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Insulin-like growth factor (IGF) binding proteins and their mRNAs in connective tissues of fasted guinea-pigs

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Fasting (with vitamin C-supplementation) and vitamin C-deficiency in guinea-pigs are associated with decreased collagen gene expression in connective tissues. Recently we presented evidence that circulating insulin-like growth factor binding protein (IGFBP)-1 and -2 that are induced during both nutritional deficiencies may be responsible for this inhibition by interfering with IGF-I action. The present objective was to determine whether circulating IGFBPs are accumulated in bone, skin and cartilage during fasting, which would support an endocrine role for them. IGFBP-1 mRNA was not detected in any of the connective tissues. The protein, as measured by ligand blotting, was not present in tissues of normal animals but accumulated early during fasting in all of the tissues. Bone and cartilage from normal animals contained IGFBP-2 and its mRNA, but only in bone did their levels increase during fasting. IGFBP-3 mRNA was not detected in connective tissues from normal or fasted guinea-pigs. Little or no IGFBP-3 was detected in normal tissue extracts, but protein accumulated during fasting and presumably was derived from the circulation. IGF-I and -II mRNAs were expressed in bone and cartilage but in skin, only IGF-II mRNA was detected. Affinity cross-linking revealed that in skin, IGFBP-3 contained relatively few unoccupied IGF-I binding sites compared to IGFBP-1 while in bone and cartilage, only IGFBP-1 contained unoccupied binding sites. IGFBP-1, acting by endocrine action, is probably the major factor responsible for inhibition of IGF-I-dependent collagen gene expression during fasting.

Keywords: autocrine/paracrine regulation; collagen; growth factors; bone; cartilage; skin

Introduction

IGFs I and II are polypeptides that are thought to mediate the effects of growth hormone. They stimulate longitudinal bone growth and collagen synthesis *in vivo* and DNA, proteoglycan and collagen synthesis in a variety of cell culture systems (Humbel, 1990; Schmid *et al.*, 1989; Goldstein *et al.*, 1989). The rates of synthesis of collagen and proteoglycans are decreased in connective tissues of guinea-pigs that have been on a vitamin C-free diet for 3–4 weeks when animals are losing weight, a period designated as phase II of scurvy (Peterkofsky, 1991). Fasted guinea-pigs receiving vitamin C respond similarly (Peterkofsky, 1991), and phase II of scurvy appears to be equivalent to fasting with respect to regulation of collagen gene expression. Sera from phase II scorbutic or fasted guinea-pigs inhibit the synthesis of collagen and other IGF-I-dependent functions in cultured cells (Oyamada *et al.*, 1990). Inhibition is caused by IGFBPs that are induced during fasting and scurvy, as demonstrated by reversal of inhibition upon addition of IGF-I (Oyamada *et al.*, 1990) or by removal of IGFBP-1 and -2 from fasted and scorbutic guinea-pig serum with specific antibodies (Peterkofsky *et al.*, 1994). Circulating IGFBP-1 and -2 are induced early enough to account for suppression of collagen gene expression in connective tissues during phase II of scurvy or fasting (Gosiewska *et al.*, 1994), providing they act by an endocrine mechanism. Induction of these IGFBPs is probably related to the rapid and severe decrease in serum insulin concentrations within 10 h after fasting begins (Gosiewska *et al.*, 1994).

The liver is the main source of circulating IGFs but many other adult tissues express mRNA for these factors and contain immunoreactive IGFs and the cellular receptor for IGF-I is widely expressed (Humbel, 1990; Cohick & Clemmons, 1993). Circulating IGFs occur mainly in a complex with IGFBP-3 and an acid labile subunit (ALS), with very little in the free form (Humbel, 1990;, Cohick & Clemmons, 1993; Rechler & Brown, 1992). Under normal circumstances, IGFBP-3 is the most abundant binding protein in the circulation, although levels of other IGFBPs may be increased during several pathological conditions (Cohick & Clemmons, 1993; Rechler & Brown, 1992). Like the IGFs, the IGFBPs are expressed mainly in the liver, except for IGFBP-5 and -6 (Shimasaki *et al.*, 1991a,b), but they also are expressed to varying extents in other tissues and are secreted by a variety of cell types in culture, including connective tissue cells (Cohick & Clemmons, 1993; Rechler & Brown, 1992). Bone or bone cells in culture produce IGFs I and II (Canalis *et al.*, 1988; McCarthy *et al.*, 1992) and also several IGFBPs, although the types vary depending on the source of the cells (Hassager *et al.*, 1992). Newborn fetal skin fibroblasts (Cohick & Clemmons, 1993) and chondrocytes (Froger-Gaillard *et al.*, 1989) in culture also secrete IGFs and IGFBPs. Therefore, it has been suggested that IGFs and IGFBPs may participate in an autocrine/paracrine system to regulate IGF action in tissues (Humbel, 1990;, Cohick & Clemmons, 1993). On the other hand, an endocrine role for the IGFs is supported by the observations that IGF-I is transported from the circulation to non-hepatic tissues (Hodgkinson *et al.*, 1991; Bar *et al.*, 1990b) and that IGF-I administered *in vivo* affects growth and other tissue-specific functions (Humbel, 1990; Schmid *et al.*, 1989; Cohick & Clemmons, 1993).

As indicated above, circulating IGFBP-1 and -2 that are induced during vitamin C deficiency and fasting could be responsible for the decrease in collagen gene expression in connective tissues by endocrine action. The purpose of the present study was to determine whether the genes for IGFs and their IGFBPs are expressed in bone, skin and cartilage *in vivo*, whether their expression is affected by fasting, and whether inhibitory IGFBP-1 and -2 that are induced during fasting are taken up from the circulation by these tissues. This would allow us to establish which type of mechanism, auto/paracrine, endocrine or both, might be responsible for the suppression of collagen gene expression in connective tissues during fasting.

Results

Expression of mRNAs for IGFBPs and IGFs in connective tissues

For those probes not previously used, specificity was evaluated by determining transcript sizes on Northern blots

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prepared with either total or poly(A)$^+$ RNA, depending on the level of expression of individual genes. Northern blots also were used to test for expression in tissues not previously analysed.

IGFBP-1 and -2 mRNAs in liver are poorly expressed in normal guinea-pigs but both are induced by fasting and vitamin C deficiency (Gosiewska *et al.*, 1994). In the present studies, we found that IGFBP-1 mRNA was not detectable in bone, skin or cartilage of normal or fasted guinea-pigs. As observed previously (Gosiewska *et al.*, 1994), the IGFBP-2 probe detected a 1.6 kb transcript in normal liver after prolonged exposure of autoradiograms (Figure 1, lane 1). A similar transcript was observed in skin and bone (Figure 1, lanes 2 and 3) but there also was a smaller transcript. In cartilage, IGFBP-2 mRNA expression was much lower (data not shown). The guinea-pig IGFBP-3 and the rat IGFBP-4 probes did not detect transcripts in skin, bone or cartilage. Since these IGFBPs are expressed mainly in the liver of other species, we ascertained whether the probes would hybridize with the appropriate transcripts in this tissue. The IGFBP-3 probe detected mainly a 2.7 kb mRNA in normal and fasted liver, (Figure 1, top band, lanes 4 and 5), although in both samples there were some smaller transcripts. The size of the major transcript is similar to the size of the IGFBP-3 transcript in liver of other species (Shimasaki and Ling, 1991).

