Endocrine, *vol. 9, no. 2, 185–192, October 1998* 0969–711X/98/9:185–192/$10.00

Involvement of Insulin-Like Growth Factor-1 and Its Binding Proteins in Proliferation and Differentiation of Murine Bone Marrow-Derived Macrophage Precursors

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Insulin-like growth factor 1 (IGF-1) and its binding proteins (IGFBPs) are involved in proliferation and differentiation of many cell types. In the present study, the involvement of IGF-1 and IGFBPs in proliferation and differentiation of murine bone marrow-derived macrophages (BMDM) was investigated. L929-conditioned media (LCM) containing abundant macrophage colony-stimulating factor CSF-1 were used to stimulate BMDM development from their bone marrow precursors. The alteration of IGF-1 and IGFBPs during LCM-induced BMDM proliferation and differentiation was first studied. The cells were cultured in RPMI complete media containing 20% LCM for different time periods and then incubated in serum-free media for 24 h. The supernatants were collected for Western ligand blotting and immunoblotting analyses, and the cell pellets for Northern blotting analyses. The mRNA level of IGF-1 increased in a time-dependent manner. An increase of IGFBP-4 accumulation in the conditioned media was also observed during this process. However the mRNA expression of IGFBP-4 remained constant, indicating a posttranscriptional regulation of IGFBP-4 secretion and/or stability. The effects of exogenous recombinant human IGF-1 (rhIGF-1) on BMDM proliferation and differentiation were further studied. Two IGF-1 analogs (long R^3 IGF-1 and des [1-3] IGF-1) were also used in parallel with regular IGF-1 to indicate the involvement of IGFBPs in BMDM development. Cells were cultured in complete media containing 20% LCM for different time periods, and then incubated in serum-free media in the presence of rhIGF-1 or its analogs for 24 h. These three forms of IGF-1 all potentiated the proliferation of freshly isolated BMDM precursors (d 0). rhIGF-1 and long R^3 IGF-1, but not des (1-3) IGF-1, continued to stimulate the cell proliferation on d 1. The effects of these three forms of IGF-1 on BMDM differentiation were investigated using mannose receptor expression as a marker. Long R^3 IGF-1 and des (1-3) IGF-1, but not rhIGF-1, enhanced BMDM differentiation on d 4. The different effects of rhIGF-1 and its analogs on BMDM differentiation suggest that the accumulation of IGFBP-4 in BMDM development might have an inhibitory effect on IGF-1 actions by sequestering free IGF-1.

Key Words: IGF-1; IGFBP-4; BMDM; LCM.

Introduction

It has been shown that insulin-like growth factor 1 (IGF-1) has pluripotent actions on a wide variety of tissues and organs. Initially, IGF-1 was considered to be an endocrine hormone mainly circulating in the plasma. However, in the past two decades, its autocrine and paracrine functions have been extensively studied. Many tissues and cell types can synthesize and secrete this hormone, which subsequently influences local cellular functions *(1)*.

The growth-promoting effect of IGF-1 has been demonstrated on various cell types, such as osteoblasts *(2)*, smooth muscle cells *(3)* and fibroblasts *(4)*. In addition, IGF-1 plays an important role in regulating cell differentiation *(5,6)*. Accumulating evidence also suggests that IGF-1 mediates the growth and development of some immune cells. For instance, IGF-1 stimulated the proliferation of human lymphocytes *(7)* and monocytes *(8)*. The involvement of IGF-1 in B-cell lymphopoiesis *(9)* and granulopoiesis *(10,11)* has been documented.

Macrophages are derived from bone marrow progenitors, which undergo several steps of proliferation and differentiation. The development of macrophages is regulated by some colony-stimulating factors (CSFs), including granulocyte-macrophage CSF (GM-CSF) and CSF-1 *(12)*. Mouse L929-conditioned media (LCM) containing abundant CSF-1 stimulated macrophage differentiation from rat

Received February 11, 1998; Revised May 13 and July 16, 1998; Accepted July 16, 1998.

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bone marrow progenitor cells cultured in vitro *(13)*. Although the exact mechanism of macrophage development has not yet been clarified, accumulating evidence suggests that IGF-1 might be one of the mediators involved in this process. IGF-1 stimulated macrophage growth in serum-free rat bone marrow cultures. This stimulatory effect could be blocked by an antibody against CSF-1, suggesting a putative relationship between the actions of CSF-1 and IGF-1 on bone marrow-derived macrophage (BMDM) development *(14)*. In addition, Arkins et al. *(15)* showed that during murine BMDM differentiation stimulated by CSF-1 and other cytokines, there was an increase in IGF-1 mRNA expression.

