Comparative Use of Immunological Methods & Ligand Blotting of Insulin-like Growth Factor Binding Proteins in Serum and Other Biological Fluids

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Abstract. There is controversy as to which methodology most accurately detects the Insulin-like growth factor binding proteins (IGFBPs). We have compared immunological and ligand binding techniques to assess the IGFBPs in serum from non-pregnant women and women in late pregnancy, fetal cord serum and amniotic fluid. A specific radioimmunoassay for IGFBP-3 measured mean levels of 3.5 and 3.7 $\mu\text{g/ml}$ for non-pregnancy and pregnancy serum, respectively, 0.7 $\mu\text{g/ml}$ for fetal cord serum and 5.1 $\mu\text{g/ml}$ for amniotic fluid. In contrast, Western-ligand blot of fluid showed IGFBP-3 as a 40 kDa doublet in non-pregnancy serum and fetal cord serum, unlike pregnancy serum or amniotic fluid, where IGFBP-3 was undetectable by standard blot analysis. However, analysis of fluids at 25-50 times more volume showed that IGFBP-3 was detectable as a 40 kDa doublet in pregnancy serum and amniotic fluid. The molecular mass of immunoreactive IGFBP-3 was examined by Western-immunoblot, probed with antibody $\alpha\text{IGFBP-3g1}$. As in Western-ligand blot, intact IGFBP-3 (40 kDa) was easily detected in non-pregnancy serum and fetal cord serum, but required greater volumes of sample for detection in pregnancy serum and amniotic fluid. In addition, smaller immunoreactive fragments were found in all fluids: a faint 27 and 22 kDa band was found in non-pregnancy serum and fetal cord serum, with more dominant bands of 31-27, 26, 25, 24 and 22 kDa in pregnancy serum and 31-26, 25, 22, 19-17 kDa in amniotic fluid. To address the obvious discrepancies between ligand and immunological analysis, samples were assessed for IGFBP-3 protease activity. Non-pregnancy serum proteolyzed 14% of the substrate, pregnancy serum 61%, fetal cord serum 33%, and amniotic fluid 50-61%. Thus, fluids with apparently low IGFBP-3 by Western-ligand blot, but high IGFBP-3 by RIA, showed high levels of protease activity. Previously, IGFBP-proteases have been shown to degrade IGFBP to smaller fragments with a reduced affinity for IGF. We conclude that the discrepancy between ligand binding techniques and immunological techniques can be explained, at least in part, by the presence of IGFBP-protease activity.

Key words: insulin-like growth factor (IGF), protease, IGF binding protein (IGFBP).

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Introduction

The insulin-like growth factors (IGFs) are polypeptides that exert a wide range of mitogenic and differentiative effects at the cellular level [1]. IGFs circulate associated with IGF binding proteins (IGFBP). To date, six IGFBPs have been isolated and characterized on the basis of amino acid and DNA sequence [2]. IGFBP-1, 2, 4, 5 and 6 are the smaller IGFBPs (24-36 kDa), whereas IGFBP-3, the major serum IGFBP of 45 kDa, circulates as a 150 kDa complex [3-5]. IGFBPs can modulate the action of IGFs in either an inhibitory mechanism (binding the IGF and preventing presentation to the cellular receptor), or by potentiating the action of IGF (enhancing cellular presentation or mediating non-receptor activation at the cell surface) [1, 6,7].

IGFBPs are generally examined by Western-ligand blot analysis. This characterization technique relies upon the stability and functionality of the IGFBPs following SDS PAGE and electrotransfer of proteins to nitrocellulose, and upon their subsequent ability to bind radiolabeled ligand [8]. Recently, the evaluation of IGFBPs in the serum of pregnant women has illustrated the inherent problems with this technique. IGFBP-3 is apparently absent by week eight of pregnancy as assessed by Western-ligand blot analysis [9, 10], but measurable by radioimmunoassay [4], as well as by indirect techniques that measure the IGF content of the 150 kDa complex [11]. This discrepancy has been attributed to a pregnancy-associated IGFBP-protease [9,12]. The protease can degrade IGFBP-3 into smaller fragments that display a reduced affinity for IGF and are subsequently difficult to detect by techniques that rely upon ligand binding (*ie*, Western-ligand blots) [13]. The physiological significance of the pregnancy-associated IGFBP-protease is still controversial. Possibly, the partial proteolysis of IGFBP-3 by the IGFBP-protease may promote the release of IGF, thereby

potentiating the bioavailability and bioactivity of IGFs.

