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A NOVEL HUMAN INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN SECRETED BY OSTEOBLAST-LIKE CELLS

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Summary: Insulin-like growth factor binding proteins (IGFBPs) modulate the cellular action of the insulin-like growth factors. Inhibition or enhancement of IGF effects by these cell-secreted binding proteins have been described. We have purified two IGFBPs (23 and 29 kDa) from media conditioned by U-2 human osteosarcoma cells using ligand-affinity chromatography and reversed phase HPLC. N-terminal amino acid analysis of the 23 kDa protein revealed a unique sequence with variable homology to IGFBPs 1-4. The 29 kDa IGFBP was found to be nearly identical to a recently reported IGFBP. Because the affinity purified U-2 IGFBPs enhanced IGF-I-stimulated osteoblast mitogenesis, we suggest that one or both of these binding proteins enhance IGF action in bone.

Insulin-like growth factors promote the growth of osteoblast-like bone cells (1-4). Since IGF-I and IGF-II are also secreted by these cells (5,6), they may function in an autocrine manner to regulate cellular proliferation and bone matrix protein synthesis. IGF-binding proteins modulate IGF actions in a positive or negative fashion (7-12) and may also have intrinsic bioactivity (13).

While multiple IGF-binding proteins have been demonstrated in media conditioned by osteoblast-like cells (14), only one has been identified (15). This inhibitory binding protein has been designated IGFBP-4 (16,17). Because the identity of the other bone cell-derived IGFBPs is unknown, we undertook the purification of IGF-binding proteins from media conditioned by the human osteoblast-like cell line, U-2 osteosarcoma.

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Abbreviations: IGF, insulin-like growth factor; IGFBP, IGF-binding protein; HPLC, high pressure liquid chromatography; FBS, fetal bovine serum; PVDF, polyvinylidine difluoride; TFA, trifluoroacetic acid; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Cell culture and media collection - U-2 human osteosarcoma cells (ATCC) were grown in McCoy's 5a medium (GIBCO) supplemented with 5% FBS until confluence. Cultures were transfered to serum-free medium for a 24 hour period to remove all traces of serum-derived binding proteins. The cells were then incubated for 72 hours in fresh serum-free medium which was saved for binding protein purification. Finally, fresh serum-containing medium was added to the cultures for 72 hours and the collection scheme was repeated. All cultures were maintained at 37 C in a humidified-air atmosphere containing 5% CO2.

Purification of IGF-binding proteins - Three liters of conditioned media were acidified to 1 M acetic acid and ultrafiltered using a YM-10 membrane (10,000 MW cutoff, Amicon). The retentate was washed twice with equal volumes of 1 M acetic acid and twice with phosphate-buffered saline. The concentrate was then applied to an IGF-I-affinity column prepared by coupling 1 mg IGF-I (Imcera) to cyanogen bromide-linked agarose (Affigel 10). After washing the column with equilibration buffer (0.05 NaCl, 0.05 Tris-HCl, 0.002% Tween-20), IGF-binding proteins were eluted with 0.5 M acetic acid. The eluate was dried in a Speed Vac concentrator and the residue dissolved in 0.1% TFA and applied to a C-8 reversed phase HPLC column (Vydac). IGF-binding proteins were eluted with a 15-60% acetonitrile gradient in 0.1% TFA, collected as 1 ml fractions in siliconized glass tubes and dried by Speed Vac.

[^{125}I]IGF-I binding activity - IGF-I binding activity was determined using the method of Busby et al (12). IGF binding is expressed as a percentage of the [^{125}I]IGF-I precipitated , divided by the total radioiodine added to each tube.

Western ligand blot - Ligand blots of IGF-binding proteins were performed according to the method of Hossenlopp et al (18). Briefly, proteins were separated by 12% SDS-PAGE under nonreducing conditions, transferred to nitrocellulose, blotted with [^{125}I]IGF-I overnight and autoradiographed at -70 C for 5 days.

Mitogenic assay - Cell proliferation was estimated by the incorporation of [^{3}H]thymidine into the DNA of normal mouse osteoblast-like cells (19). Primary cultures were subcultured into 96-well plates in Dulbecco's modified Eagles medium containing 10% FBS. After allowing the cells to adhere overnight, the cultures were incubated with serum-free media for 24 hours. The medium was replaced with serum-free media containing 0.1% BSA and 50 ng/ml IGF-I (Bachem) without and with the binding protein preparation from the IGF-affinity column. After 18 hours [methyl-^{3}H]thymidine (Amersham) was then added for an additional 4 hours and thymidine incorporation was determined by liquid scintillation counting.

Protein sequencing - Affinity-purified IGF-binding proteins, separated by SDS-PAGE, were transfered to PVDF filters according to the method of Matsudaira (20). Protein bands identified by Coumassie stain and ligand blots were excised and the N-terminal amino acid sequences determined by automated Edman degradation using an Applied Biosystems Model 477A Protein Sequencer with an on-line Model 120A Analyzer (kindly performed by Brad McMullen, Department of Biochemistry, University of Washington, Seattle). Sequence determinations were made with and without tributyl phosphene reduction and 4-vinyl pyridine alkylation of the membrane-bound protein.