IGF-1 binds its receptors (IGF-1R) on cell surfaces, and freshly isolated mouse bone marrow cells express high levels of IGF-1R mRNA, as demonstrated by Arkins et al. *(15)*. Since it has been extensively proven that IGF-1 exerts autocrine effects on various cell types, it is plausible to investigate whether the IGF-1 expressed by BMDM progenitors also regulates cellular functions in an autocrine fashion.

Apart from IGF-1R, a family of closely related proteins bind IGF-1 with high affinities, which are called IGF binding proteins (IGFBPs). Up to now, at least six IGFBPs have been identified. They can indirectly influence cellular functions by interaction with IGF-1 or directly mediate cell responses independent of IGF-1. A series of studies showed that IGFBPs were expressed in different amounts by cells in distinct physiological stages, and the alteration of IGFBPs affected the signaling pathways involved in cell growth and differentiation *(5,16,17)*. However, it is still unknown whether IGFBPs also influence the development of the immune system, especially macrophage differentiation.

In the present study, we investigated the expression and involvement of IGF-1 and IGFBPs in the proliferation and differentiation of BMDM progenitors induced by LCM. We report that the endogenous IGF-1 and IGFBP-4 were elevated during the BMDM development, and that the exogenous IGF-1 influenced the growth and differentiation of BMDM progenitors.

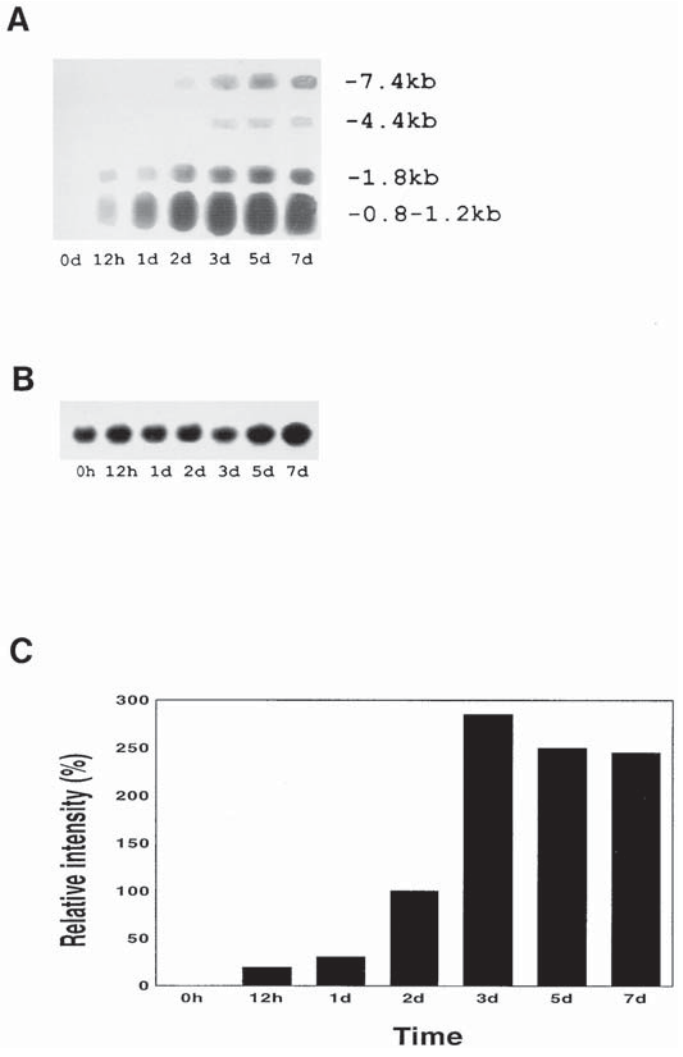

Fig. 1. Expression of IGF-1 mRNA during BMDM differentiation induced by CSF-1. Freshly isolated bone marrow cells were cultured in complete media with 20% LCM for different time periods, and then incubated with serum-free media for 24 h. Total RNA was extracted from the cells, and IGF-1 mRNA was detected by Northern blotting analysis using rat IGF-1 cDNA probe, as described in Materials and Methods. **(A)** The autoradiograph of IGF-1 expression. **(B)** Shows the autoradiograph of β-actin expression. Panel **C** displays the results of densitometric analysis of IGF-1 expression corrected for β-actin (data shown as the percentages of β-actin expression).

