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Effects of Age and Estrogen Status on the Skeletal IGF Regulatory System

Studies with Human Marrow

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Human marrow was obtained as material discarded during total hip replacement and was established in culture with phenol red-free α-MEM with 10% fetal bovine serum (FBS) and antibiotics. Insulin-like growth factor I (IGF-I) and its binding proteins were secreted by human marrow cells, in amounts that increased with time in culture. Western ligand blotting showed that insulin-like growth factor binding protein -3 (IGFBP-3) accounted for the majority (~75%) of the secreted binding proteins. Evidence for marrow secretion of BP-3 protease was found by electrophoretic analysis of mixtures of radiolabeled IGFBP-3 and marrow-conditioned media. The amount of constitutive secretion of IGFBP-3 increased with age of the subject ($r = 0.97$, $p = 0.0058$). A notable exception was marrow from a postmenopausal women on estrogen replacement therapy (ERT) at the time of surgery; her marrow secreted 89.3 ng/mL after 14 d in vitro, only 38% of the IGFBP-3 that was secreted by cultures from two age-matched peers (208.8 and 285.2 ng/mL). This in vivo effect of estrogen was matched by an in vitro experiment in which $10^{-8}M$ 17-β estradiol suppressed IGFBP-3 to 60% of the constitutive level. In all cultures of marrow from postmenopausal women, IL-1β suppressed IGFBP-3 secretion to either undetectable levels or levels between 11% and 35% of control. Thus, human bone marrow cultures demonstrate components of the skeletal IGF regulatory system: IGF-I, IGF-binding proteins, and evidence of IGFBP-3 proteolysis. These results provide evidence of regulation by both systemic (age, estrogen status) and cytokine (IL-1β) factors.

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Introduction

The insulin-like growth factor (IGF) regulatory system is an important component of bone remodeling. Current understanding of this system in bone recognizes the ligands (IGF-I/II), their receptors (Types 1 and 2), secreted IGF-binding proteins (BP-2 through 6), and proteases that cleave IGF-binding proteins (BPs) to enhance IGF bioavailability *(1,2)*. Much of the information in humans has been obtained from serological studies in metabolic bone disorders and from in vitro studies with transformed cell lines. Less is known about the biology of these components in normal human tissue. The purpose of this study was to test the hypotheses that the components of IGF system are present in normal human marrow, and that they are regulated by local and systemic factors.

Materials and Methods

Marrow was procured as material discarded during the course of total hip reconstruction for noninflammatory osteoarthritis. Single cell suspensions were prepared by mechanical dispersion and density centrifugation with Ficoll-Histopaque 1077. Low density cells were cultured in phenol red-free α-MEM with 10% heat-inactivated fetal bovine serum (FBS), in most cases. Media were changed and collected twice each week.

Insulin-like growth factor-I was extracted from the media by an acid-ethanol cryoprecipitation method, described by Breier et al. *(3)*. IGF-I was quantified from the extract by using the double-antibody RIA competitive binding principle (Nichols Institute Diagnostics, San Juan Capistrano, CA). A rabbit-derived monoclonal anti-IGF antibody was added to the samples and incubated at room temperature for 1h. ^{125}IGF-I was added and incubated at 4° C for 16 h. The bound antigen-antibody complex was

precipitated by the addition of a goat-antirabbit antibody to IGF-I and was incubated at room temperature for 20 min. Samples were centrifuged 1500*g* at 4°C, the supernatants were discarded, and the pellets were retained. The concentration of IGF-I present in the sample pellet was inversely proportional to the amount of [^{125}I]-labeled-IGF-I bound to the antibody complex in the pellet. The sensitivity of this assay was 6.3 ng/mL; cross-reactivity with IGF-II was < 0.5%.

For analysis of binding proteins, conditioned media were electrophoresed through 12.5% SDS PAGE, transferred to nitrocellulose filters, and probed with [^{125}I]-IGF-I. Band intensity was quantified by scanning densitometry. Western immunoblot for IGFBP-3 was performed as above followed by the addition of a polyclonal antibody to IGFBP-3.

Insulin-like growth factor binding protein 3 (IGFBP-3, nonglycosylated, 29 k) was measured by a two-site immunoradiometric assay (Diagnostic Systems Laboratories, Inc., Webster, TX). Media were diluted 1:200 in PBS assay buffer and added to tubes coated with anti-IGF BP-3, to which an [^{125}I]-labeled second antibody to IGFBP-3 was added. The mixture was incubated at room temperature for 4 h. Unbound radiolabel was removed by repeated washing and the radioactivity of the bound components was determined. The sensitivity of this assay was 0.5 ng/mL; cross-reactivity with IGFs and other IGF-BPs was < 0.01%.

