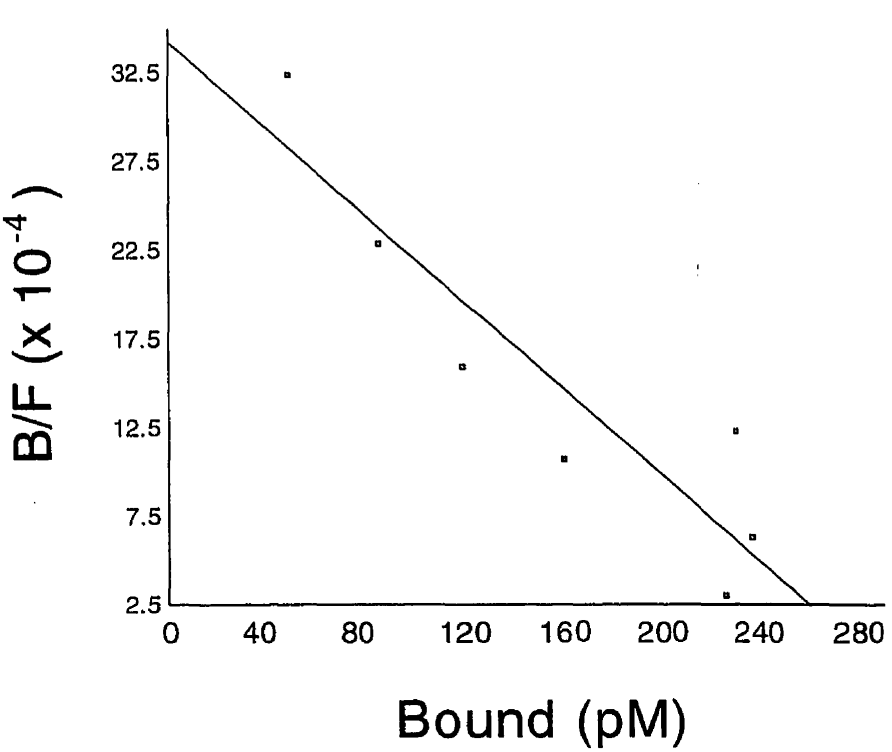
A


16.5
14.5
12.5
10.5
8.5
6.5
4.5
2.5
B/F (x 10-4)
0
20
40
60
80
Bound (pM)

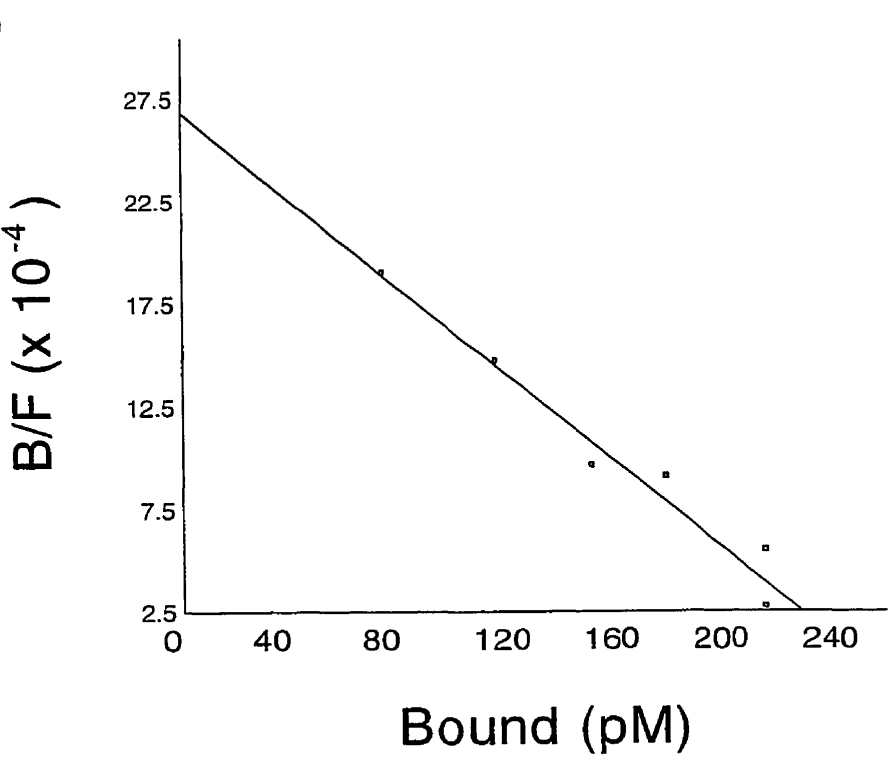
B


25
20
15
10
5
0
B/F (x 10-4)
0
20
40
60
80
100
120
140
Bound (pM)