Results

Alteration of Endogenous IGF-1 mRNA Expression During BMDM Development

BMDM progenitors were collected from female Balb/c mice, and cultured in complete media (RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum [FBS]), in the presence of 20% LCM. Cells were collected at different time-points, and IGF-1 mRNA level was measured by Northern blotting analysis. IGF-1 mRNA was undetectable in freshly isolated bone marrow cells (0 h), but its expression was increased in a time-dependent manner until d 3 and maintained at a constant level thereafter. In the early stages of culture (12 and 24 h), only two IGF-1 transcripts (0.8–1.2 and 1.8 kb) were detected. Starting from d 2, another transcript of 7.4 kb was observed. Four transcripts (0.8–1.2, 1.8, 4.4, and 7.4 kb, respectively) were detected in the samples collected after d 3 (Fig. 1).

Alteration of Endogenous Expression of IGFBPs During BMDM Differentiation

To determine whether IGFBP secretion is altered during BMDM differentiation, Western ligand blotting (WLB) analysis was performed. No IGFBP was observed

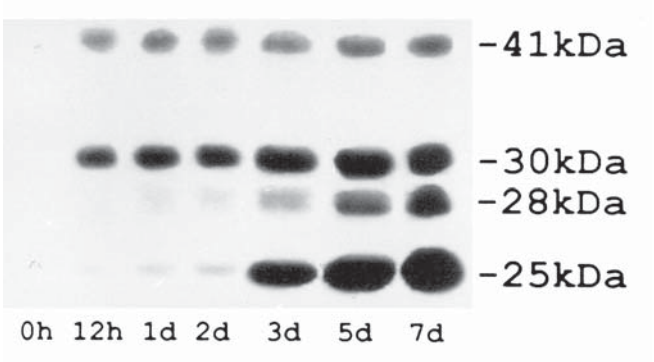

Fig. 2. WLB analysis of IGFBP secretion during BMDM differentiation induced by CSF-1. Freshly isolated bone marrow cells were cultured in the complete media with 20% LCM for different time periods, and then incubated in serum-free media for 24 h. Conditioned media were collected and WLB analysis was performed as described in Materials and Methods.

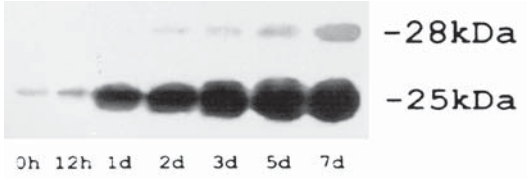

Fig. 3. Immunoblotting analysis of IGFBP secretion during BMDM differentiation induced by CSF-1. Freshly isolated bone marrow cells were cultured in complete media with 20% LCM for different time periods and then incubated with serum-free media for 24 h. Conditioned media were collected, and immunoblotting analysis was performed using anti-IGFBP-4 antibody.

in freshly isolated bone marrow cells. Two bands, with approximate molecular weights of 41 and 30 kDa, were detected in the samples collected from 12 h to 7 d, with constant expression levels. Two additional proteins, with molecular weights of approx 28 and 25 kDa, were not detectable at the early culture stages, but accumulated in a time-dependent manner at later stages. Comparatively, the 25-kDa protein was expressed at a higher level than the 28-kDa protein (Fig. 2).

To identify the IGFBPs detected in WLB analysis, immunoblotting analysis was performed. Anti-IGFBP-4 antibody recognized one 25-kDa and one 28-kDa band. The 25-kDa band could be detected in all samples, although it was faint in the samples collected at 0 and 12 h. The intensity of this band increased in a time-dependent manner. The 28-kDa band was detected from d 2. Compared to the intensity of the 25-kDa band, this band was much weaker, but also increased in a time-dependent manner (Fig. 3). These data indicate that both the 28- and the 25-kDa bands detected by WLB analysis are IGFBP-4, presumably in different glycosylated forms.