The IGFBP-4 probe detected a transcript of approximately 2.2 kb (Figure 1, lane 6), which is similar in size to the rat IGFBP-4 transcript (Shimasaki & Ling, 1991). Transcripts for IGFBP-5 and -6 were not detected on blots with liver, bone, cartilage or skin RNA, even with prolonged autoradiographic exposure. Previous reports showed very low expression of these mRNAs in liver, in contrast to the expression of IGFBPs 1-4 (Shimasaki & Ling, 1991; Shimasaki *et al.*, 1991a; Shimasaki *et al.*, 1991b). Failure to detect these mRNAs is probably not due to the use of heterologous probes. The rat IGFBP-5 cDNA was used to select human IGFBP-5 cDNA clones (Shimasaki *et al.*, 1991a) and a porcine probe was used to select rat and human IGFBP-6 cDNA clones (Shimasaki *et al.*, 1991b), although there are some differences between rat and human DNA sequences, especially for IGFBP-6. As described here and previously (Gosiewska *et al.*, 1994), rat probes for IGFBP-1, -2, and -4 hybridized well with the corresponding guinea-pig mRNAs, corroborating the high degree of interspecies homology amongst IGFBPs (Rechler & Brown, 1992).

The IGF-I probe was used previously and detected transcripts in guinea-pig liver, bone and cartilage, but not in skin (Gosiewska *et al.*, 1994). The guinea-pig IGF-II probe prepared by RT-PCR was sequenced and found to be identical to the previously reported sequence for the 164-178 region (Levinovitz *et al.*, 1992), except for a C instead of the reported T at position 178. The distribution of transcripts hybridizing with this probe differed in liver and nonhepatic tissues (Figure 2). In liver, the major transcript was slightly smaller than 5 kb (28S rRNA), while in bone and cartilage there were major transcripts of 6 kb and 5.3 kb with lesser amounts of a 4.8 kb species. Expression was low in skin, but after prolonged exposure the 6 kb and 5.3 kb transcripts were observed. The transcripts observed in these tissues are similar in size to those observed in other species (Sussenbach *et al.*, 1992).

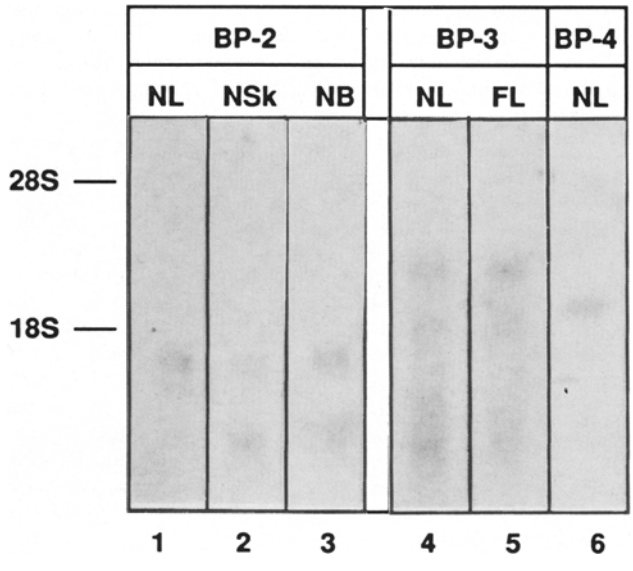

Figure 1 Northern blot analysis of IGFBP-2, IGFBP-3 and IGFBP-4 mRNAs. Total RNA (10 μg, lanes 1–3) or poly(A)$^+$ RNA (5 μg, lanes 4–6) from liver (L), skin (Sk) and bone (B) of normal (N) or 4 day-fasted (F) guinea-pigs were analysed by Northern blotting as described in Materials and methods. The positions of 28S and 18S rRNA were determined by ethidium bromide staining of the gel containing total RNA and a separate lane containing RNA markers on the other gel. Exposure times for autoradiograms were: IGFBP-2, 4 days; IGFBP-3, 1 day; IGFBP-4, 12 h

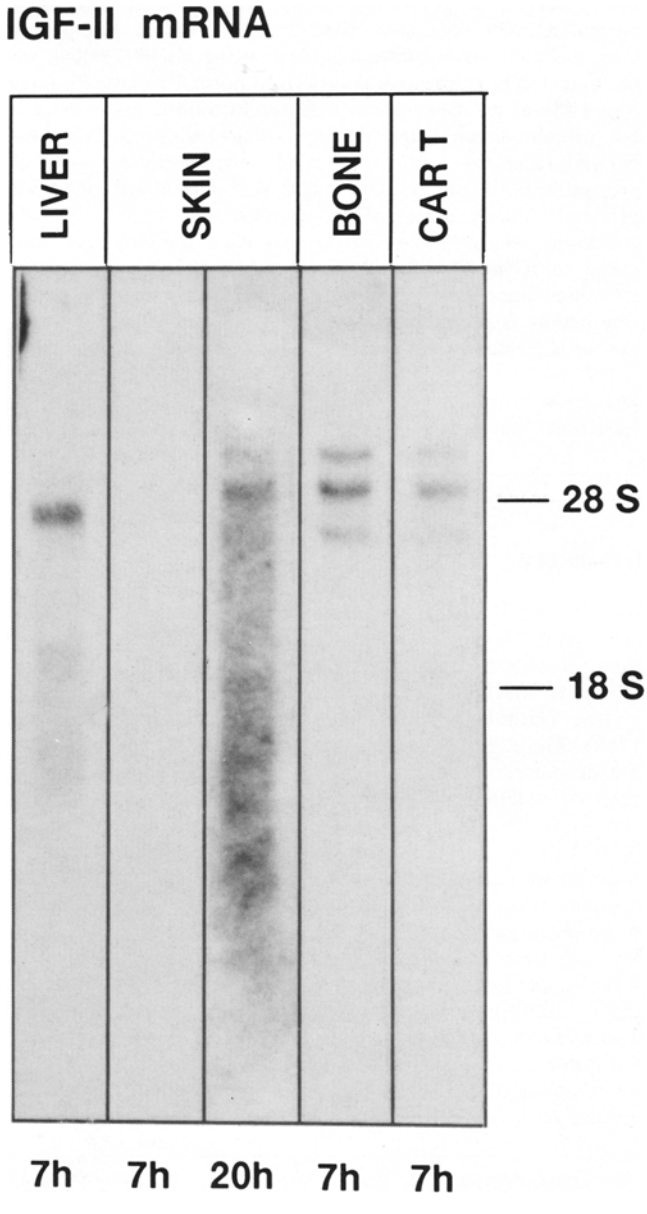

Figure 2 Northern blot analysis of IGF-II mRNA. Poly (A)$^+$ RNA (3 μg) from liver and connective tissues was analysed as described in the legend to Figure 1. Exposure times are indicated in the figure

Densitometric analysis of slot-blot hybridizations were used to quantitate concentrations of expressed mRNAs for IGFs and IGFBPs in skin, bone and cartilage during fasting. Results are shown only for those mRNAs whose concentrations changed significantly during fasting, which included mRNAs for IGF-II and IGFBP-2 in bone (Figure 3). IGF-II mRNA decreased to approximately 70% of normal ($P = 0.06$) after 72 h of fasting (Figure 3A). For comparison, the time-course is shown for the decrease in type I collagen gene expression in bone that we observed previously in the same group of animals (Gosiewska *et al.*, 1994) and this decrease slightly preceded the reduction in IGF-II mRNA concentration. Although IGF-I mRNA is expressed in bone (Gosiewska *et al.*, 1994), it was not affected by fasting. Expression of mRNA for IGFBP-2 in bone was induced by 24 h of fasting to approximately twice the normal level and remained at that level ($P < 0.1$) throughout fasting (Figure 3B). In cartilage, IGF-I and -II mRNA concentrations were as high as in bone but both were unaffected by fasting. As mentioned above, IGF-I was not expressed in skin and the low levels of IGF-II and IGFBP-2 mRNAs that were expressed were not affected by fasting.