We have measured and characterized IGFBP-3 in serum from non-pregnant and pregnant women, fetal cord serum and amniotic fluid. Specifically, we have: 1) measured the concentration of immunoactive IGFBP-3 by means of a newly established specific radioimmunoassay; 2) characterized the ligand binding-form of IGFBP-3 by Western-ligand blot; 3) characterized the immunoreactive form(s) of IGFBP-3 by western-immunoblot; and 4) quantitated the IGFBP-protease activity using two different sources of IGFBP-3.

Methods

IGFBP-3 Radioimmunoassay

Samples were assessed for immunoreactive IGFBP-3 as previously described [14]. In brief, assay samples (500 μ l in total) consisted of: 50 μ l of a diluted sample, α IGFBP-3g1 antiserum (50 μ l of 1/1000 dilution), 125 I-IGF-IGFBP-3 complex (10,000 cpm/50 μ l), and 350 μ l of buffer containing BSA. Following an incubation for 18 h at 4C, samples were incubated with 50 μ l of both 4% normal rabbit serum and goat anti-rabbit IgG for 30 min at 22C, after which 1 ml of 6% PEG was added. The samples were centrifuged immediately at 3000 rpm for 30 min at 4C, the supernatant was aspirated and the pellets counted.

The mean specific and non-specific binding were $42 \pm 3\%$ and $7 \pm 2.5\%$, respectively. The minimal detectable concentration was 0.66 ng/tube or 3.5 ng/ml, and the intra- and inter-assay co-efficients of variation were 4.4% and 12%, respectively.

Western Analysis

1) Ligand blot

Samples were subjected to Western-ligand blot analysis as described by Hossenlopp *et al.* [8]. Samples were subjected to SDS-polyacrylimide gel electrophoresis, transferred

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to nitrocellulose and probed with radiolabel IGF-I and IGF-II. The treated nitrocellulose was exposed to x-ray film for 3-7 days at -70°C .

2) Immunoblot using chemiluminescence

As previously described [14], samples were subjected to Western-ligand blot analysis as outlined above, but were probed with $\alpha\text{IGFBP-3g1}$ antiserum. The secondary detection system utilized a goat anti-rabbit IgG conjugated with horseradish peroxidase and chemiluminescent reagents purchased from Amersham (ECL kit). Nitrocellulose was exposed to X-ray film for 20-40 min at room temperature.

IGFBP-3 Protease Assay

Fluids were assessed for IGFBP-3 protease activity [12]. Following an incubation with iodinated E. coli-derived IGFBP-3, fluids were electrophoresed and the gels dried under vacuum. The gels were exposed to X-ray film for 12-18 h at -70°C . The autoradiographic intensity of the bands was determined with a Laser densitometer scanner (Pharmacia, LKB). The % proteolysis was calculated as the optical density of IGFBP-3 fragments/total IGFBP-3-related densities.

Results

The measurement of immunoreactive IGFBP-3 in serum (non-pregnancy, pregnancy and fetal cord) and amniotic fluid required parallelism between the purified peptide and fluid; the slopes of the curves were virtually superimposable, ranging from -0.39 to -0.42 (Figure 1, upper panel). The concentrations of IGFBP-3 in the various fluids are shown in Figure 1 (lower panel). Non-pregnancy serum contained 3556 ± 508 ng/ml (mean $\pm$ SEM); pregnancy serum, 3718 ± 842 ng/ml; fetal cord serum, 753 ± 101 ng/ml; and amniotic fluid, 5150 ± 688 ng/ml.

In order to characterize the molecular mass of the IGFBPs, fluids were assessed by

Fig 1. Radioimmunoassay (RIA) of IGFBP-3
Panel A: Parallelism between glycosylated IGFBP-3 and biological fluids.

Samples were serially diluted and measured in the IGFBP-3 RIA. The y-axis represents the sample binding divided by the total specific binding, and the x-axis, the sample dilution ($\mu\text{l/ml}$) versus IGFBP-3 (ng/ml).

Analysis were repeated in five independent experiments at 7-8 dilutions, and each dilution was measured in triplicate.

Non-pregnancy and pregnancy serum (16-21 weeks) are represented by a mean of pools containing 3 individual sera. Fetal cord serum is represented as a mean of 3 individuals, and amniotic fluid (15-21.5 weeks) represents a mean of 5 individual samples.

Panel B: Concentration of immunoreactive IGFBP-3.

Data represent means $\pm$ SEM for an average of 3 experiments, where samples were measured at three concentrations in triplicate.

