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Inhibition of IGFBP-5 Binding to Extracellular Matrix and IGF-I–Stimulated DNA Synthesis by a Peptide Fragment of IGFBP-5

C. Rees and D.R. Clemmons*

Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

Abstract Insulin-like growth factor binding protein-5 (IGFBP-5) is synthesized and secreted by smooth muscle cells (SMC). IGFBP-5 synthesis is stimulated five- to sixfold by IGF-I, and IGFBP-5 has been shown to augment IGF-I–stimulated DNA synthesis in this cell type. The ability of IGFBP-5 to augment the SMC response to IGF-I is dependent upon its binding to extracellular matrix. A highly charged region of IGFBP-5 that contains amino acids in positions 201–218 has been shown to mediate binding of IGFBP-5 to human fibroblast extracellular matrix (ECM), and a synthetic peptide containing this sequence inhibits IGFBP-5 binding to fibroblast ECM. In this study we show that exposure of SMC cultures that are constituitively synthesizing IGFBP-5 to a synthetic peptide (termed peptide A) containing this sequence has no effect on its synthesis but reduces its abundance within the ECM. The addition of increasing concentrations of the peptide to SMC cultures resulted in a concentration-dependent reduction in ECM-associated IGFBP-5. In contrast, a control peptide (peptide B), which contained the region of amino acids in positions 131–141 and had a similar charge-to-mass ratio, caused a minimal decrease in ECM binding. This effect was functionally significant since the addition of 10 μg/ml of peptide A inhibited the cellular replication response to 10 ng/ml IGF-I by 51%, and peptide B had no effect. The effects of peptide A were not due to nonspecific cytotoxicity since it had no inhibitory effect on the response of these cells to human serum and was associated with only minimal inhibition of the cellular response to platelet-derived growth factor. The findings suggest that inhibiting IGFBP-5 binding to porcine SMC ECM results in reduced cellular responses to IGF-I. J. Cell. Biochem. 71:375–381, 1998.

Key words: insulin-like growth factor-I; insulin-like growth factor binding protein-5; smooth muscle cells; atherosclerosis; substratum

Insulin-like growth factor binding protein-5 (IGFBP-5) is unique among members of the IGFBP family in that it contains two highly basic regions [Rechler, 1993; Shimasaki et al., 1991]. Although one of these regions is contained in IGFBP-3, the affinity of IGFBP-3 for extracellular matrix (ECM) is substantially less than that of IGFBP-5 [Jones et al., 1993]. The binding of IGFBP-5 to ECM results in an important functional change since its affinity for IGF-I and -II is lowered substantially [Jones et al., 1993). Likewise, when IGFBP-5 is bound to ECM, it is protected from proteolysis [Camacho-Hubner et al., 1992]. Therefore, ECM-associated IGFBP-5 represents a stable, low affinity reservoir for the IGFs.

In previous studies, we have demonstrated that when ECM contains increasing amounts of this low affinity form of IGFBP-5, it results in significant enhancement of human fibroblast and porcine smooth muscle cell (pSMC) replication responses to IGF-I [Jones et al., 1993; Zheng et al., 1998]. Therefore, it appears that this stable, low affinity reservoir may provide a mechanism for sequestering IGF-I that can be utilized during cell migration or tissue remodeling. Previously we have used in vitro mutagenesis to show that critical basic residues within the region between amino acid positions 201 and 218 of IGFBP-5 mediate its binding to human fibroblast ECM [Parker et al., 1996]. One peptide containing residues 201–218 was particularly effective in competing with

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*Correspondence to: David R. Clemmons, Division of Endocrinology, CB #7170, 6111 Thurston Bowles Bldg., University of North Carolina, Chapel Hill, NC 27599.

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IGFBP-5 for binding to fibroblast ECM, and mutagenesis of two of the basic residues within this peptide resulted in a decrease in its capacity to compete for binding with the intact protein [Parker et al., 1996]. Because this peptide had been shown to competitively inhibit the binding of IGFBP-5 to fibroblast extracellular matrix, we determined whether it could inhibit the deposition of IGFBP-5 that was being synthesized constitutively by pSMC into the ECM and whether this resulted in any change in the ability of IGF-I to stimulate DNA synthesis.