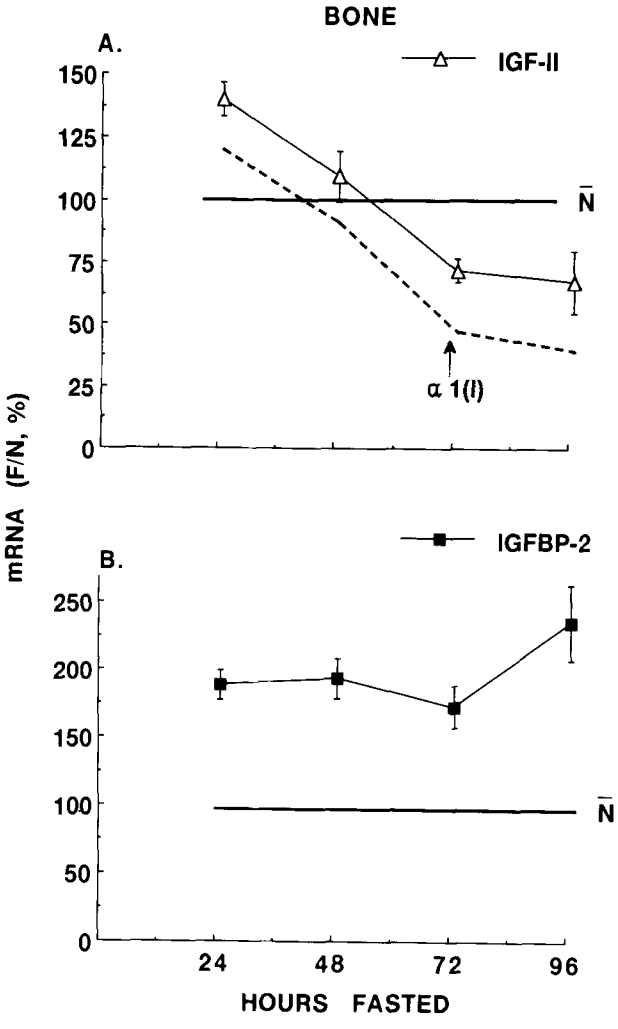

Figure 3 Relative concentrations of IGF-II (**A**) and IGFBP-2 (**B**) mRNAs. Results from densitometric analysis of slot-blots are expressed as the values for fasted guinea-pigs as a percentage (F/N%) of the mean value from four normal animals (horizontal line, 100%; IGF-II SEM = ± 7.1; IGFBP-2 SEM = ± 7.7). They are representative of several similar analyses. The same blot was used for both probes and was stripped between hybridizations. For the fasted samples, each point represents the mean ± SEM from two different RNA concentrations (0.25 μg and 1 μg) and at least two scans. The 96 h value for IGFBP-2 was not significantly different than the 24–72 h fasted samples ($p > 0.5$). Data for α1(I) collagen mRNA obtained previously (Gosiewska *et al.*, 1994) is represented by a dashed line (**A**)

Characterization of the IGFBPs in extracts from connective tissues

The method used to prepare tissue extracts was similar to that of Gelato *et al.* (1992). They showed that the IGFBP profile in extracts was the same whether or not tissues were perfused with normal saline prior to extraction, indicating that peripheral blood was not the source of the IGFBPs. The observation that little or no IGFBP-3 was found in tissue extracts from normal guinea-pigs (see below), although it is the major circulating IGFBP (Peterkofsky *et al.*, 1991; Peterkofsky *et al.*, 1994) supports this conclusion. In addition, a comparison of extracts and guinea-pig serum on protein stained gels showed the absence of major serum proteins such as albumin and immunoglobin (data not shown). Preliminary ligand blotting analysis with [^{125}]IGF-I detected IGFBPs in tissue extracts from fasted guinea-pigs. The major species were 29, 35 and 44 kDa and there were variable amounts of a 40-kDa form (Figure 4, lanes 4, 7, 10). These proteins (Figure 4, lane 1) were similar in size to IGFBPs in FGPS (fasted guinea-pig serum) which have been identified previously as IGFBP-3 (40 and 44 kDa), IGFBP-2 (35 kDa) and IGFBP-1 (Peterkofsky *et al.*, 1994). A trace amount of a 24 kDa binding protein that is probably unglycosylated IGFBP-4 is observed in NGPS (normal guinea-pig serum) and FGPS after long exposure of ligand blots (Peterkofsky *et al.*, 1994). Antisera to purified rat IGFBP-1 and -2 that we had prepared previously (Peterkofsky *et al.*, 1994) were used to identify binding proteins in tissue extracts. These antibodies react with guinea-pig IGFBP-1 and -2, respectively, but not with IGFBP-3 or the presumptive IGFBP-4 in guinea-pig sera (Peterkofsky *et al.*, 1994). We confirmed that IGFBP-1 and 2 were specifically immunoprecipitated from FGPS (Figure 4, lanes 2, 3). Anti-IGFBP-2 also precipitated a binding protein migrating similarly to IGFBP-1 (lane 3), as observed previously (Peterkofsky *et al.*, 1994). Since the antibody does not react with IGFBP-1, this band may represent proteolysed IGFBP-2. A proteolytic activity that cleaves IGFBP-2 has been reported (Wang *et al.*, 1988; Gockerman *et al.*, 1995). Immunoprecipitation of tissue extracts with anti-IGFBP-1 identified the 29 kDa IGFBP in bone, cartilage and skin from fasted animals as IGFBP-1 (Figure 4, lanes 5, 8, 11) and anti-IGFBP-2 identified the 35 kDa IGFBP in bone and cartilage as IGFBP-2 (Figure 4, lanes 6, 9). Note that fasted bone and skin extracts contained none of the faster migrating band that precipitated from FGPS with the IGFBP-2 antibody, although both extracts contained IGFBP-1, while cartilage contained only a small amount. Using N-glycanase, we confirmed previous observations that the 40 and 44 kDa bands in guinea-pig serum are the differentially N-glycosylated forms of IGFBP-3 (Peterkofsky *et al.*, 1994). N-glycanase treatment of proteins in tissue extracts from fasted animals prior to ligand blotting resulted in deglycosylation of the 44 kDa-protein (Figure 5), so it appears to correspond to the most higly glycosylated form of IGFBP-3. As expected, the migration of IGFBP-1 and -2, which were most concentrated in cartilage, were not altered by N-glycanase treatment (Figure 5).