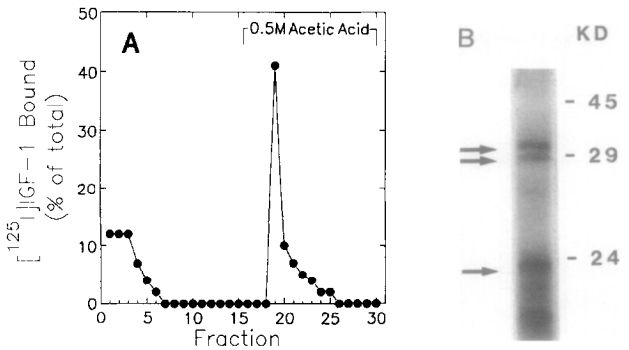

Figure 1. Affinity-purification of IGFBPs from U-2 cell conditioned media. A. Following removal of endogenous IGFs by ultrafiltration, the crude binding protein preparation was applied to an IGF-I-affinity column and IGFBPs were eluted with 0.5 M acetic acid. B. SDS-PAGE of affinity-purified IGFBPs. Proteins were separated by 12% SDS-PAGE under non-reducing conditions and the gel was silver-stained. Arrows indicate IGFBPs identified on [^{125}I]IGF-I ligand blot (data not shown). Molecular weight standards are shown on the right.

RESULTS AND DISCUSSION

Osteoblast-derived IGF-binding proteins that eluted from the IGF-I-affinity column (Figure 1A.) appeared as 3 bands on SDS-PAGE (Figure 1B.) as determined by Western ligand blot (data not shown). Their sizes, 23, 29 and 31 kDa are similar to those previously identified by Western ligand blotting of untreated conditioned media (data not shown). As shown in Figure 2, incubation of the affinity column eluate with normal mouse osteoblasts resulted in enhanced IGF-I-stimulated mitogenesis. This stimulatory effect would not be due to other growth factors because of the exclusive adsorption properties of the affinity column. The response is in contrast to the inhibitory effect of IGFBP-4 on osteoblast mitogenesis (15), and from the inhibition of cell proliferation demonstrated for IGFBP-2 (21). It also differs from the mitogenic enhancing effects of IGFBP-1 (11,12) and IGFBP-3 (22) in two ways. First, in contrast to IGFBP-1 (23), the enhancing action of the U-2 cell-derived binding proteins is achieved without the need for platelet-poor plasma or any serum in the assay. Secondly, preincubation of the U-2 IGFBPs was not necessary, whereas a minimum 8 hour preincubation period is required for IGFBP-3 to enhance IGF-stimulated cell proliferation (22). Because of these functional differences, we reasoned that U-2 cells probably secrete at least one IGF-binding protein that differed from IGFBPs 1-4.

To identify the 23 kDa IGFBP (Figure 1B.), affinity purified IGFBPs were separated by SDS-PAGE, transfered to PVDF membranes and the 23 kDa Coumassie stained band was sequenced. The resulting N-terminal amino acid sequence, Phe-Val-His-Cys-Glu-Pro-Cys-Asp-Glu-Lys-Ala-Leu-Ser-Met-X-Pro-Pro-Ser, was found to be distinct from IGFBPs 1-4 (Figure 3).[1] As shown, there are 5 residues that

[1] A similar N-terminal sequence has been reported for a rat serum and porcine follicular fluid IGF-binding protein (S. Shimasaki et al, Second International IGF Symposium, San Francisco, CA, January 15, 1991; Abstract C54).

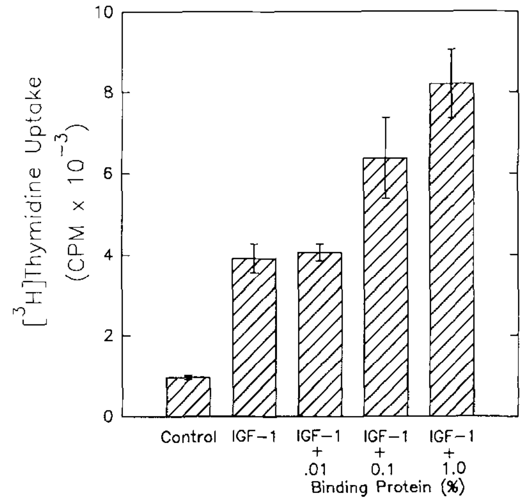

Figure 2. Mitogenic response to affinity-purified IGFBPs by normal mouse osteoblasts. Cultures maintained in serum-free medium containing 0.1% BSA were incubated with the IGFBPs (at 0.01, 0.1 and 1.0% v/v) with 50 ng/ml IGF-I. Control cultures contained neither IGF-I nor binding proteins. Values are the mean ± SE of 6 replicate wells.

have identical position alignment, including the 2 cysteines. Sequence identity with IGFBP-3 was 56%, 39% with IGFBP-4, 38% with IGFBP-1 and 28% with IGFBP-2. It has been noted that the amino- and carboxy-terminal regions of IGFBP 1-4 are the most homologous, in contrast to their internal sequences (16). In addition, the cysteine residues, which are located in the amino- and carboxy-terminal portions of IGFBP 1-4, show identical alignment, with the exception that IGFBP-4 has an additional 2 cysteines within its internal region (17).