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

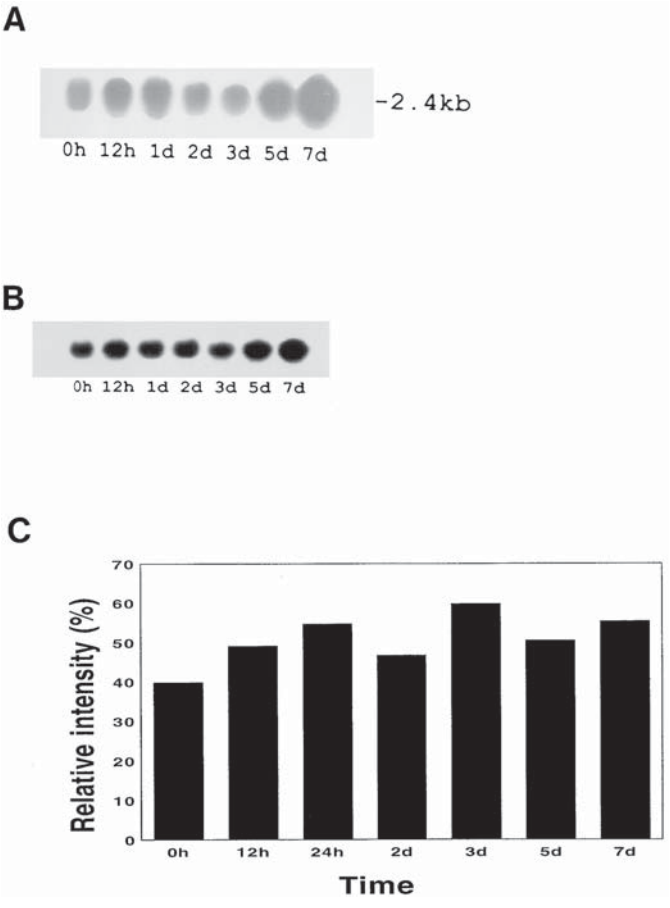

Fig. 4. Expression of IGFBP-4 mRNA during BMDM differentiation induced by CSF-1. Freshly isolated bone marrow cells were cultured in complete media with 20% LCM for different time periods, and then incubated with serum-free media for 24 h. Total RNA was extracted from the cells and IGFBP-4 mRNA was detected by Northern blotting analysis using rat IGFBP-4 cDNA probe, as described in Materials and Methods. **(A)** The autoradiograph of IGFBP-4 gene expression. **(B)** The autoradiograph of β-actin expression. Panel **C** displays the results of densitometric analysis of IGFBP-4 expression corrected for β-actin (data shown as the percentages of β-actin expression).

The IGFBP-4 mRNA level was measured by Northern blotting analysis. One 2.4-kb transcript was detected in all the samples with a constant expression level (Fig. 4). The mRNA levels of IGFBP-1, 2, and 5 were undetectable by Northern blotting analyses (data not shown).

Effects of Exogenous IGF-1 and Its Analogs on Cell Proliferation

To investigate whether IGF-1 has a physiological role in bone marrow cell proliferation, cells were stimulated with exogenous recombinant human IGF-1 (rhIGF-1). To determine whether IGFBPs could interfere with IGF-1, two IGF-1 analogs, long R^3 IGF-1 and des(1-3)IGF-1, which have much lower affinities for IGFBP, were also used as stimulants. Freshly isolated bone marrow cells were cultured in complete media with 20% LCM. At different timepoints, cells were incubated with rhIGF-1 or its analogs in

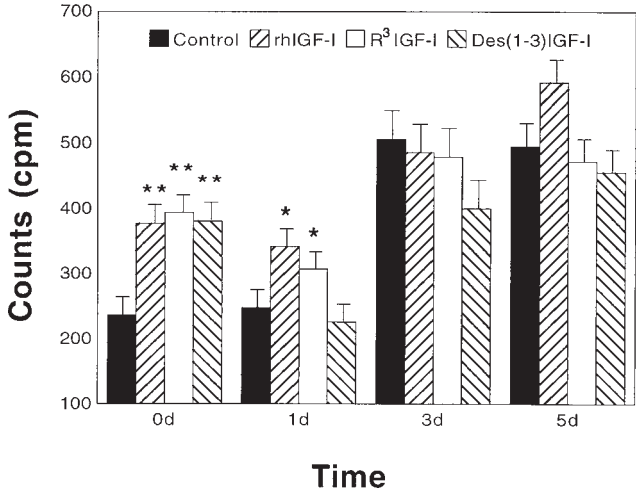

Fig. 5. Effects of rhIGF-1 and its analogs on [^{3}H] thymidine incorporation during BMDM differentiation. Freshly isolated bone marrow cells were cultured in complete media with 20% LCM for different time periods, and then incubated with rhIGF-1, long R^3 IGF-1 or des(1-3)IGF-1 in serum-free media for 24 h. [^{3}H] thymidine incorporation was measured as described in Materials and Methods. *$p < 0.05$ compared with the control; **$p < 0.01$ compared with the control.

serum-free media for 24 h, and cell proliferation was measured by [^{3}H] thymidine incorporation thereafter. For freshly isolated bone marrow cells incubated with rhIGF-1, long R^3 IGF-1, and des(1-3)IGF-1 for 24 h (referred as 0 d), [^{3}H] thymidine incorporation increased 59.7, 66.7, and 61.1%, respectively, compared to that of control. When cells were incubated in complete media with LCM for 1 d and then stimulated with one of the three forms of IGF-1 for 24 h (referred as 1 d), rhIGF-1 and long R^3 IGF-1 continued to enhance cell proliferation, although 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 the control. However, des(1-3)IGF-1 was ineffective. For cells incubated in complete media with LCM for 3 or 5 d, none of the stimulants had an effect on cell proliferation (Fig. 5).