Western-ligand blot (Figure α IGFBP-3g1 2A) and Western-immunoblot using α IGFBP-3g1 antiserum (Figure 2B). Sera from non-pregnant women Western-ligand blotted as a 44–35 kDa doublet with a similar molecular mass to glycosylated IGFBP-3, as well as faint

Fig 2. Characterization of IGFBPs

Panel A: Western-ligand blot

Samples were incubated with SDS loading buffer, and molecular weight markers were incubated with reducing SDS-loading buffer, and then subjected to electrophoresis and to electrotransfer, and probed with radiolabelled IGF-I and IGF-II. The treated nitrocellulose was autoradiographed for 8 days.

The molecular mass markers are indicated. The fluids were pools of 'n' individual samples: lane 1, NP = non-pregnancy serum (n=3, 2 μ l); lane 2, P = pregnancy serum (16-21 weeks of gestation, n=3, 2 μ l); lane 3, FC = fetal cord sera (n=2, 2 μ l); and AF = amniotic fluid (18 weeks gestation, n=3, 1 μ l).

All samples were treated on one gel. This is one representative gel of these biological fluids and has been repeated 5 times.

Panel B: Western-immunoblot with α IGFBP-3g1 antisera

Samples were electrophoresed, electrotransferred, and incubated with antiserum. Binding was detected following incubation with goat antirabbit, horse radish peroxidase conjugated IgG and fluorescent substrates. The nitrocellulose was exposed to X-ray film at room temperature for 20 min.

The molecular mass markers are indicated. The fluids were pools of 'n' individual samples: lane 1, NP = non-pregnancy serum (n=3, 2 μ l); lane 2, P = pregnancy serum (16-21 weeks of gestation, n=3, 2 μ l); lane 3, FC = fetal cord sera (n=2, 2 μ l); and AF = amniotic fluid (18 weeks gestation, n=3, 1 μ l).

All samples were treated on one gel. This is one representative gel of the fluids and has been repeated twice.

32, 28–27 and 22 kDa IGFBPs (Figure 2A, lane 1). Of these IGFBPs, the 44–35 kDa doublet and to a lesser extent the 28 kDa band reacted with α IGFBP-3g1 antisera (Figure 2B, lane 1). Western-ligand blot of fetal cord serum (2 μ l) produced a similar pattern to non-pregnancy serum (Figure 2A, lane 3): a 44–35 kDa IGFBP-3 doublet, as well as 32, 28, 26 and 22 kDa IGFBPs. Western-immunoblot with IGFBP-3 antisera showed reactivity with the 40 kDa doublet, as well as the 28 kDa IGFBP band (Figure 2B, lane 3). Thus, intact 44–35 kDa IGFBP-3, as well as a 28 kDa immunoreactive fragment was seen in non-pregnancy and fetal cord serum. In contrast, sera from women between weeks 16–18 of pregnancy (2 μ l) contained little 44–35 kDa IGFBP.

Instead, very faint 32 and 29–26 kDa IGFBPs were visible by ligand blot (Figure 2A, lane 2). Of these bands, only the broad 28–26 kDa IGFBP reacted with α IGFBP-3g1 antisera: no intact IGFBP-3 was detected (Figure 2B, lane 2). Likewise, amniotic fluid Western-ligand blotted as a faint 32 kDa and 42–37 kDa doublet, as well as a dominant band at 30–24 kDa. Only the 28 kDa band reacted with the α IGFBP-3g1 antiserum (Figure 2A & B, lane 4). These results appeared to contradict the high IGFBP-3 levels detected in amniotic fluid by RIA.

To address the possibility that pregnancy serum and amniotic fluid contained IGFBP-3, possibly at low concentrations or with reduced affinity for ligand, we analysed increasing

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Fig 3. Increasing concentration of fluid for IGFBP characterization

Panel A: Western-ligand blot

Samples were treated as described for Figure 2A. The nitrocellulose was exposed to X-ray film for three days.

The molecular mass markers are indicated. The fluids were pools of 'n' individual samples: lanes 1-3, non-pregnancy serum (n=3) at 0.5, 1, and 5 μ l; lanes 4-6, pregnancy serum (16-21 weeks of gestation, n=3) at 1, 5, 10 μ l; and lanes 7-9, amniotic fluid (18 weeks gestation, n=3) at 1, 5, 10 μ l.

All gels were prepared and treated identically. This is one representative set of experiments and has been repeated three times.

Panel B: Western-immunoblot with α IGFBP-3g1

Samples were treated as described in Figure 2B.