MATERIALS AND METHODS

Porcine aortic smooth muscle cells (pSMC) were isolated as previously described [Ross, 1971]. The cells were maintained in Dulbecco's modified eagle medium (DMEM) (Life Technologies, Grand Island, NY) containing streptomycin, 100 µg/ml, and penicillin, 100 U/ml, supplemented with 10% fetal bovine serum (Life Techologies). For determination of ECM binding, the cells were plated in six-well plates (Falcon Labware Division, Becton Dickinson Co., Franklin Lakes, NJ) at a density of 1.0×10^4 cells/cm^2. The cultures were maintained for 7 days with one medium change. At that time, they were changed to serum-free DMEM and incubated with increasing concentrations of the test peptides ranging between 2.0 and 10 µg/ml for 14 h at 37°C. The cultures were washed twice, and the ECM was prepared by incubating the cells in PBS containing 2 M urea (Sigma Chemical Co., St. Louis, MO) for 20 min. This removed the cells and the cytoskeletal proteins but left substantial quantities of ECM, as proven by fibronectin staining [Jones et al., 1993]. The remaining ECM was extracted with 0.5 cc Laemmli sample buffer. Insoluble material was removed by centrifugation at 15,000*g* for 10 min; then the supernatants were heated to 95°C, electrophoresed through a 12.5% SDS polyacrylamide gel, and transferred to Immobilon PSQ (Millipore, Bedford, MA) membranes. The protein extracts were analyzed by Western ligand blotting [Hossenlopp et al., 1986] using ^{125}I-IGF-I as previously described [McCusker et al., 1989]. The ^{125}I-IGF-I (specific activity 125 µCi/µg) was added using 400,000 cpm/ml of incubation buffer and was a gift of Dr. Louis Underwood. It was prepared as described previously [Copeland et al., 1980]. Band intensity was determined by Phosphor Image analysis and quantified using Image Quant Software (Molecular Dynamics, Sunnyvale, CA). To confirm that the band that was detected in the ECM extracts was IGFBP-5, we also analyzed the extracts by immunoblotting using a 1:1,000 dilution of anti-IGFBP-5 antiserum using a previously described method [Nam et al., 1994].

Cell Proliferation Studies

For determination of ^{3}H-thymidine incorporation into DNA, the cells were seeded in 96-well plates (Falcon 3004) at a density of 15,000 cells/cm^2 in DMEM supplemented with 10% fetal bovine serum and penicillin, 100 U/ml, and streptomycin, 100 µg/ml. After 5 days without a medium change, the cells were washed 2× in serum-free DMEM and then exposed to DMEM containing 0.2% human platelet-poor plasma that had been prepared as described previously [Clemmons and Gardner, 1990] and 0.5 µCi ^{3}H-thymidine (16 Ci/mmol) (ICN Biochemical Inc., Cosa Mesa, CA). Increasing concentrations of IGF-I (a gift from Genentech Inc., South San Francisco, CA) (2–50 ng/ml) or the test peptides were also added. Control cultures were exposed to increasing concentrations of human serum (0.5–10%) or platelet-derived growth factor (PDGF) (1–20 ng/ml) (Intergen, Purchase, NY) with or without the peptides. Following a 30 h incubation, the reaction was stopped by the addition of 1 M ascorbic acid, the DNA was extracted, and ^{3}H-thymidine incorporation was determined as described previously [Clemmons and Gardner, 1990]. To determine if peptide A could inhibit the response to IGF-I by competing with IGF-I for binding to IGFBP-5, we determined its ability to compete by adding increasing concentrations (1–10 µg/ml) with ^{125}I-IGF-I (20,000 cpm/ml) and IGFBP-5 (1 ng/ml), and the binding capacity of IGFBP-5 was determined as described previously [McCusker et al., 1989]. Peptide A did not compete with IGF-I for binding at any concentration tested.