Further purification of the 29 kDa IGFBP, necessitated by insufficient quantities of affinity-purified binding protein on SDS-PAGE, was performed by reversed phase HPLC (Figure 4). The UV peak eluting at 62 minutes contained a single 29 kDa IGFBP as determined by SDS-PAGE and Western ligand blotting and by a single peak when rechromatographed by reversed phase HPLC run (data not shown). Moreover, it gave the single N-terminal amino acid sequence, X-X-Pro-Gly-(Pro)-Gly-Gln-Gly-Val-Gln-Ala-(Gly)-X-Pro-(Gly). This sequence is nearly identical (Figure 5) to the N-terminal sequence of the IGFBPs purified from human

h U-2 IGFBP										F	V	H	C	E	P	C	D	E	K	A	L	S	M	X	P	P	S	X	L	
hIGFBP 1									A	P	W	Q	C	A	P	C	S	A	E	K	L	A	L	C	P	P	V	S		
rIGFBP 2								E	V	L	F	R	C	P	P	C	T	P	E	R	L	A	A	C	G	P	P	P	V	P
hIGFBP 3	G	A	S	S	G	G	L	G	P	V	V	R	C	E	P	C	D	A	R	A	L	A	Q	C	A	P	P	P		
hIGFBP 4								D	E	A	I	H	C	P	P	C	S	E	E	K	L	A	R	C	R	P	P	V	G	C

Figure 3. Comparison of the N-terminal amino acid sequence of the 23 kDa U-2 cell-derived IGFBP with IGFBPs 1-4. Alignment was made to maximize sequence identity.

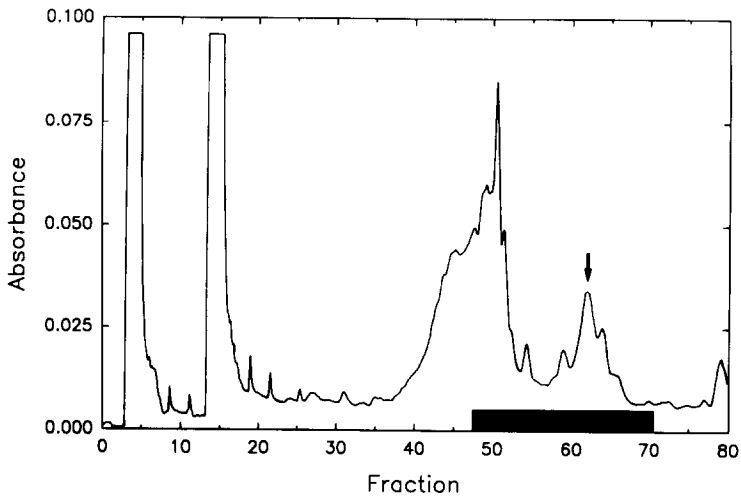

Figure 4. Reversed phase HPLC purification of the 29 kDa IGFBP. IGFBPs eluted from the IGF-I-affinity column were concentrated and applied (2.6 mg.) to a C-8 reversed phase column. IGF-binding activity is depicted by the solid bar. The arrow over the peak eluting at 62 minutes is the 29 kDa IGFBP.

cerebrospinal fluid (24), human transformed fibroblasts (25) and the He[39]L fibroblast cell line (26).

Although multiple osteoblast-derived IGF-BPs have been identified, the function of only one of these has been evaluated (15). Mohan et al demonstrated that IGFBP-4 isolated from the TE-89 osteosarcoma cell line inhibited IGF-stimulated mitogenesis in chick osteoblasts. Our findings in a different human osteosarcoma cell line suggest that osteoblast-like cells may secrete at least one type of enhancing IGFBP which could have an important role in the autocrine regulation of osteoblast proliferation and function. The balance between inhibitory and enhancing effects could in part be determined by growth factor and hormonal regulation of these binding proteins. For example, Schmid et al demonstrated that IGF-I stimulates the secretion of at least three osteoblast-derived IGFBPs (14) and Mohan et al have shown that cAMP stimulates IGFBP-4 secretion in osteoblast-like cells (15). Whether there is growth-factor upregulation of the U-2 enhancing IGFBPs remains to be determined.

N-Terminal Sequence

U-2	X	X	P	G	(P)	G	Q	G	V	Q	A	(G)	X	P	(G)
CSF	L	A	P	G	X	G	Q	G	V	Q	A	G	A	P	G
He[39]L	L	A	P	G	P	G	Q	G	V	Q	A	G	X	P	G
transformed fibroblasts	R	A	P	G	C	G	Q	G	V	Q	A	G			

Figure 5. N-terminal amino acid sequence of the 29 kDa U-2 cell-derived IGFBP compared to IGFBPs isolated from other sources. Residues in parentheses indicate incomplete assignments.

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