The nitrocellulose was exposed to x-ray film at room temperature for 40 min.

The molecular mass markers are indicated. The fluids were pools of 'n' individual samples: lanes 1-3, non-pregnancy serum (n=3) at 0.5, 1, and 5 μ l; lanes 4-6, pregnancy serum (16-21 weeks of gestation, n=3) at 1, 5, 10 μ l; and lanes 7-9, amniotic fluid (18 weeks gestation, n=3) at 1, 5, 10 μ l.

All gels were prepared and treated identically. This is one representative set of experiments and has been repeated three times.

volumes of fluid by Western analysis (Figure 3). Western-ligand blots of non-pregnancy serum showed that the 41 and 38 kDa doublet was detectable with 0.5 μ l, while the smaller bands of 28, 27 and 23 kDa were only seen with 1 or 5 μ l (Figure 3A, lanes 1–3). Similarly, Western-immunoblot with α IGFBP-3g1 recognised the 44–33 kDa doublet with 1 μ l, as well as 31, 28–26 and 22 kDa immunoreactive bands (Figure 3B, lanes 1–3). Detection of intact IGFBP-3 in pregnancy serum required 10-fold higher volumes of sample, causing the problem of distortion of the IGFBP profile by albumin. Western-ligand blotting revealed bands of 37–32 kDa, as well as 28–27 kDa and a faint 23 kDa IGFBPs (Figure 3A, lanes 4–6). Western-immunoblot analysis using α IGFBP-3g1 recognised the 41 and 34 kDa IGFBP-3 doublet, a broad 31–27 kDa and a 24 kDa band, as well as two non-ligand binding bands of 26 and 25 kDa (Figure 3B, lanes 4–6). Thus, at high volumes of sample, pregnancy serum contained detectable and immunoreactive intact IGFBP-3, and smaller immunoreactive fragments, some undetectable by Western-ligand blot. Western-ligand blot analysis showed amniotic fluid to be similar to pregnancy serum. Intact IGFBP-3 (42 and 38 kDa bands) was only detectable at 10–20 times more sample than required for detection of non-pregnancy serum IGFBP-3 (Figure 3A, lanes 7–9). In addition, major IGFBPs of 34, 32–24 and 23 kDa were intensely labelled at these higher loadings. A single immunoreactive band of 27–28 kDa was detected at 1 μ l, but at higher sample volumes, the 43–37 kDa IGFBP-3 doublet, as well as a faint 33 kDa band, a dominant 31–26 kDa, 25 kDa, 22 kDa and 19–17 kDa bands were visible (Figure 3B, lanes 7–9). Thus, amniotic fluid also contained low levels of intact 40 kDa IGFBP-3, as well as many smaller immunoreactive fragments.

The discrepancies between western-ligand blot and RIA may be due to the presence on an IGFBP-protease, which has been shown to degrade IGFBP-3 into smaller fragments that

Fig 4. Proteolysis of iodinated IGFBP-3 by biological fluids

Samples (20 μ l of a 1:10 dilution in buffer) were incubated with 10 μ l of iodinated IGFBP-3 for 5h at 37C, before being subjected to SDS-PAGE. Gels were dried under vacuum and exposed to X-ray film overnight at -70C.

The molecular mass markers are indicated. The fluids were pools of 'n' individual samples: C=control, iodinated IGFBP-3 alone; NP=non-pregnancy serum (n=3, 2 μ l); P=pregnancy serum (16–21 weeks of gestation, n=3, 2 μ l); FC=fetal cord sera (n=2, 2 μ l); and AF1–5=amniotic fluid (15, 16, 18, 19, 21.5 weeks gestation respectively, n=1 per lane, 2 μ l).

The autoradiographic intensities of the bands were determined by scanning with a laser densitometric scanner. The amount of proteolysis was calculated as a percentage of the optical density of fragmented IGFBP-3 over the sum of all IGFBP-3-related optical densities in that lane. The percentage proteolysis is presented beneath the autoradiograph.