IGFBP levels in protein extracts during fasting

Proteins in extracts from guinea-pigs fasted for varying time intervals and normal controls were analysed by ligand blotting and the amounts of each IGFBP were quantitated by densitometry of autoradiograms. Equal amounts of extract protein were compared on each gel. After ligand blotting, representative membranes for each tissue were stained for protein and results confirmed that similar amounts of protein were applied to gels (data not shown).

In bone extracts from normal guinea-pigs, mainly IGFBP-

2 and a trace of IGFBP-3 could be detected (Figure 6A). In other gels, no IGFBP-3 was present in extracts from normal bone (data not shown). Normal guinea-pig serum, however, contains mainly IGFBP-3 and no IGFBP-2. In bone extracts from guinea-pigs fasted for 10-96 h (Figure 6A), IGFBP-1 appeared at the earliest time point (10 h) and accumulated in a biphasic manner with peak levels at 24 and 96 h (Figure 6, A and B). This pattern of accumulation differed only slightly from that of circulating IGFBP-1 levels, which peaked initially at 10 h (Gosiewska *et al.*, 1994). IGFBP-3 concentrations remained low until 48 h of fasting when it was approximately five times the normal level (Figure 6, A and B). IGFBP-3 in fasted bone consisted mainly of the most highly glycosylated 44 kDa form (Figure 6A), although in fasted guinea-pig serum the 40 and 44 kDa bands are present in about a 2:1 ratio (Figures 6A, 7A and 8A). The level of IGFBP-2 increased to about twice the normal level after 24 h of fasting and remained there (Figure 6A and B), a pattern similar to induction of its mRNA (Figure 3B).

Cartilage extracts from normal animals also contained mainly IGFBP-2 and some IGFBP-3 (Figure 7A). A band that migrated similarly to IGFBP-1 was present but was not observed in two other analyses of this same sample (data not shown). A similar band that was precipitated with anti-

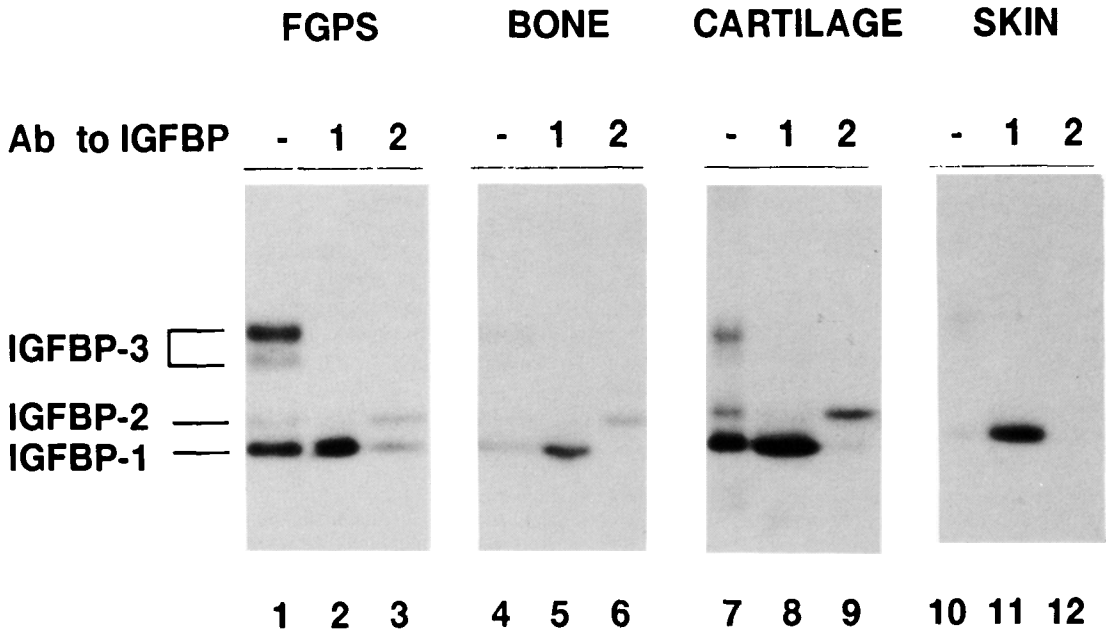

Figure 4 Immunoprecipitation of IGFBP-1 and -2 from serum and tissue extracts. Serum from guinea-pigs fasted for 96 h (FGPS, 0.5 μl) and fasted guinea-pig tissue extracts from skin (96 h), bone (96 h) and cartilage (96 h) were untreated (lanes 1, 4, 7, 10) or immunoprecipitated with antibody to rat IGFBP-1 (lanes 2, 5, 8, 11) or IGFBP-2 (lanes 3, 6, 9, 12). For untreated samples, extract containing 40 μg of protein was used but five times that amount was used for immunoprecipitations. Gel electrophoresis and ligand blotting were performed as described in Materials and methods. Positions of IGFBP-3, -2 and -1 in FGPS are indicated on the left. Extracts are from the same animals used in experiments shown in Figures 6–8

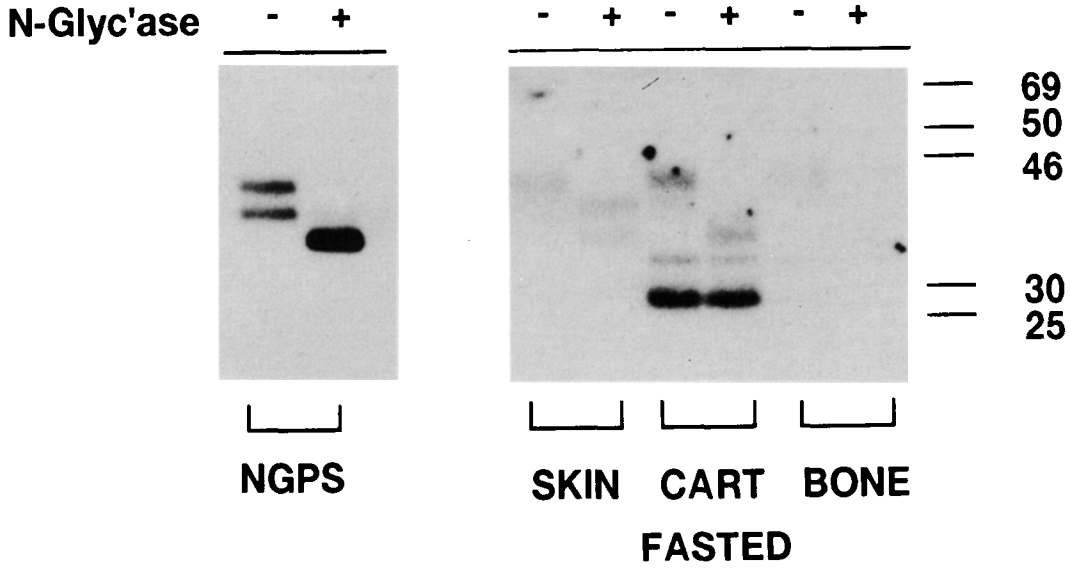

Figure 5 Treatment of serum and tissue extracts with N-glycanase. Serum (1 μl) from normal guinea-pigs (N) and fasted guinea-pig extracts from skin (24 h, 50 μg protein), cartilage (cart, 96 h, 25 μg protein) and bone (48 h, 50 μg protein) were incubated with (+) or without (−) N-glycanase prior to electrophoresis and ligand blotting. Molecular weight markers (in kDa) are shown on the right. Extracts are from the same animals used in experiments shown in Figures 6–8

IGFBP-2 was observed in fasted cartilage and may be a degradation product of IGFBP-2. IGFBP-1 appeared in cartilage at a relatively high level by 10 h of fasting and remained elevated (Figure 7A and B). IGFBP-3 in cartilage, as in bone, occurred mainly as the 44 kDa form (Figure 7A) and its concentration was increased after 10 h of fasting to approximately twice the normal level (Figure 7B). The content of IGFBP-2 in cartilage decreased slightly during fasting (Figure 7A and B).