Synthetic Peptides

The synthetic peptides were prepared and purified as described previously [Clemmons and Van Wyk, 1985]. The correct mass of each product was confirmed by mass spectroscopy. The peptide A sequence was $_{201}$ RKGFYKRKQCKPSRGRK$_{218}$, and peptide B was $_{131}$ VKKDRRKKLTQ$_{141}$. An altered form of peptide A that contained two substitutions for basic amino acids R207A and 214A but was otherwise identical to peptide A was also syn-

thesized, purified, and determined to have the correct mass estimate.

IGFBP-5 Synthesis

Confluent pSMC cultures were plated in 35 mm dishes (Falcon 3006) and grown to confluency. They were washed twice with serum-free DMEM and exposed to 1.0 ml of low methionine (10^{-6} M) DMEM containing 50 µCi of ^{35}S-methionine (1,206 Ci/mmol). Duplicate cultures contained peptide A added at 1.0 or 10 µg/ml. After 6 h, 100 µl of media was removed and incubated with 1.0 ml of 0.05 M Tris pH 7.4 containing a 1:1,000 dilution of anti-IGFBP-5 antiserum for 14 h at 4°C. The immune complexes were precipitated by the addition of protein A sepharose, and then the plated proteins were analyzed by SDS-PAGE (12.5% gel), followed by flourography and autoradiography [Imai et al., 1997].

RESULTS

SDS-PAGE followed by Western ligand blotting of pSMC ECM revealed two bands that had M_r estimates of 31 and 34 kDa (Fig. 1). Immunoblotting showed that both bands reacted with a specific anti-IGFBP-5 antiserum and did not react with IGFBP-2 antiserum (Fig. 1). Most importantly, following a 14 h exposure to 10 µg/ml of peptide A, there was a significant reduction in the signal intensity of the IGFBP-5 band, and the extent of the reduction was dependent upon the concentration of peptide A that was added (Table 1). Phosphor Image analysis showed that the signal intensity was reduced by approximately 54 ± 8% (n = 3 separate experiments) when 10 µg/ml of peptide A was added. When a control peptide that contained the region of IGFBP-5 from positions 131–141 and had a similar charge-to-mass ratio was added, it had no effect on IGFBP-5 abundance (Fig. 2). Importantly, a form of peptide A that had substitutions for R207 and R214 also had no effect in reducing IGFBP-5 signal intensity. Repetition of this experiment three times showed similar results. To control for potential effects on IGFBP-5 synthesis, we used ^{35}S-methionine labelling followed by immunoprecipitation. In the presence of 1.0 or 10 µg/ml of peptide A, the IGFBP-5 band intensities were equal to those in control cultures, indicating that neither concentration had an effect on IGFBP-5 synthesis (data not shown).