Effects of IGF-1 and Its Analogs on Mannose Receptor Binding

Expression of mannose receptors was examined for the effect of IGF-1 and its analogs on BMDM differentiation with [^{125}I]-Man-BSA as the ligand *(18)*. Freshly isolated bone marrow cells were cultured in complete media with 20% LCM for 2 or 4 d, and then treated with different stimulants (rhIGF-1, long R^3 IGF-1 or des[1-3]IGF-1) in serum-free media for 24 h. Mannose receptor expression was then investigated by the receptor binding assay. For d 2 samples, data could not be used for analysis, presumably because of insufficient receptors on the cells (data not shown). For d 4 samples, the rhIGF-1 group was not significantly different from the control in both K_d and B_{max} values. However, the maximal number of binding sites (B_{max}) for the long R^3 IGF-1 and des(1-3)IGF-1-treated groups were increased by 260 and 228% compared to the control, respectively. K_d values for these two groups increased to a lesser extent (Table 1).

Discussion

It is well known that IGF-1 and its binding proteins exist in a variety of cells and are indispensable in regulating many cellular functions, including cell growth and differentiation. In the present study, the role of IGF-1 and IGFBPs in LCM-induced BMDM proliferation and differentiation was investigated.

It is commonly known that the expression of IGF-1 alters during cell growth and differentiation. For instance, increased IGF-1 mRNA expression was associated with mouse C2 myoblast differentiation *(19)*. By Northern blotting analysis, we demonstrated that mRNA level of IGF-1 was upregulated in a time-dependent manner until d 3, and remained constant thereafter.

Our results have shown that four IGF-1 transcripts with different sizes exist in fully differentiated BMDM. Three of the transcripts (0.8–1.2, 1.8, and 7.4 kb) corresponded to results obtained from a previous study on BMDM *(20)*. In addition, a 4.4-kb mRNA was observed, although its expression was not as abundant as the other three transcripts. These four bands were also observed by Scharla et al. *(21)* 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.

We also investigated the expression of IGFBPs during LCM-induced BMDM differentiation, since alteration of IGFBP expression has been found in the differentiation of many cell types. Four bands with sizes varying from 25 to 41 kDa were detected by WLB analysis. Immunoblotting analysis showed that the 28 and 25-kDa protein reacted with an anti-IGFBP-4 antibody. Northern blotting analysis confirmed the presence of IGFBP-4 mRNA. Based on their molecular masses, it can be assumed that the 25-kDa protein represents the nonglycosylated form, whereas the 28-kDa band is glycosylated. This is in agreement with a study done on B104 rat neuroblastoma cells, where a 28- and a 24-kDa band was detected, the former being *N*-glycosylated, and the latter not glycosylated *(22)*. Whether the two forms of IGFBP-4 have distinct physiological functions in BMDM development remains unclear.

During BMDM development, the secretion of IGFBP-4 increased in a time-dependent manner and peaked at d 7. However, IGFBP-4 mRNA level remained constant during the process of LCM-induced BMDM differentiation. This suggests that the synthesis and secretion of IGFBP-4 are controlled at the posttranscriptional level rather than at the transcriptional level, although the exact mechanisms involved are unclear. Another possible explanation for increased IGFBP-4 protein could be downregulation of

Table 1
Effects of rhIGF-1 and Its Analogs on Mannosylated-BSA Binding to the Cell Receptors[a]

	Control	rhIGF-1	Long R^3 IGF-1	Des(1-3) IGF-1
K_d (nM)[b]	47.8 ± 16.9[d]	71.1 ± 20.2[d,e]	73.5 ± 14.1[d,e]	89.5 ± 8.63[e]
B_{max} (pM)[c]	74.1 ± 14.7[d]	103.1 ± 34.6[d]	267.2 ± 24.4[e]	243.1 ± 9.94[e]

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

[b]K_d stands for the equilibrium binding constant of dissociation.

[c]B_{max} stands for receptor site concentration.

[d,e]Values in the same row with different letters indicate statistical difference from one another ($p < 0.05$).

an IGFBP-4 protease activity in a time-dependent manner. A previous study by Fowlkes and Freemark *(23)* showed that the IGFBP-4 specific protease could degrade this protein.