Figure 8 shows the time course for the changes in IGFBPs in skin extracts. It should be noted that the scale is different for IGFBP-1 and -2 compared to IGFBP-3. Skin extracts from normal animals contained only a low level of IGFBP-2 which decreased slightly during fasting (Figure 8A and B). IGFBP-1 accumulated rapidly during fasting, reaching a peak after 10 h (Figure 8B). The time-course of IGFBP-1 accumulation in skin was almost identical to the profile of circulating IGFBP-1 (Gosiewska *et al.*, 1994). IGFBP-3 appeared in skin after 10 h of fasting and by 24 h, it reached a level about four-times higher than the peak IGFBP-1 level (Figure 8B). As in bone and cartilage, it was present mainly as the 44 kDa form (Figure 8A). After 48 h, its level decreased, but still remained higher than the other IGFBPs (Figure 8B). IGFBP-3 also accumulated to a high level in skin from scorbutic guinea-pigs losing weight (data not shown).

Characterization of IGFBPs for ligand occupancy by cross-linking analysis

[^{125}I]IGF-I that is bound to free sites on IGFBPs can be cross-linked to prevent dissociation during subsequent analysis on SDS–PAGE (D'Ercole & Wilkins, 1984). We previously found that results from gel filtration of [^{125}I]IGF-I bound, but not cross-linked, to guinea-pig serum IGFBPs were similar to those obtained using affinity cross-linking (Peterkofsky *et al.*, 1991). Those results showed that IGFBP-3 in NGPS, SGPS or FGPS does not bind radioactive ligand, since it is present in these serum in a complex with IGF-I, but IGFBP-1 and -2 that are induced during fasting and scurvy bind radioactive ligand since they contain mainly unoccupied binding sites. To determine IGF-I ligand occupancy of IGFBPs in tissue extracts, representative extracts with the highest levels of IGFBPs were incubated with [^{125}I]IGF-I, cross-linked and electrophoresed. NGPS and FGPS were used as controls. Specific binding was evaluated by incubating a duplicate sample with an excess of unlabeled IGF-I (Figure 9, + lanes). Non-specific binding is due mainly to bovine serum albumin added to the assay system (Peterkofsky *et al.*, 1991). We previously identified the IGFBP-1:IGF-I complex by immunoprecipitation (Peterkofsky *et al.*, 1994) and the IGFBP-3:IGF-I complex by crosslinking of

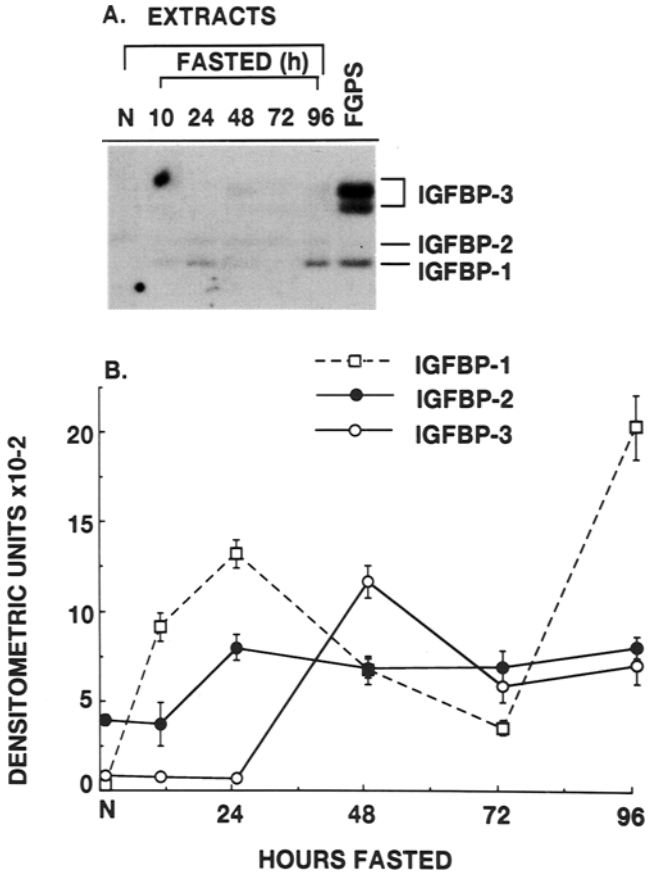

Figure 6 IGFBP concentrations in bone extracts. (**A**) Bone extracts (20 μg protein per lane) from normal (N) and fasted (10–96 h) guinea-pigs and serum (0.25 μl) from 96 h-fasted animals were electrophoresed and ligand blotted as described in Materials and methods. The positions of IGFBPs are indicated on the right. Exposure time for the autoradiogram was 18 days. (**B**) Relative concentrations of IGFBPs 1–3 determined from densitometric analysis of the ligand blot. The data are expressed as mean values in densitometric units × 10_{-2} *vs* time of fasting (10–96 h). The mean value for normal guinea-pig is indicated on the X-axis as N. Mean values ± SEM for each point were determined from several scans

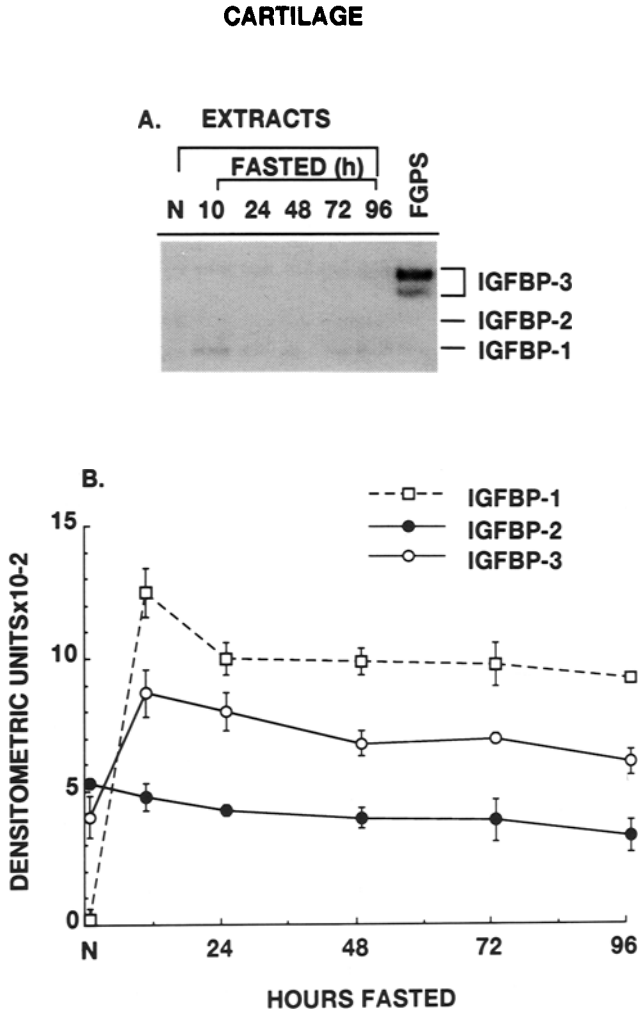

Figure 7 IGFBP concentrations in cartilage extracts. The procedures followed and the designations used were the same as described in the legend for Figure 6 except that 30 μg per lane of extract protein was applied to each lane