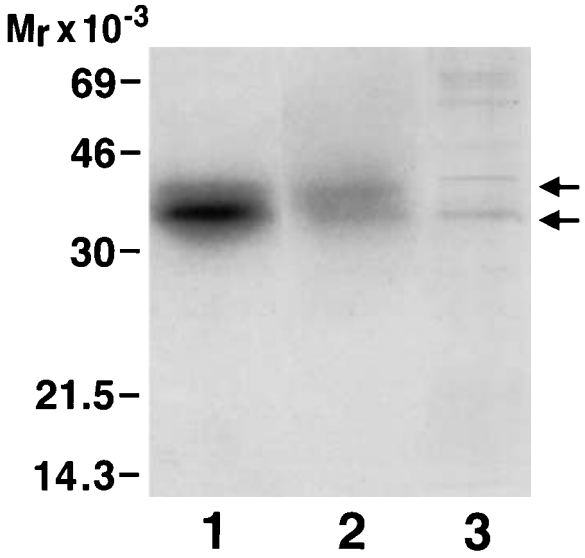

Fig. 1. Peptide A inhibits IGFBP-5 abundance in ECM. Smooth muscle cell cultures were grown to confluency as described in Materials and Methods. The cultures were exposed to either serum-free medium (**lanes 1,3**) or medium containing 10 µ/ml of peptide A (**lane 2**). Following a 14 h incubation at 37°C, the ECMs were harvested as described in Materials and Methods and analyzed by SDS-PAGE; then the IGFBP-5 was detected by Western ligand blotting (lanes 1,2) or immunoblotting (lane 3) using anti-IGFBP-5 antiserum (1:500 dilution). The band intensities expressed in arbitrary units were lane 1, 75,040 (lane 1) and 28315 (lane 2). The results show two IGFBP-5 bands noted by the arrows whose abundance is decreased in the ECM from the cultures exposed to peptide A and that these bands are immunoreactive.

TABLE I. Extracellular Matrix Content of IGFBP-5

Test conditions	Densitometric units[a]
Medium alone	58,372 ± 5,434
Peptide A, 10 µg/ml	26,844 ± 3,110*
Peptide A, 5 µg/ml	44,997 ± 6,003*
Peptide A, 2 µg/ml	53,397 ± 5,291**

[a]Each value represents the mean ± S.D. from three separate experiments.
*$P < 0.01$ compared to medium alone.
**$P < 0.05$ compared to medium alone.

To determine if this reduction in IGFBP-5 in the ECM resulted in a change in smooth muscle cell responsiveness to IGF-I, we added increasing concentrations of IGF-I with 10 µg/ml of peptide A, a concentration which had been shown to significantly reduce IGFBP-5 abundance in the ECM. Peptide A consistently inhibited the effect of each concentration of IGF-I, although its effects were greatest when added with 5 and 10 ng/ml IGF-I and were less when

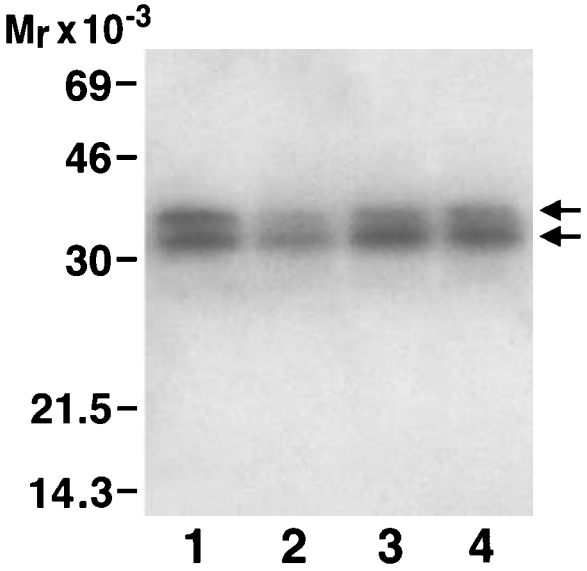

Fig. 2. Porcine aortic smooth muscle cultures were prepared as described in Materials and Methods. They were then exposed to 10 µ/ml of each peptide. **Lane 1:** Serum-free medium. **Lane 2:** Peptide A. **Lane 3:** Peptide B. **Lane 4:** Peptide A with amino acid substitutions, R207A, R214A. Following a 14 h incubation, the ECMs were extracted, and IGFBP-5 abundance was determined by SDS-PAGE followed by Western ligand blotting. Phosphor Image analysis showed the following values expressed as arbitrary units: serum-free medium, 52,574 units; peptide A 35,709 units; peptide B, 53,397 units; peptide A mutant, 52,889 units. The results show that peptide A results in significant inhibition of IGFBP-5 abundance within the ECM and that the control peptides have no effect.