Several studies have shown that IGFBP-4 inhibits the effects of IGF-1 on the target cells. In B104 neuroblastoma cells, IGF-1-stimulated [^{3}H]-thymidine incorporation was inhibited by IGFBP-4 *(22)*. In rat osteosarcoma cells, IGFBP-4 decreased the effect of IGF-1 on DNA and glycogen synthesis *(24)*. Therefore, the accumulation of IGFBP-4 during BMDM development might also exert a negative effect on the actions of IGF-1.

Although the 28- and the 25-kDa proteins were confirmed to be IGFBP-4, the 41- 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 *(25,26)*. Thus, the 41-kDa protein could be IGFBP-3. Unfortunately, rodent anti-IGFBP-3 antibody was unavailable to confirm this hypothesis.

Antibodies against IGFBP-1, 2, and 5 were used in the immunoblotting analysis, but they failed to react with any binding proteins. It could be argued that the antibodies used in this study may not have been able to crossreact strongly with mouse samples. However, Northern blotting analyses also failed to detect any mRNA for these IGFBPs. Therefore, it is unlikely for these binding proteins to be synthesized and secreted at a detectable level during BMDM development induced by LCM. In addition, IGFBP-6 has a low affinity for IGF-1 and, thus, was impossible to be detected in the WLB analysis, where [^{125}I] IGF-1 was used as the tracer. Therefore, the 30-kDa protein detected in the WLB analysis does not seem to be any of the above-mentioned binding proteins. The possibility that it is IGFBP-4 can also be eliminated, since it did not react with the anti-IGFBP-4 antibody. Although it could be the nonglycosylated or a truncated form of IGFBP-3 based on its size, there is no report that either of these two forms of IGFBP-3 exist in media and maintain a high binding affinity for IGF-1. Taken together, the identity of this 30-kDa band remains unknown.

The effect of IGF-1 in growth and differentiation has been widely studied in various cell types. IGF-1 was demonstrated to increase DNA synthesis of human peripheral blood monocytes and lymphocytes *(8)*. It also affects lymphopoiesis *(9)*, granulopoiesis *(10,11)*, and erythropoiesis *(27)*. In our study, the effect of exogenous rhIGF-1 on BMDM proliferation and differentiation was investigated. Since WLB and immunoblotting analyses demonstrated the alteration of IGFBP profiles during macrophage development, long R^3 IGF-1 and des(1-3)IGF-1 were also used as stimulants to prevent the sequestering effect of the binding proteins. These two analogs bind IGF-1R with an affinity similar to that of rhIGF-1, but have much lower affinity for IGFBP. The results showed that for freshly isolated bone marrow cells (0 d), the three different forms of IGF-1 significantly increased DNA synthesis of bone marrow cells ($p < 0.01$). For cells cultured in the complete media with LCM for 1 d (1 d), IGF-1 and long R^3 IGF-1 continued to stimulate DNA synthesis, although to a lesser extent. Unexpectedly, des(1-3)IGF-1 did not potentiate cell proliferation. The exact reason for the different effects of long R^3 IGF-1 and des(1-3)IGF-1 on cell proliferation on d 1 is not clear. Presumably it is owing to their different binding affinity for IGF-1R. A similar phenomenon has been shown on bovine neutrophils and monoclear cells *(28)*. For cells incubated in the complete media with LCM for 3 or 5 d, none of these three forms of IGF-1 had any influence on cell proliferation. This result shows that IGF-1 and its analogs only have a stimulatory effect on bone marrow cell proliferation at the early stages.

It has been reported previously that expression of the mannose receptor increases in a time-dependent manner on bone-marrow-derived cells cultured with LCM, suggesting this receptor to be a sensitive and quantitative marker of BMDM differentiation *(18)*. Accordingly, we investigated the effect of IGF-1 and its analogs on BMDM differentiation using the [^{125}I]-Man-BSA binding assay. It was not possible to analyze the results obtained on d 2 owing to low binding at that stage. On d 4, Scatchard analysis showed no significant differences for binding affinities (K_d) and the