purified IGFBP-3 (Peterkofsky *et al.*, 1991). As observed previously (Peterkofsky *et al.*, 1991), NGPS (N) exibited very low specific IGF-I binding activity, whereas specific binding in FGPS (F) increased due to the formation of the IGFBP-1:IGF-I complex. A small proportion of IGFBP-3 in FGPS also appeared to have unoccupied binding sites since a band corresponding to 55-kDa was detected and disappeared in the presence of unlabeled IGF-I (Figure 9). Analysis of skin extract revealed that IGFBP-1 and, to a lesser extent, IGFBP-3 contained unoccupied sites for IGF-I, since bands corresponding to approximately 36 and 55-kDa proteins, respectively, were eliminated by excess unlabeled IGF-I (Figure 9, skin). The ratio of complexed IGFBP-3 to IGFBP-1 determined by densitometric scanning was 0.25, although there was seven times more total IGFBP-3 than IGFBP-1 in skin extracts at this time point based on ligand blots (Figure 8). Similar results were obtained with skin extracts from vitamin C-deficient guinea-pigs (data not shown). IGFBP-1 in extracts from cartilage and bone also contained free sites for IGF-I, but IGFBP-3 did not (Figure 9).

Discussion

The present studies support our previous proposal that circulating IGFBPs induced during fasting are responsible for inhibition of collagen gene expression in connective tissues. Our results suggest that regulation of cellular functions by IGF-I in skin of normal guinea-pigs, as well as modulation of IGF-I action by the IGFBPs in skin during fasting, occurs almost entirely by endocrine action. In bone and cartilage, however, both endocrine and autocrine/paracrine mechanisms appear to be involved.

We previously found that IGF-I was not expressed in guinea-pig skin (Gosiewska *et al.*, 1994) and in the present study we found only low levels of IGF-II mRNA in this tissue. These results are similar to those found for adult rat skin using the PCR detection method (Gartner *et al.*, 1992). Skin tissue is a target for IGF regulation since skin fibroblasts contain the type I IGF receptor (Clemmons & Shaw, 1986) and we have detected the receptor on membrane preparations from guinea-pig skin (Gosiewska & Peterkofsky, unpublished observations). Collagen gene expression is an IGF-I-dependent function as evidenced by its stimulation by IGF-I in fibroblast cultures (Goldstein *et al.*, 1989) and *in vivo* (Schmid *et al.*, 1989), and by the ability of IGF-I to reverse the inhibition of collagen synthesis by IGFBPs in skin fibroblasts and other cultured cells (Oyamada *et al.*, 1990; Peterkofsky *et al.*, 1991). In fasting, however, regulation by IGF-I in skin is probably indirect. We found previously (Gosiewska *et al.*, 1994), with the same group of animals used in the present studies, that collagen gene expression is decreased almost maximally by 24 h while total circulating IGF-I levels do not begin to decrease until later. In an earlier study (Palka *et al.*, 1989) we found that IGF-II levels are unchanged during fasting.

Modulation of IGF-I action in skin is likely to be accomplished by IGFBPs. Neither IGFBP-1 nor IGFBP-3 mRNAs were expressed in skin of normal or fasted guinea-pigs. Nevertheless, both proteins accumulated in skin during fasting, suggesting that they were derived from the circulation. For IGFBP-1, the kinetics of accumulation in skin paralleled the induction of circulating IGFBP-1 that we reported previously for this group of guinea-pigs (Gosiewska *et al.*, 1994). Although IGFBP-3 accumulated in skin to a greater extent than IGFBP-1, it probably plays a less important role in regulation of IGF action since it has a smaller proportion of free IGF binding sites. IGFBP-2 probably does not play a major role in regulation of IGF-dependent functions in skin during fasting. Its mRNA was expressed in skin and the protein was present, but their levels did not change during fasting while circulating IGFBP-2 is induced several fold during fasting. Therefore, most of the IGFBP-2 in skin must

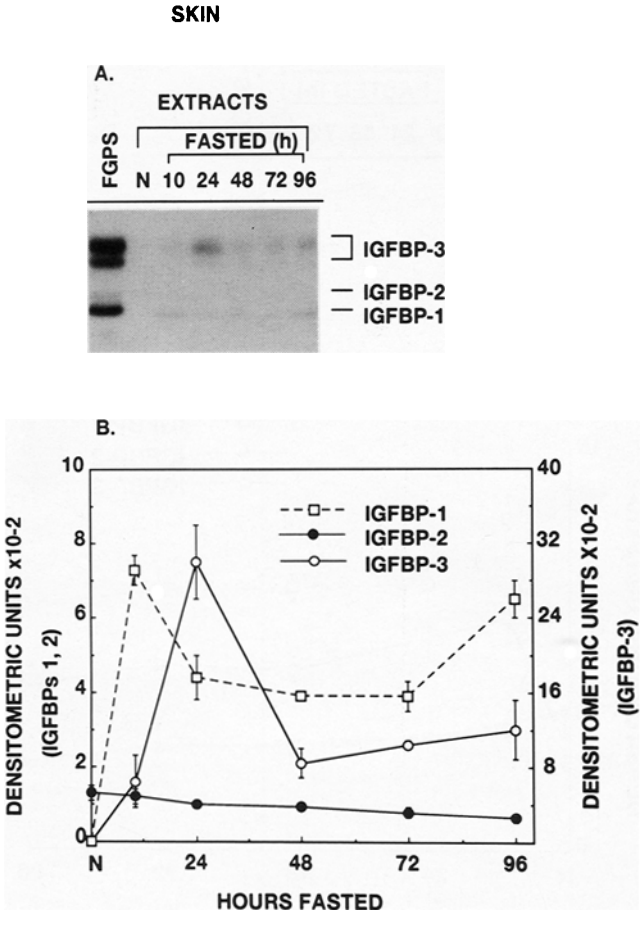

Figure 8 IGFBP concentrations in skin extracts. The procedures followed and the designations used were the same as described in the legend for Figure 6, except that 40 μg per lane of extract protein was applied to each lane, the exposure time was 12 days and the scale for IGFBP-3 is different