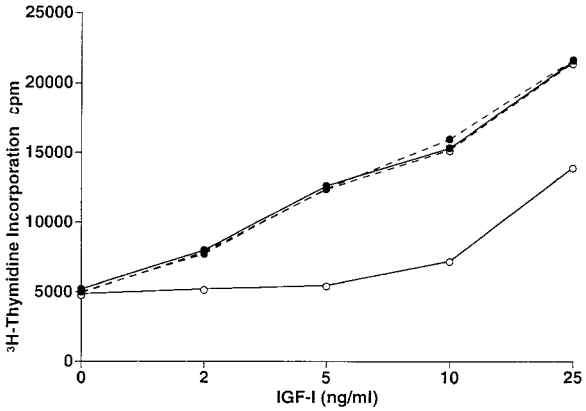

Fig. 3. Smooth muscle cell replication response to IGF-I. Increasing concentrations of IGF-I were added to quiescent smooth muscle cell cultures. After 36 h incubation, the amount of ^{3}H-thymidine incorporated into DNA was determined. Triplicate cultures received either IGF-I alone (●—●), IGF-I plus 10 µg/ml peptide A (○—○), IGF-I plus 10 µg/ml peptide B (○- -○), or IGF-I plus 10 µg/ml mutant peptide A(●- -●).

25 ng/ml was used (Fig. 3). To determine the specificity of the peptide A effect, we incubated 10 µg/ml of the mutant form of peptide A and 10 µg/ml of peptide B, peptides that did not compete for binding with IGFBP-5 to ECM, with the smooth muscle cells, and the ability of IGF-I to stimulate ^{3}H-thymidine incorporation into DNA was assessed. Neither peptide B nor the mutant form of peptide A resulted in significant attenuation of IGF-I responsiveness.

The addition of increasing concentrations of peptide A resulted in a progressive decrease in the cellular DNA synthesis response to IGF-I (Fig. 4). When peptide A was added at concentrations between 1 and 10 µg/ml, there was a concentration-dependent decrease in ^{3}H-thymidine incorporation in response to IGF-I. In order to exclude the possibility of nonspecific cytotoxicity, we performed two types of experiments. First, increasing concentrations of peptide A were added with human serum. This resulted in minimal attenuation of the human serum response that was not statistically significant (Table 2). Similarly, when increasing concentrations of PDGF were coincubated with peptide A, there was no inhibition of the cellular replication response to this growth factor.

DISCUSSION

The results of this study clearly demonstrate that a highly basic peptide in which 10 of 18 amino acids are positively charged can compete with IGFBP-5 that is being synthesized constitutively for binding to extracellular matrix. In contrast, a control peptide that contained a similar charge-to-mass ratio did not result in a significant reduction in ECM-associated IGFBP-5. Similarly, a mutant peptide that contained substitutions for two of the ten basic residues in peptide A had no effect. This finding suggests that this region of IGFBP-5 is an important determinant of its binding to pSMC extracellular matrix and that specific residues within this sequence motif (e.g. R207 and R214) are very important for ECM association.

Importantly, the addition of this peptide resulted in significant attenuation of the cellular DNA synthesis response to IGF-I. The specificity of this effect was further confirmed by showing that peptide A did not have a nonspecific cytotoxic effect since it had minimal effects on the capacity of serum or PDGF to stimulate DNA synthesis. Since the cellular DNA synthesis response was attenuated by exposure to peptide A but not by peptide B or the mutant