C


32.5
27.5
22.5
17.5
12.5
7.5
2.5
B/F (x 10^{-4})
0
40
80
120
160
200
240
280
Bound (pM)

D


27.5
22.5
17.5
12.5
7.5
2.5
B/F (x 10^{-4})
0
40
80
120
160
200
240
Bound (pM)

VI. Discussion

It is well known that IGF-I and its binding proteins exist in a variety of cells and are indispensable in regulating many cellular functions, including cell growth and differentiation. In this study, the potential role of IGF-I and IGFBP in CSF-1-induced BMM proliferation and differentiation was investigated.

The WLB analysis revealed that four IGFBP with different sizes (41 kDa, 30 kDa, 28 kDa, and 25 kDa, respectively) were detected in the culture medium of bone marrow cells stimulated by CSF-1. The 41-kDa and the 30-kDa bands were observed from a very early stage, while the other two bands were observed only at a later stage. In contrast, no single IGFBP was detected in freshly isolated bone marrow cell conditioned medium. Immunoblotting analysis proved that the 28-kDa and 25-kDa bands are IGFBP-4. Furthermore, Northern blotting analysis showed that IGFBP-3 and IGFBP-4 mRNA were present in the samples collected at all time points. In addition, an increase in IGF-I gene expression was observed during the process of BMM development induced by CSF-1. IGF-I transcripts with different sizes were expressed in well-differentiated BMM.

Exogenous IGF-I and its analogs displayed a stimulatory effect on cell proliferation only if added at very early stages. IGF-I did not exert any effect on macrophage differentiation, whereas both long R^3 IGF-I and des(1-3)IGF-I had a stimulatory action on macrophage differentiation when the mannose receptor expression was used as a specific marker. When using the morphology and NSE-1 staining as the criteria for differentiation, however, no difference could be observed among the control group and the groups treated with either IGF-I or its analogs.

1. Expression of IGF-I

Expression of IGF-I can be modulated by many factors. During differentiation of many cell types, endogenous IGF-I level changes significantly. For instance, increased IGF-I mRNA expression was associated with mouse C2 myoblast differentiation (Tollefsen et al., 1989). By Northern blotting analysis, we demonstrated that mRNA level of IGF-I was up-regulated in a time-dependent manner until day 3, and down-regulated thereafter. Using a ribonuclease protection assay, Arkins et al. (1995) reported that IGF-I transcription was enhanced during BMM differentiation, and reached a plateau on day 4. This is in accordance with our results, since our "day 3 sample" was actually collected after three days of culture in the complete medium with 20% LCM, plus one day in serum-

free medium. Nonetheless, the results obtained by Arkins et al. (1995) did not display any downregulation after day 4.

In differentiated macrophages, four IGF-I transcripts with different sizes were detected. Three of the transcripts (0.8-1.2 kb, 1.8 kb, and 7.4 kb, respectively) corresponded to the results obtained from a previous study on BMM (Arkins et al., 1993). In addition, a 4.4-kb mRNA was also observed, albeit its expression was not so abundant as the other three transcripts. These four bands were also observed by Scharla et al. (1991) using mouse osteoblasts. The size difference probably comes from alternate splicing from the same gene, or from the use of distinct polyadenylation sites. Its physiological significance still needs to be elucidated in further studies.

2. Expression of IGFBP-4

Alteration of IGFBP expression was connected to the differentiation of a wide range of cells. In CSF-1-induced BMM differentiation, four bands with sizes varying from 25- to 41-kDa were detected by WLB analysis. By immunoblotting analysis, it was shown that the 28-kDa and 25-kDa protein reacted with an anti-IGFBP-4 antibody. Northern blotting analysis also confirmed the existence of IGFBP-4 mRNA. Therefore, it is obvious that these two bands are both IGFBP-4. Based on their

molecular masses, it can be assumed that the 25-kDa protein represents the non-glycosylated form while the 28-kDa band is glycosylated. This is in agreement with the situation in B104 rat neuronal cells, where a 28-kDa and a 24-kDa band were detected, the former being N-glycosylated, and the latter not glycosylated (Cheung et al., 1991). During the early stages of bone marrow cell culture, the secretion of IGFBP-4 was almost undetectable, but it started to accumulate in the culture medium on day 3, and increased continuously thereafter, and the increase is correlated with the extent of BMM differentiation.