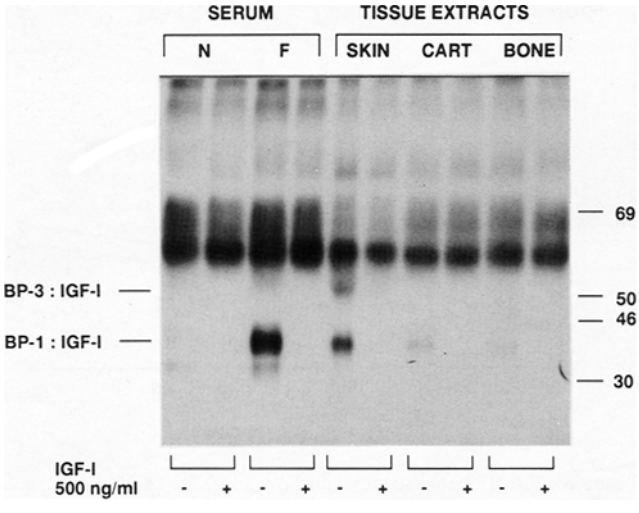

Figure 9 Affinity cross-linked IGFBPs in serum and tissue extracts. IGFBPs in serum (1μl) from normal (N) or fasted (F) guinea-pigs and tissue extracts (30 μg of protein) from fasted guinea-pigs were cross-linked to bound [^{125}I]IGF-I and analysed on SDS-PAGE. Extracts containing the highest concentration of IGFBPs were used. Samples in the lanes marked '+' contained 500 ng/ml of unlabeled IGF-I in the binding reaction and represent nonspecific binding. Positions of IGFBPs cross-linked to IGF-I are indicated on the left and markers (in kDa) are indicated on the right

be derived from local production. In agreement with our results, *in situ* hybridization analysis of adult human skin also detected IGFBP-2 and not IGFBP-1 mRNA (Batch *et al.*, 1994). In contrast to our results, however, mRNAs for IGFBP-3, -4, and -5 also were observed in human skin. It is possible that the use of whole skin is less sensitive than *in situ* hybridization, but our ability to detect IGFBP-2 mRNA argues against this since in the *in situ* study IGFBP-2, 3, -4, and -5 appear to be expressed to about the same extent.

Our results in bone and cartilage of normal guinea-pigs agree with previous studies showing that these tissues express IGF-I and IGF-II mRNAs (McCarthy *et al.*, 1992; Canalis *et al.*, 1993). Therefore, guinea-pig bone and cartilage have the potential to produce the proteins. Other studies have shown that bone and cartilage, or cells derived from them, in culture produce the proteins (Canalis *et al.*, 1988; McCarthy *et al.*, 1993; Froger-Gaillard *et al.*, 1989; Bautista *et al.*, 1990). Where both parameters have been measured, the levels of the mRNAs seem to determine the extent of protein produced (Leaman *et al.*, 1990; Schmid *et al.*, 1992; Canalis *et al.*, 1993). It is unlikely that collagen gene expression in these tissues is inhibited directly because of a decrease in local levels of the IGFs. In bone, IGF-II expression decreased later in fasting and IGF-I mRNA levels did not change at all, while in cartilage neither IGF-I nor IGF-II expression was affected by fasting. Thus, in bone and cartilage, as in skin, IGF activity is probably modulated by IGFBP-1, and to a lesser extent IGFBP-3, that accumulate early in fasting and are derived mainly from the circulation. Neither IGFBP-2 nor IGFBP-3 in bone and cartilage contained free IGF binding sites, which would make them less effective inhibitors of IGF-I action.

The accumulation of IGFBP-3 in tissues during fasting in the absence of its mRNA, is surprising. Circulating IGFBP-3 is not increased during fasting or vitamin C deficiency in the guinea-pig (Peterkofsky *et al.*, 1991 and 1994) and, as shown here, fasting did not affect the level of its mRNA in liver. Furthermore, almost all of circulating IGFBP-3 is complexed with ALS and IGFs and this complex is not transported to the extravascular space (Binoux & Hossenlopp, 1988). IGFBPs complexed only with IGF-I (Bar *et al.*, 1990b) or free IGFBPs (Bar *et al.*, 1990a) can be transported through the vascular wall. Therefore IGFBP-3 bound to IGF-I but lacking ALS might be formed during fasting and transported to tissues. In all of the tissues examined, the most highly glycosylated form of IGFBP-3 was the major species present, which suggests the possibility that this form may be preferentially transported from blood vessels or accumulated by tissues.

Some of our results differed from those observed in cell culture studies. Only IGFBP-2 was significantly expressed in guinea-pig bone but bone cells in culture display considerable variation in the types of IGFBPs they produce. Normal human osteoblasts secrete IGFBP-3, -4, and -5 (Hassager *et al.*, 1992; Mohan *et al.*, 1992) while osteosarcoma cell lines vary in the types that they produce (Hassager *et al.*, 1992; Conover & Kiefer, 1993). The types of IGFBPs produced are influenced by cell density and the presence of IGF-I and other hormones (Hassager *et al.*, 1992; Mohan *et al.*, 1992; Conover & Kiefer, 1993). Adult bovine chondrocytes produce what appears to be IGFBP-4, but IGF-I treatment leads to secretion of IGFBP-2 (Olney *et al.*, 1993). Most cell culture studies were carried out with serum-free medium and perhaps even the addition of growth factors does not reproduce the *in vivo* environment of tissues.

These studies offer insight into the regulation of IGF-I action *in vivo* during fasting, and similar results have been found in scorbutic guinea-pigs (Gosiewska & Peterkofsky, unpublished observations). In all of the tissues studied, it appears that the accumulation of IGFBP-1 derived from the circulation can play a major role in regulating IGF action and consequently in inhibition of collagen gene expression during fasting. IGFBP-1 could sequester IGFs derived either from the circulation or produced locally and limit their availability for the IGF-I receptor. In skin, IGFBP-3 could contribute to this inhibition. Although several IGFBPs have been found to potentiate IGF-I action in cells under some culture conditions (Cohick & Clemmons, 1993), IGFBP-1 and -2 in fasted and scorbutic guinea-pig sera have only inhibitory effects on IGF-I-dependent DNA, collagen and proteoglycan synthesis in connective tissue cells (Palka *et al.*, 1989; Oyamada *et al.*, 1990; Peterkofsky *et al.*, 1991 and 1994). A strictly inhibitory role for IGFBP-1 is supported by a study showing that its injection into hypophysectomized rats inhibits the action of either administered or endogenously produced IGF-I (Cox *et al.*, 1994).

Materials and methods

Serum and tissue preparation

The procedures for fasting 5 week-old female strain 2 guinea-pigs and collecting calvarial bone, articular cartilage, and skin from the back have been described in detail (Gosiewska *et al.*, 1994) and were in accordance with NIH guidelines. The group of animals described here is the same group used in a previous study (Gosiewska *et al.*, 1994). Fasted guinea-pigs and normal controls that were fed *ad libitum* received vitamin C orally (25 mg/100 g body weight) daily. Guinea-pigs were fasted for 10, 24, 48, 72 and 96 h and lost 3.3 ± 0.2, 7.7 ± 0.8, 14.6 ± 0.4, 15.7 ± 1.3, and $20 \pm 2.4\%$ of their body weight, respectively. Normal animals were gaining weight at approximately 7.8 g/day. Two fasted and control animals were killed at each time point, except for 24 h, when four animals were studied. Animals were killed by decapitation and blood was drained from the trunk and collected as described previously (Gosiewska *et al.*, 1994). Tissues were removed, rinsed in saline to remove residual blood, frozen in dry ice and stored in liquid nitrogen.

Tissue extracts

Frozen tissue samples (bone, skin and cartilage) weighing approximately 20–50 mg were immersed in 500 µl of extraction buffer (20 mM Tris-HCl pH 7.4 and 2% Triton-X 100) at 0°C and were minced with scissors. Minced tissue was transferred to a glass homogenizer, homogenized with 20 strokes and the homogenates were centrifuged for 5 min at 12 000 *g* at 4°C. Protein content of the supernate (extract) was determined using the BCA protein assay (Pierce Chemical Co., Rockford, Il, USA). Extracts were stored in small aliquots at −20°C.