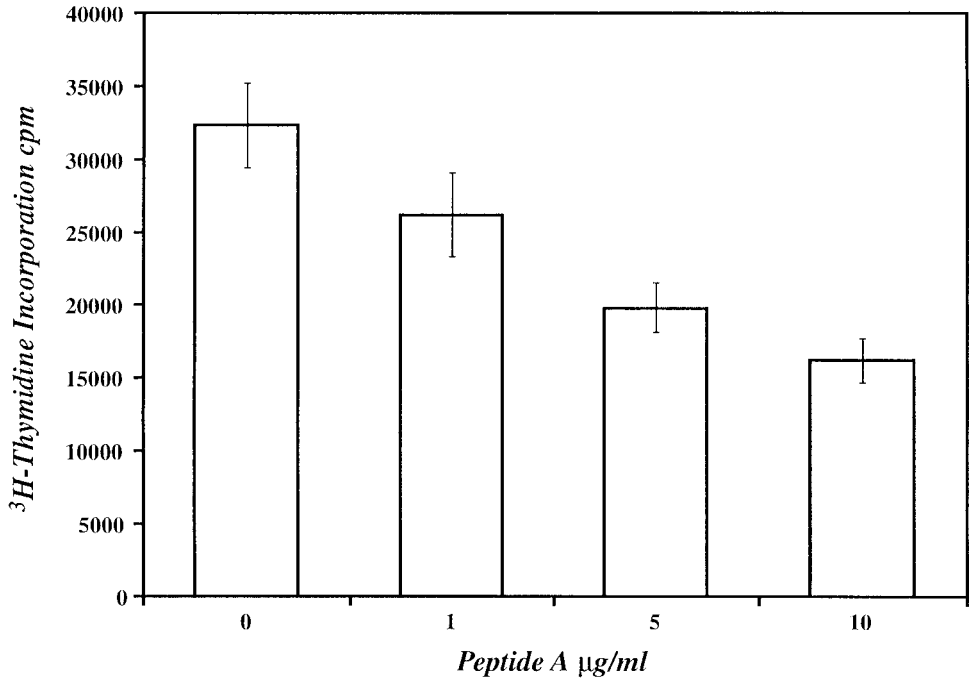

Fig. 4. Smooth muscle cell replication response to peptide A. Increasing concentrations (1–10 μg/ml) of peptide A were incubated with pSMC cultures that were also exposed to 20 ng/ml of IGF-I. After 36 h, the amount of ^{3}H-thymidine incorporated into DNA was quantified as described in Materials and Methods. The results are the mean ± S.D. of triplicate determinations.

TABLE II. Effect of Peptide A on Stimulation by Other Growth Factors[a]

Peptide A	Human serum %				PDGF (ng/ml)			
(mg/ml)	0.5	2	5	10	2	5	10	25
0	12,110 ± 290	24,430 ± 1,830	39,860 ± 3,990	53,330 ± 6,600	6,200 ± 700	12,790 ± 1,380	18,960 ± 1,990	26,030 ± 2,730
10	11,990 ± 470	22,970 ± 1,980	36,070 ± 3,540	47,660 ± 6,830	5,990 ± 550	12,660 ± 1,440	18,470 ± 1,360	24,190 ± 2,650

[a]Each value represents the mean counts per minute ± SEM of triplicate determinations of ^{3}H-thymidine incorporated into DNA.

form of peptide A, this further suggests that the effort of peptide A is specific and that the ability of pSMC to respond to IGF-I is partially dependent upon the amount of IGFBP-5 within the ECM. We have previously reported that augmentation of the amount of IGFBP-5 in either human fibroblast or pSMC ECM results in potentiation of the cellular responsiveness of these cell types to IGF-I [Jones et al., 1993; Zheng et al., 1998]. This quantitative relationship is supported by the observation that the addition of increasing amounts of peptide A resulted in parallel reductions in ^{3}H-thymidine incorporation and in the abundance of IGFBP-5 within the ECM. This suggests that the amount of IGFBP-5 remaining in the ECM is a major determinant of DNA synthesis response to IGF-I.

Previous studies have shown that IGFBP-5 binds preferentially to ECM and that the amino acids between positions 201 and 218 mediate this binding [Parker et al., 1996; Arai et al., 1994a]. In addition, the affinity of ECM proteins for IGFBP-5 is high, in the range of 10^{-9} M. In contrast, IGFBP-3 has an approximately thirtyfold lower affinity for ECM even though its sequence between positions 234 and 252 contains 17 of the 18 amino acids that are present in peptide A [Shimasaki et al., 1991; Jones et al., 1993]. We and others [Booth et al., 1996] have reported that IGFBP-3 and IGFBP-5 also bind to heparin, but IGFBP-3 binds with much lower affinity to heparin compared to IGFBP-5. The peptide A region of IGFBP-5 also mediates heparin binding [Arai et al., 1994a, 1996]. These results further suggest that

there is decreased surface exposure of the peptide A region of IGFBP-3 and that this also accounts for its reduced binding to heparin and to ECM compared to IGFBP-5.