IGFBP-3 protease activity was measured by a modified blotting technique. Maternal serum (mid-term, peak-gestational age) or seminal plasma was used as a positive control for BP-3 protease activity. The controls and samples were diluted 1:10 and prepared for electrophoresis on a 12.5% non-reducing SDS-PAGE gel by mixing 1:1 with [^{125}I]-IGFBP-3. Following SDS-PAGE, the gels were dried and autoradiographed on Kodak X-Omat AR film for up to 10 d at –70° C. The resultant bands were observed and compared to control lanes, which represent 100% proteolytic activity, by laser scanning densitometry to determine the relative percent of BP-3 protease activity.

Results

Marrow cultures from seven postmenopausal women secreted elements of the skeletal IGF regulatory system (Table 1). The amount of IGF-I increased with time after seeding of cultures (data not shown). Western ligand blots revealed that IGFBP-3 represented approx 75% of the total binding in conditioned media (with correction for background binding because of FBS constituents, Fig. 1). IGFBP-3 was present as a major 43 k and a minor 32 k protein. IGFBP-4 and 5 were also found. Evidence for marrow secretion of BP-3 protease was found by electrophoretic analysis of mixtures of radiolabeled IGFBP-3 and marrow-conditioned media, in this case serum-free for 3 d (Fig. 2). Proteolytic fragments (18k and 30k) were not detected in samples that contained PMSF, a protease inhibitor.

Table 1
IGF-I and IGFBP-3 Secretion by Human Marrow after 7 d *In Vitro* in the Absence and Presence of IL-1β (20 U/mL)

Age of subject (Y)	Conditions	IGF-I (ng/mL)	IGFBP-3 (ng/mL)
46	Basal	4.5	und
	IL-1b	3.5	und
47	Basal	13.7	5.5
	IL-1b	13.6	6.8
52	Basal	13.8	32.8
	IL-1b	11.7	und
52	Basal	13.5	25.6
	IL-1b	12.5	13.3
58	Basal	13.4	115.1
	IL-1b	14.4	78.9
61	Basal	14.1	148.5
	IL-1b	12.4	69.0

und = undetectable

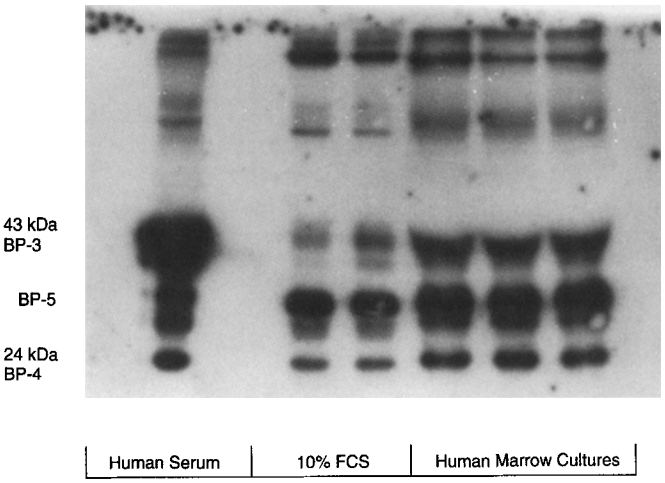

Fig. 1. This ligand blot using IGF-I as a radiolabeled probe demonstrates the distribution of IGFBPs in human marrow cultures. In the first lane is normal human serum showing IGFBP-3 (43 kDa), BP-5 (30kDa) and BP-4 (24kDa). Lane 2 is blank, lanes 3 and 4 represent 10% fetal calf serum without cell cultures; lanes 5–7 represent cm from human marrow cell culture. IGFBP-3, BP-2, and BP-5 (30 kDa) and IGFBP-4 (24 kDa) are noted in these cultures.

Conditioned media contained from 4.5–14.1 ng/mL IGF-I after 7 d in vitro, showing no relationship with age of the donor ($r = 0.11, p = 0.44$, Table 1). In contrast, secreted IGFBP-3 ranged from undetectable to 148.5 ng/mL, with a striking age-dependence ($r = 0.97$, $p = 0.0058$, Fig. 3). A notable exception was marrow from a 53-yr old postmenopausal women on ERT at the time of surgery; her marrow secreted 89.3 ng/mL after 14 d in vitro, only 38% of the IGFBP-3 that was secreted by cultures from two age-

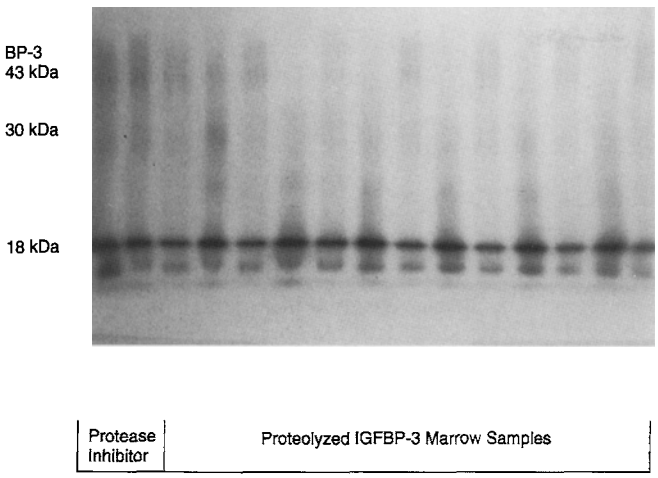

Fig. 2. IGFBP-3 radiolabeled was incubated with and without protease inhibitors to determine if IGFBP-3 protease activity is present in CM from human marrow cells. In lanes 1 and 2, a protease inhibitor was added to CM showing intact IGFBP-3 and few fragments at 30 and 18 kDa. Lanes 3–13 are CM from human marrow cells without protease inhibitor showing that there is minimal IGFBP-3 and a significant proportion of IGFBP fragments of mol wt 18 and 30 kDa. These data suggest active proteolysis of IGFBP-3 in the CM from human marrow cells.