Ligand blotting

Proteins in serum and tissue extracts were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide (10%) gels. Proteins were electroblotted to an Immobilon-P membrane (Millipore) and were analysed by [^{125}I]IGF-I (Amersham, Arlington Heights, Il, USA) ligand binding as described previously (Peterkofsky *et al.*, 1991). Several analyses of tissue extracts were carried out and autoradiograms of representative blots are shown in figures. The relative amounts of each IGFBP was quantitated by scanning densitometry using the Image program (W Rasband, NIH) for the Macintosh computer. Three or four scans of each autoradiogram were used to calculate means and SEM for each point.

Immunoprecipitation

The specificities of antisera against rat IGFBP-1 (anti-QA, second bleed) and rat IGFBP-2 (anti-SA, fourth bleed) that cross react with the corresponding guinea-pig IGFBPs were described previously (Peterkofsky *et al.*, 1994). Sera or tissue

extracts were incubated for 16 h at 4°C with diluted antibodies in 0.1 M Tris-HCl, pH 7.6. Dilutions of 1:350 and 1:1200 were used for IGFBP-1 and -2 antisera, respectively. Immune complexes that bound to Protein A-Sepharose were isolated and analysed by ligand blotting as described above.

N-Glycanase treatment

For analysis of N-glycosylation, serum samples and tissue extracts were denatured, treated with 30 units/ml N-glycanase (Genzyme Corp, Boston, MA, USA) for 3 h at 25°C and analysed by ligand blotting.

Affinity cross-linking

[^{125}I]IGF-I bound to IGFBPs in tissue extracts was cross-linked with disuccinimidylsuberate (Peterkofsky *et al.*, 1991). Duplicate samples containing 500 ng/ml of nonradioactive IGF-I (Bachem, Torrance, CA, USA) were prepared to evaluate specific binding. Cross-linked samples were analysed by SDS-polyacrylamide (10%) gel electrophoresis under nonreducing conditions (Peterkofsky *et al.*, 1991).

RNA preparation

Total RNA was extracted from bone, skin and cartilage and poly(A)$^+$ RNA was prepared from total RNA with an oligo(dT)-cellulose column, as described previously (Gosiewska *et al.*, 1994).

cDNA probes

cDNA probes for rat IGFBP-1 and -2, and guinea-pig IGF-I were prepared by the polymerase chain reaction (PCR) amplification of plasmid inserts, as described previously (Gosiewska *et al.*, 1994). A 30-mer oligonucleotide (Takeda *et al.*, 1992) was used as a probe for 28S rRNA. Rat IGFBP-4, -5, and -6 probes (Shimasaki & Ling, 1991) were generously provided by Dr S. Shimasaki (The Whittier Institute, La Jolla, CA, USA) and they were amplified by PCR with specific oligonucleotides (Table 1). Guinea-pig cDNAs for IGF-II and IGFBP-3 were generated by reverse transcriptase-PCR (RT-PCR). Briefly, 1 μg of poly(A)$^+$ RNA derived from guinea-pig liver was incubated for 1 h at 42°C in a 25 μl reaction mixture containing: 1 × RT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 3 μM specific antisense oligonucleotide (Table 1), 2.5 mM of each dNTP, 100 μg/ml of acetylated BSA and 200 units of superscript RNase H-free reverse transcriptase (BRL, Gaithersburg, MD, USA). The reaction was terminated by the addition of an equal volume of cold water and 10 μl of RT reaction was used for PCR. The PCR mixture (100 μl final volume) contained: 16 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM of each dNTP, 1 μM of each antisense and sense oligonucleotide (Table 1), and 2.5 units of Taq DNA polymerase (USB, Cleveland, OH, USA). DNA was amplified for thirty five cycles, each consisting of: denaturation, 1 min at 95°C; annealing at a temperature specific for the melting point of each pair of oligonucleotides, 2 min; elongation at 72°C, 3 min. For the final cycle, elongation was 7 min. Reaction products were separated by agarose gel electrophoresis and visualized by ethidium bromide. The identity of the PCR product was confirmed by its size and formation of the expected cleavage products after restriction enzyme digestion based on the sequence of the cDNA. Identification of IGF-II cDNA was confirmed by cloning of the PCR product into pGEM-T vector (Promega) and sequencing analysis with a Sequenase Version 2 T7 DNA Polymerase sequencing kit (USB, Cleveland, OH, USA) and an internal antisense oligonucleotide (acacgtccctctcggactt). The protocol provided with the sequencing kit was followed with some modifications. Briefly, approximately 140–200 ng of cDNA (purified from low-melting temperature agarose gels) and 1 pmol/μl of internal oligonucleotide were annealed by boiling for 90 sec, chilling rapidly in a dry ice-ethanol bath and thawing on ice for 2 min. Labeling mixture containing 0.1 M DTT, 5 μCi of [α-^{35}S]dATP (Amersham), 3 units of DNA polymerase and 1 μl of Mn buffer provided with the kit were added and the standard protocol was followed. Samples were loaded onto a 6% polyacrylamide gel and electrophoresis was performed in TBE buffer at 1600 V using a sequencing apparatus (Bio-Rad, Hercules, CA).

Table 1 Oligonucleotide primers used in PCR

Probe (species)	*1* *2*	*Sense 5'* *Antisense 5'*	*Size (base pairs)*	*Annealing temp. (C)*
IGF-II (GP)	1	gaccgcggcttctacttca	199	50
	2	caagcccacggggtatct		
IGFBP-3 (GP)	1	agccagcgctacaaagtt	226	55
	2	tttggaagggcgacactg		
IGFBP-4 (R)	1	ctgcgcacattgatgcac	420	50
	2	tgtcttccgatccacaca		
IGFBP-5 (R)	1	gtttgcctcaacgaaaag	261	55
	2	ctgtgtcatctcaggtgca		
IGFBP-6 (R)	1	agaacgaagagacacctt	232	58
	2	tgcagtactgaatccaag		

GP: guinea-pig, R: rat

Radiolabeling of cDNA probes by nick translation, hybridization and quantitation procedures were similar to those used previously (Takeda *et al.*, 1992; Gosiewska *et al.*, 1994), except that blots were prehybridized in a solution containing 10% dextran sulphate, 2 × Denhardt's solution, 50% formamide, 5 × SSPE, 0.1% SDS and 250 μg/ml of sheared salmon testis DNA.

Hybridization was carried out in the same solution, except that ^{32}P-labeled DNA probe and 150 μg/ml of salmon testis DNA were included. Washing steps were carried out as follows: twice for 15 min with 2 × SSC at 65°C, once for 30 min with 2 × SSC/0.1% SDS at 65°C and once for 10 min with 0.1 × SSC at 65°C. After blots containing total RNA had been probed for IGFs or IGFBPs, they were hybridized with the probe for 28S rRNA. When the same blot was used for more than one probe, it was stripped between hybridizations. Blots were exposed to X-ray film with intensifying screens for varying times. The autoradiograms from slot blots were scanned using the Image program. The arbitrary absorbance units for fasted samples were compared to the mean value for normal samples from the same blot to obtain relative amounts of mRNA. Significance of differences were determined by *t*-tests using a Stat Soft program.

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