The specific amino acids within the 201–218 region that mediate fibroblast ECM binding appear to be residues 202, 207, 214, and either 217 or 218 [Camacho-Hubner et al., 1992; Parker et al., 1996]. These studies reinforce the importance of residues R207 and R214 since a mutant peptide that contained neutral substitutions for these residues was ineffective in reducing the abundance of IGFBP-5 within pSMC ECM.

The exact molecular mechanism by which IGFBP-5 enhances the response to IGF-I in smooth muscle cells and the reason inhibition of constitutively synthesized IGFBP-5 binding to ECM results in inhibition of DNA synthesis were not definitively determined in these studies. However, we have shown previously that increasing the amount of IGFBP-5 within the ECM results in enhancement of the ability of IGF-I to stimulate fibroblast growth or smooth muscle cell replication [Jones et al., 1993; Zheng et al., 1998]. In studies that are under consideration, we have shown that mutation of specific amino acids within the peptide A region of IGFBP-5 and transfection into pSMC followed by expression of these ECM binding-deficient mutant forms results in attenuation of IGF-I responsiveness. Therefore, it appears that a defined amount of ECM-associated IGFBP-5 is required for IGF-I to optimally stimulate pSMC DNA synthesis and that reductions in this level of ECM-associated IGFBP-5 result in attenuation of cellular responsiveness. Further studies to determine the mechanism have shown that ECM-associated IGFBP-5 has a markedly lower affinity for IGF-I than IGFBP-5 in the culture medium [Jones et al., 1993]. High concentrations of intact, high affinity IGFBP-5 in extracellular fluid can inhibit cellular responses to IGF-I [Imai et al., 1997]. However, with standard cell culture conditions, almost all of the IGFBP-5 that is synthesized by smooth muscle cells [Duan and Clemmons, 1996], fibroblasts]Nam et al., 1996], or osteoblasts [Kanzaki et al., 1994] is degraded proteolytic fragments, and these fragments have very low affinity for IGF-I. These fragments do not inhibit IGF-I actions [Andress and Birnbaum, 1991]. Therefore, ongoing proteolysis of intact IGFBP-5 in the medium appears to be required for optimal smooth muscle cell responsiveness. In contrast, the effect of intact, ECM-associated IGFBP-5 is concentration-dependent, suggesting that there is a quantitative relationship between the amount of IGF-I associated with this low affinity binding site and its ability to stimulate replication.

All of the extracellular matrix proteins that bind IGFBP-5 have not been identified. We have reported that tenascin [Parker et al., 1996] and other heparan sulfate–containing proteoglycans bind IGFBP-5 and that a peptide containing the peptide A sequence motif of IGFBP-5 inhibits binding [Arai et al. 1994a, 1996]. Similarly, we have shown that type IV collagen and plasminogen activator inhibitor-I bind to IGFBP-5 and that peptide A specifically inhibits PAI-1 binding [Nam et al., 1997]. We are currently determining if other proteins that are present in pSMC/ECM can bind IGFBP-5 and whether the peptide A sequence domain is important for IGFBP-5 binding.

These results have major practical significance. This peptide can be used to alter the amount of ECM-associated IGFBP-5 in cell types that contain high concentrations of IGFBP-5 within their ECM. Therefore, the peptide itself may have great utility in probing the effects of IGFBP-5 and IGF-I on connective tissue cells that incorporate IGFBP-5 into their ECM. Other investigators have shown that both osteoblasts and chondrocytes synthesize IGFBP-5 and that it binds to their ECM [Mohan et al., 1995; Olney et al., 1996]. Therefore, it may be possible to utilize this peptide to further probe these molecular interactions in these cell types and to determine whether the mechanism that appears to be operative in smooth muscle cells and fibroblasts is also operative therein.

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