number of receptors (B_{max}) between the control and IGF-1 groups. Both long R^3 IGF-1 and des(1-3)IGF-1 significantly increased the B_{max} values compared to the control, but enhanced the K_d values to a much lesser extent. This indicates that the IGF-1 analogs do have a stimulatory effect on BMDM differentiation. Accumulation of IGFBP-4 was shown to occur during BMDM differentiation, and in most situations, IGFBP-4 blocks the action of IGF-1 by sequestering free IGF-1. Therefore, it is suggested that IGF-1 does enhance BMDM differentiation, yet the effect of rhIGF-1 is inhibited by the sequestering effect of IGFBP-4. IGFBP-4 does not have a high affinity for IGF-1 analogs. Therefore, the stimulatory effect of these analogs on BMDM differentiation was not compromised. This phenomenon is comparable to a previous study on myoblast differentiation. Myogenesis of rat L6E9 skeletal muscle cells was induced by both IGF-1 and des(1-3)IGF-1, but the latter was more potent. Meanwhile, IGFBP-4 and IGFBP-6 were found to accumulate during the differentiation of these cells. Because of the relative abundance of IGFBP-4 and its higher affinity for IGF-1 than IGFBP-6, it was suggested that the accumulation of IGFBP-4 antagonized the differentiation induced by IGF-1. Des(1-3)IGF-1 could overcome the sequestering effect of IGFBP-4 and was more efficient in stimulating myogenesis *(5)*.

In summary, the present study has demonstrated that during BMDM development induced by LCM, the expression of IGF-1 and IGFBPs is altered. IGF-1 stimulates bone marrow cell proliferation at early culture stages, but it probably enhances BMDM differentiation at later stages. Accumulation of IGFBP-4 occurs during the late stages of BMDM differentiation. This binding protein may act as a negative regulator by binding with IGF-1, in order to maintain BMDM differentiation at a certain pace. IGFBP-3 may also be expressed during BMDM development, but its presence and function need to be further investigated.

Materials and Methods

Materials

Female Balb/c mice (4–6 wk old) were obtained from Charles River (Montreal, Que., Canada). L929 fibroblasts and β-actin cDNA probe were kindly provided by K. Chadee (Institute of Parasitology, McGill University, Montreal, Que, Canada). Rat IGF-1 and IGFBP-1, 2, 4, and 5 cDNA probes were obtained from H. T. Huynh (Lady Davis Research Institute, Montreal, Quebec, Canada). Rabbit antibovine IGFBP-2 polyclonal antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). According to the information provided by the supplier, this antibody crossreacts with mouse IGFBP-2. Rabbit antirat IGFBP-1, 4, and 5 polyclonal antibodies were kindly provided by S. Shimasaki (Whittier Institute for Diabetes and Endocrinology, La Jolla, CA). These antibodies were tested to be highly specific for their antigens *(29)*. Except for those specified elsewhere, all reagents were purchased from Sigma (St. Louis, MO).

Preparation of LCM

L929 fibroblasts were cultured in T75 flasks (Nunc, Naperville, IL) with 50 mL of the RPMI complete media (RPMI 1640 media containing 10% heat-inactivated FBS [Gibco-BRL, Grand Island, NY]) until confluence. The LCM was collected by the passage of the supernatant through a 0.22-μm sterile filter (Costar, Cambridge, CA) and used as a source of CSF-1 *(15)*.

Bone Marrow Cell Isolation and Culture

Bone marrow cells were flushed from the femurs and tibia of 4- to 6-wk-old female Balb/c mice with RPMI 1640 media, and the erythrocytes were lysed. For mRNA and protein analyses, the remaining cells were cultured in 100-mm^2 nonadherent culture dishes (Baxter, Mississauga, Ont., Canada) at a density of 10^7 cells/dish in complete media in the presence of 20% LCM for different time periods. Then the cells were incubated for 24 h in serum-free media. Afterward, cells were scraped and centrifuged (1000*g*, 8 min, 4°C). The supernatants were collected for the WLB and the immunoblotting analyses, while the pellets were dissolved in TRIzol reagent (Gibco-BRL) for mRNA analysis by Northern blotting. For the other assays, cells were put in adherent Petri dishes (Nunc) with the same media as above. After overnight incubation, fibroblastic cells and mature macrophages were attached to the bottom of the plates, and the nonadherent cells (more than 99% of which belonged to macrophage precursors) were transferred to 24-well plates (Becton Dickinson, Lincoln Park, NJ) and cultured at a density of 10^6 cells/mL/well for further use.

WLB Analysis of IGFBPs

Samples were concentrated 10 times by 10-kDa cutoff filters (Costar) and electrophoresed on a nonreducing 12% SDS-polyacrylamide gel (Bio-Rad, Mississauga, Ont., Canada). Size-separated proteins were electrotransferred to a nitrocellulose membrane (Bio-Rad) at 30 V overnight. 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. Afterward, the membrane was blocked in TBS containing 1% bovine serum albumin (BSA) for 2–3 h, and then hybridized overnight with [^{125}I]IGF-1 (Amersham, Oakville, Ont., Canada) in TBS containing 0.1% Tween-20 and 1% BSA. The following day, free [^{125}I]IGF-1 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, Markham, Ont., Canada) 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.