Recently, Li et al. (1996) described the synthesis and secretion of a 25-kDa IGFBP-4 by CSF-1-induced murine BMM. The present study substantiated their findings, and also demonstrated the existence of another form of IGFBP-4, with a molecular weight of 28-kDa. In the current study, samples were concentrated 10-fold before the WLB and immunoblotting analyses were performed. This might have improved the sensitivity of the assays, so that the existence of the 28-kDa IGFBP-4 could be observed, its intensity however being much weaker compared to the 25-kDa protein.

During BMM development, the secretion of IGFBP-4 was induced only at a late stage (day 3), then increased in a

time-dependent manner, and peaked at the end of differentiation. However, the mRNA level of IGFBP-4 remained unchanged during the process of CSF-1-induced BMM differentiation. This suggests that the translation of IGFBP-4 was controlled at the posttranscriptional level rather than at the transcriptional level, even though the exact mechanisms involved are unclear. Presumably, proteolysis might be involved in this process.

Up to now, no potentiating effect of IGFBP-4 on target cells has been reported. In several cases, it inhibited IGF-I actions on the cells. In B104 neuroblastoma cells, IGF-I-stimulated thymidine incorporation was inhibited by IGFBP-4 (Cheung et al., 1991). In human rat osteosarcoma cells, IGFBP-4 decreased the effect of IGF-I on DNA and glycogen synthesis (Kiefer et al., 1992). Therefore, the accumulation of IGFBP-4 during BMM development might also exert negative effects on IGF-I actions.

3. Expression of other IGFBP

While the 28-kDa and the 25-kDa proteins are confirmed to be IGFBP-4, the 41-kDa and the 30-kDa proteins still need to be identified. Previous studies have suggested that it is common for IGFBP-3 to be glycosylated in various cells and to have a molecular weight of around 40 kDa or higher (Bradshaw

et al., 1993, Loret et al., 1991). Thus, we assume the the 41-kDa protein to be IGFBP-3. Unfortunately, rodent anti-IGFBP-3 antibody was unavailable to confirm this assumption. Nevertheless, when IGFBP-3 cDNA probe was used in the Northern blotting analysis, IGFBP-3 mRNA was detected. Taken together, although not completely verified, it is reasonable to believe that IGFBP-3 is synthesized and secreted during BMM differentiation. This protein exists in a glycosylated form, with a molecular weight of 41 kDa.

Surprisingly, the Northern blotting analysis revealed a unique pattern for gene expression of IGFBP-3. It was first downregulated and then upregulated, which is hard to interpret at this stage. While IGFBP-3 mRNA was detected in freshly-isolated bone marrow cells, its protein could not be detected in the culture medium. Therefore, some inhibitory factors of IGFBP-3 translation are suggested to exist in these cells. With the addition of CSF-1, these factors could be inactivated, followed by the production of IGFBP-3 protein. Also, IGFBP-3 might already be synthesized in these cells, but its secretion into the medium could be blocked. Addition of CSF-1 might help IGFBP-3 move to the extracellular space.

IGFBP-3 could have either stimulatory or inhibitory actions on cells, depending on different cell types and situations. In chick embryo fibroblasts, DNA synthesis induced

by IGF-I was inhibited by IGFBP-3 (Blat et al., 1989). IGF-I-stimulated glycogenolysis and glucose oxidation was inhibited by porcine IGFBP-3 in porcine fat cells (Walton et al., 1989). These inhibitory effects could be neutralized by the addition of higher concentrations of IGF-I, indicating that IGFBP-3 inhibits cellular responses by sequestering IGF-I. On the other hand, the inhibitory effect of IGFBP-3 might also be independent of IGF-I in some cells. Recombinant IGFBP-3 inhibited MCF-7 breast cancer cell proliferation induced by estradiol, and growth inhibition induced by ICI 182780 was correlated with the accumulation of IGFBP-3 in these cells (Huynh et al., 1996).

When IGFBP-3 were preincubated with human fibroblasts and then replaced with IGF-I, the IGF-I-induced DNA synthesis was potentiated. However, when IGFBP-3 was coincubated with IGF-I, the effect of IGF-I on cell growth was inhibited. It was postulated that this potentiating effect caused by preincubation with IGFBP-3 was related to the cell association of this binding protein (DeMellow and Baxter, 1988).

The potentiating effect of IGFBP-3 on target cells might be independent of IGF-I actions. IGFBP-3 increased growth hormone receptor binding and proliferation in rat osteoblast cells. This effect was suggested to be unrelated to IGF-I functions (Slootweg et al., 1995).