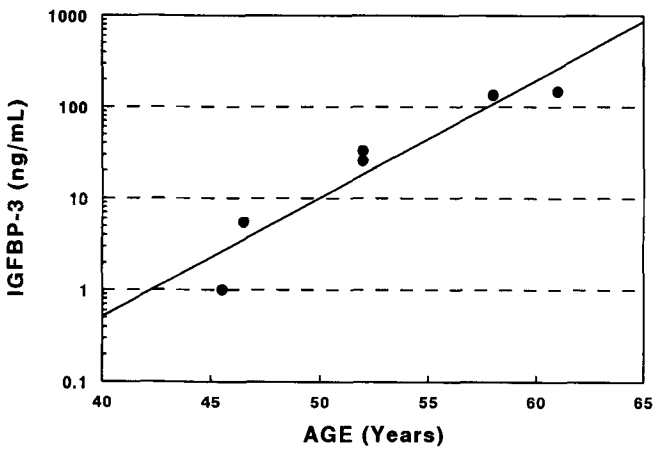

Fig. 3. Effect of age of subject upon secretion of IGFBP-3 by marrow cultured for 7 d. The two symbols at 52 yr represent repeat cultures from a woman who had bilateral hip replacements 1 wk apart.

matched peers (208.8 and 285.2 ng/mL). This in vivo effect of estrogen was matched by an in vitro experiment in which $10^{-8}M$ 17-β estradiol suppressed IGFBP-3 to 60% of the constitutive level.

Additional evidence of in vitro regulation of IGFBP-3 secretion was found in cultures treated with IL-1β (20 U/mL). In all cultures of marrow from postmenopausal women, IL-1β suppressed IGFBP-3 secretion to either undetectable levels or levels between 11% and 35% of control. Conditioned media from 7 d cultures showed equal magnitudes of specific cleavage of IGFBP-3 into 30 and 18 k fragments, either in the presence or absence of IL-1β. From this result, the authors conclude that IL-1β inhibition of IGFBP-3 is not mediated through increased proteolysis.

Discussion

From this preliminary study, we report that human marrow cultures produce components of the IGF system, that secretion of IGFBP-3 is dependent upon age and estrogen status of the subject, and that secretion of IGFBP-3 can be modulated in vitro by IL-1β and estrogen.

Marrow from subjects undergoing total hip reconstruction was previously shown to secrete Interleukin-6 (IL-6) with a striking age-dependence ($r = 0.98$, $p < 0.001$) *(4)*. More recently, the authors reported that constitutive secretion of IL-6 and IL-11 was suppressed in cultures from women receiving ERT at the time of surgery, compared to age-matched estrogen-deficient women *(5)*. Thus, this culture system demonstrates several alterations in marrow biology dependent upon age or estrogen status. Furthermore, in contrast to supression of IGFBP-3 secretion by IL-1β, stimulation of IL-6 secretion was found in cultures supplemented with IL-1β. In addition, not all marrow products are secreted in association with age of the subject; age-independent factors include IL-11, GM-CSF, and IL-1β. Finally, we reported that marrow from subjects with rheumatoid arthritis secreted IL-1β constitutenly, whereas that from subjects with osteoarthritis did not *(6)*. Other studies of human marrow cultures use conditions different from these. An investigation using autologous serum showed a decline in cytokine production with time in culture *(7)*. Another report concerned marrow aspirates from the iliac crest *(8)*. Studies of human marrow often use 1,25-dihydroxyvitamin D3 to enhance differentiation of cells of the macrophage lineage *(9)*, dexamethasone and b-glycerolphosphate to enhance differentiation of osteoblasts *(10)*, or horse serum and hydrocortisone to promote hematopoiesis *(11)*. In summary, IGF-I, IGFBPs, and IGFBP-3 protease are made by human marrow cells. Western ligand blotting showed that IGFBP-3 accounted for the majority (~75%) of the secreted binding proteins. The amount of BP-3 was increased with time in culture and with age of the subject. Furthermore, the lower level of IGFBP-3 secretion by marrow from an estrogen-treated woman suggests that the marrow cultures maintain the effects of in vivo regulation, at least for 2 wk in vitro. Suppression of IGFBP-3 by exogenous IL-1β or estrogen supports the paracrine mechanism of modulation of growth factor bioactivity.

Acknowledgments

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