Immunoblotting Analysis of IGFBPs

Samples were concentrated, electrophoresed, and transferred onto a nitrocellulose membrane, and immunoblotting analysis was performed using the enhanced chemiluminescence (ECL) method 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 rabbit antirat IGFBP antibody (1:5000 dilution) in TBS-T containing 1% BSA for 1 h, followed by one wash for 15 min and two washes for 5 min in TBS-T. Subsequently, the membrane was incubated with the goat antirabbit 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 15 s. The developed film was scanned, and the intensity of the bands was analyzed using NIH image 1.60 program.

RNA Extraction and Northern Blotting Analysis

Extraction of total RNA was performed based on the guanidium thiocyanate-phenol-chloroform method *(30)*. Briefly, 1×10^7 cells were lysed in 1 mL of TRIzol reagent by pipeting 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 s. After sitting at room temperature for 2–3 min, samples were centrifuged at 12,000*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*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)-treated water. RNA concentration was determined by spectrophotometry. RNA (15 μg/sample) was denatured by glyoxal for 1 h at 50°C, separated by 1% agarose gel electrophoresis (Bio-Rad) in 10 m*M* sodium phosphate buffer (pH 7.0), and then blotted onto Hybond-N nylon membrane (Amersham) by capillary transfer overnight. The membrane was baked at 80°C for 2 h to fix the RNA. Prior to hybridization, the membrane was prehybridized in a solution including 5X Denhardt (0.1% Ficoll, 0.1% polyvinylpyrodilone, 0.1% BSA), 5X SSPE (0.75 *M* NaCl, 0.05 *M* NaH_2PO_4, 0.05 *M* EDTA), 50% formamide (Gibco-BRL), 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 labeled by ^{32}P incorporation using a nick-translation kit (Amersham) and purified by spinning in a Sephadex G-50 (Pharmacia, Uppsala, Sweden) column. The labeled rat IGF-1 and IGFBP cDNA probes were added into the above-mentioned solution and incubated with the membrane for 12–18 h at 42°C. Afterward, the membrane was washed once with 0.5X SSC (0.075 *M* NaCl and 7.5 m*M* sodium citrate) for 30 min at room temperature, twice with 0.5X SSC containing 0.1% SDS for 30 min at 55°C, and once or twice with 0.1X SSC containing 0.1% SDS for 30 min at 65°C, and 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 analyzed by NIH image 1.60 program. β-actin was used as a reference for the relative quantitation of RNA.

Cell Proliferation

Cells (10^6/well/mL) were cultured in complete media in the presence of 20% LCM for different time periods, and then incubated with serum-free media for 24 h in the presence of 50 ng/mL of rhIGF-1. In order to investigate whether there is any interference of IGFBP on IGF-1 effect, two IGF-1 analogs, des(1-3)IGF-1 (50 ng/mL) and long R^3 IGF-1 (50 ng/mL), which bind poorly with IGFBP, but otherwise have similar effects to that of normal IGF-1, were also used as stimulants. The dosage of rhIGF-1 was chosen based on the previous published study *(15)*. Cell proliferation was determined by [^{3}H] thymidine incorporation into DNA as previously described *(15)* with slight modifications. One microcurie of [^{3}H] methyl thymidine (ICN-Biomedical, Montreal, Que., Canada) was added into each well, and cells were incubated for 6 h. The media were then removed, and the cells 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). Radioactivity was counted in a scintillation counter.

Cell Differentiation

Cells (10^6/well/mL) were cultured in the same conditions as those for cell proliferation, and mannose receptor expression was used as a marker for BMDM differentiation. Iodination of mannosylated-BSA was performed as described by Clohisy et al. *(18)*. Briefly, 100 μg of mannosylated-BSA (EY Laboratories, San Mateo, CA) and 300 μg of chloramine-T were added to 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 190 μL of potassium iodide (10 mg/mL) were added to stop the reaction. Sephadex G-50 column chromatography (1 × 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/tube and the labeled protein fractions were identified by γ-counting *(18)*. Stimulated cells were washed with the Hank's balanced salt (HHBG) solution (Gibco-BRL), 10 m*M* HEPES, 10 m*M* Tris, 0.1% glucose, 10 mg/mL of BSA, pH 7.1) three times and then incubated with [^{125}I]-mannosylated 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 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 Scatchard analysis *(31)*.

Statistical Analyses

All data were obtained from at least three independent experiments. Statistical analyses were 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 *(32)*.

Acknowledgment

Financial support by the Natural Sciences and Engineering Research Council of Canada (NSERC) is greatly appreciated (OGP0155423 to X. Z.).

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