The complex functions of IGFBP-3 makes it difficult to assume its effect during BMM proliferation and differentiation. However, the secretion of this protein has been proved to be constant in the process of BMM development. Thus, IGFBP-3 might be a less important regulator than IGFBP-4 in this event.

Antibodies against IGFBP-1, -2, and -5 have been used in the immunoblotting analysis, but they failed to react with any binding proteins. Expression of IGFBP-2 and -5 mRNA were not detected by the Northern blotting analysis, either. Therefore, it is unlikely for these binding proteins to be synthesized and secreted at a detectable level during BMM development induced by CSF-1. In addition, IGFBP-6 has a relatively lower affinity for IGF-1, and thus is impossible to be detected in the WLB analysis, when [^{125}I] IGF-I is used as the tracer. Therefore, the 30-kDa protein detected in the WLB analysis seems not to be any of the above-mentioned four binding proteins. The possibility of it being IGFBP-4 can also be disregarded since it did not cross-react with the anti-IGFBP-4 antibody. Although it could be the non-glycosylated or a truncated form of IGFBP-3 based on its size, there is no report that either of these two forms of IGFBP-3 exists in the medium and maintains a high binding-affinity for IGF-I. Taken together, the identity of this 30-kDa band remains unknown. Another possibility is that this band might result from IGFBP-

2 contamination by FBS when the assay was performed. This IGFBP-2 of bovine origin could have a high binding affinity for IGF-I from all sources, but only recognize the antibody from its own species. As a result, it could be detected in IGF-I-traced WLB analysis, but not in the rodent-antibody-probed immunoblotting analysis.

4. Effects of IGF-I and IGFBP on cell proliferation and differentiation

The effect of IGF-I in growth and differentiation has been widely studied in various cells. IGF-I was proved to increase DNA synthesis of human peripheral blood monocytes and lymphocytes (Kooijman et al., 1992b). It also affects lymphopoiesis (Jardieu et al., 1994), granulopoiesis (Merchav et al., 1988 and 1993) and erythropoiesis (Kurtz et al., 1988). Expression of IGF-I mRNA was increased during BMM differentiation, and IGF-IR mRNA was detected on freshly isolated bone marrow cells (Arkins et al., 1995). Since IGF-I can act in autocrine or paracrine modes, it is likely that IGF-I may exert some action on bone marrow cells during the process of macrophage proliferation and differentiation. Arkins et al. (1995) demonstrated that exogenous IGFBP-3 could inhibit DNA synthesis of macrophage precursors, probably due to its sequestration of endogenous IGF-I, and exogenous IGF-I could overcome the inhibitory effect of IGFBP-3. They also

proved that recombinant IGF-I enhanced DNA synthesis of freshly isolated bone marrow cells by 65% in the presence of CSF-1.

To better understand the effect of IGF-I on bone marrow cell proliferation, DNA synthesis was measured by [^{3}H] thymidine incorporation. Since WLB and immunoblotting analyses demonstrated changes of IGFBP profiles during macrophage development, long R^3 IGF-I and des(1-3)IGF-I as well as rhIGF-I were used as stimulants to prevent the interaction of the binding proteins in the subsequent DNA synthesis measurement. These two IGF-I analogs bind with IGF-IR normally but have much lower affinity for IGFBP. At different time points, the stimulants were added in the serum-free medium for 24 h incubation, as described in Materials and Methods. The results showed that on day 0, three different IGF-I significantly increased DNA synthesis of bone marrow cells ($P<0.01$). This is in agreement with what Arkins et al. reported (1995). On day 1, IGF-I and long R^3 IGF-I still stimulated DNA synthesis, though to a lesser extent. Surprisingly, des(1-3)IGF-I did not have any effect. Starting from day 3, no significant stimulatory effect of these three IGF-Is was detected. This result shows that IGF-I and its analogues only have a stimulatory effect on bone marrow cell proliferation at the early stages. IGFBP-3 might help IGF-I to exert its stimulatory effect on day 1, since rhIGF-I enhanced cell

proliferation to a larger extent than long R^3 IGF-I, and des(1-3)IGF-I did not even have any effect.

The effects of IGF-I and its analogues on BMM differentiation were investigated. The morphology of cells was observed and NSE-1 assay was performed. From day 0 to day 4, the percentage of macrophages increased gradually, according to their morphology and the intensity of NSE-1 staining. However, NSE-1 stains positively not only for macrophages, but also for all cells belonging to the lineage of mononuclear phagocytes, including the precursors of macrophages. Besides, there are no obvious and strict criteria to determine the extent of macrophage differentiation by its appearance under the light microscope. Indeed, in the present study, on the same day, the external appearance and NSE-1 staining feature of cells in different treatment groups appeared to be similar. Neither morphology nor NSE-1 staining could reflect the effect of IGF-I on BMM differentiation.

It has been reported previously that expression of the mannose receptor increased with time on the bone-marrow derived cells cultured with CSF-1, suggesting this receptor to be a sensitive and quantitative marker of BMM differentiation (Clohisy et al., 1987). Accordingly, we investigated the effect of IGF-I and its analogues of BMM differentiation using [^{125}I]-Man-BSA binding assay. It was not possible to analyse

the results obtaned on day 2 because there was not enough receptor expression at that stage. On day 4, Scatchard analysis showed that no significant differences for the binding affinities (indicated as K_d) and number of receptors (indicated as B_{max}) between the control and IGF-I groups. However, both long R^3 IGF-I and des(1-3)IGF-I significantly increased the number of receptor expression compared to the control, but enhanced the K_d values to a much lesser extent, indicating that these IGF-I analogs do have a stimulatory effect on BMM differentiation. It is worthwhile to point out that our data must be interpreted with caution, since the variation for K_d and B_{max} values in the control and IGF-I groups were relatively large. Further experimentation is needed to confirm the results.

In light of the different binding affinities for IGFBP among regular IGF-I and its analogues, the divergence of the effect of IGF-I and its analogues on BMM differentiation could be interpreted as their interaction with IGFBP. Accumulation of IGFBP-4 was proved to occur during BMM differentiation, and in most situations, IGFBP-4 blocks the action of IGF-I by sequestering the free IGF-I. Therefore, it is suggested that IGF-I does enhance BMM differentiation, yet the effect of regular IGF-I is inhibited by IGFBP-4 by its binding with this protein. The analogs hardly bind with IGFBP-4 so that their stimulatory effects have not been compromised. This phenomenon

is comparable to a previous study on myoblast differentiation (Silverman et al., 1995). Myogenesis of rat L6E9 skeletal muscle cells was induced by both IGF-I and des(1-3)IGF-I, but the latter was more potent. Meanwhile, IGFBP-4 and IGFBP-6 were found to accumulate during the differentiation of these cells. Due to the relative abundance of IGFBP-4 and its higher affinity for IGF-I than IGFBP-6, it was suggested that the accumulation of IGFBP-4 antagonized the differentiation induced by IGF-I. Des(1-3)IGF-I could overcome the sequestering effect of IGFBP-4 and was more efficient in stimulating myogenesis (Silverman et al., 1995).

In summary, the present study has clearly demonstrated that during BMM development induced by CSF-1, the expression of IGF-I and IGFBP is altered. IGF-I stimulates bone marrow cell proliferation at early culture stages, while it probably enhances BMM differentiation at later stages. Accumulation of IGFBP-4 occurs during the late stages of BMM differentiation. This binding protein may act as a negative regulator by binding with IGF-I, so as to keep BMM differentiation at a certain pace. IGFBP-3 is also possibly expressed during BMM development, but its function still needs to be investigated.

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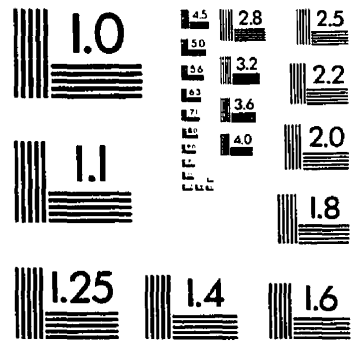

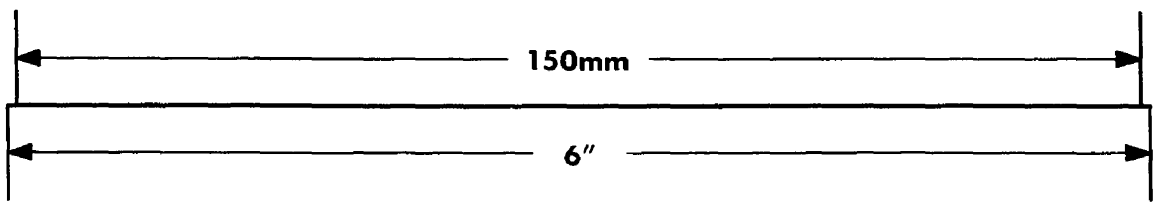

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