All samples were treated on one gel. This is one representative gel of these fluids and has been repeated three times.

display reduced affinity for IGFs [13]. Consistently, both amniotic fluid and pregnancy serum displayed high immunoreactive IGFBP-3, but intact IGFBP-3 was difficult to detect, and only smaller immunoreactive fragments were observed. Thus, to assess for proteolytic activity, fluids were incubated with iodinated IGFBP-3, electrophoresed, dried and autoradiographed (Figure 4). Serum from non-pregnancy serum (NP) showed the least proteolysis and yielded frag-

ments of 23, 18 and 15 kDa. In contrast, pregnancy serum (P) and amniotic fluid (AF1-5) contained proteases that degraded 50–60% of the IGFBP-3 substrate. The proteolytic pattern also differed from that of non-pregnancy serum. Pregnancy serum produced 21.5, 20, 18 and 15 kDa bands, whereas amniotic fluid produced bands of 20 and 18 kDa with faint 16 and 15 kDa fragments. Fetal cord serum resulted in 33% proteolysis with a 23, 21.5 and 20 kDa triplet, as well as 18 and 15 kDa fragments. These data suggest the presence of one or more types of protease capable of degrading IGFBP-3.

Discussion

Regulation of the IGFBP and IGF axis has been recently complicated by the presence of an IGFBP-protease. To-date, documented serum proteases include a pregnancy-associated protease in human serum [9,10] and rodent serum [15,16], a protease associated with human serum of severely ill patients [17], and a protease associated with growth-hormone deficient rats [unpublished observations]. These IGFBP-proteases appear to be selective for IGFBP-3, resulting in degradation of IGFBP-3 into smaller molecular weight fragments. These fragments display a reduced affinity for radioligand [13], but are detectable by immunological analysis. The discovery of these proteases has made it necessary to reassess our conventional analysis of IGFBPs. We have, therefore, compared the ability of ligand-dependent and immunological techniques, as well as the recently developed IGFBP-protease assay, to detect the IGFBPs in non-pregnancy, pregnancy and fetal cord serum, as well as amniotic fluid.

In non-pregnancy serum, we have found that IGFBP-3 is detected by RIA at 3.5 $\mu\text{g/ml}$, similar to levels reported by Blum and co-worker [18], but lower than reported by Baxter and Martin [6]. IGFBP-3 in non-pregnancy serum is found on Western-ligand blot as a 40 kDa doublet, and immunoblot

analysis with $\alpha\text{IGFBP-3g1}$ detects the majority of IGFBP-3 as an intact 40 kDa doublet. In addition, little IGFBP-3 proteolytic activity was measured in non-pregnancy serum. These data agree with previous reports [5,6, 11]. Similarly, fetal cord serum contained intact IGFBP-3, as assessed by Western-ligand, consistent with our previous data [19]. Western-immunoblot analysis showed IGFBP-3 to be predominantly intact, and again little proteolytic activity was observed. Consequently, a good relation between RIA and Western-ligand blot was found. In contrast, intact IGFBP-3 in pregnancy serum was difficult to detect by either Western-ligand blot or Western-immunoblot analysis, but IGFBP-3 was readily immunoassayable. Previous results have shown IGFBP-3 in pregnancy serum to be undetectable by Western-ligand blot [9,10], but detectable by RIA [6] or by indirect techniques that measure the IGF content of IGFBP [11]. In agreement, we have shown that IGFBP-3 was undetectable by Western-ligand or -immunoblot analysis when compared with equivalent volumes of non-pregnancy serum. However, when increased sample volumes were loaded (up to 10 times more), intact IGFBP-3 could be detected. Western-immunoblot analysis of pregnancy serum at low serum volumes displayed 31–27 kDa bands, but at greater sample volumes we detected intact IGFBP-3 and smaller IGFBP-3 immunoreactive fragments, some of which did not bind IGF by Western-ligand blot. Consistently, we and others have shown that pregnancy serum contains a protease capable of fragmenting IGFBP-3 *in vitro* [9,12]. Thus, the discrepancy between RIA and Western-ligand blot can be explained, at least in part, by the presence of an IGFBP-3 protease. This phenomenon was not limited to pregnancy serum. Amniotic fluid displayed very low levels of intact IGFBP-3 by western-ligand or western-immunoblot analysis, yet RIA measured IGFBP-3 at 5 $\mu\text{g/ml}$. In agreement, Baxter and Martin have previously reported high levels of immunoassayable IGFBP-3 in amniotic fluid, and have purified

IGFBP-3 from this fluid [20]. Analysis with greater sample loading demonstrated the presence of intact IGFBP-3, as well as smaller immunoreactive fragments, some of which were only detected by Western-immunoblot analysis. Further, amniotic fluid was found to contain high levels of IGFBP-3 proteolytic activity. Discrepancies between Western-ligand, Western-immunoblot and RIA thus appear to be readily explained by the presence of IGFBP-proteases. In both pregnancy serum and amniotic fluid: (a) IGFBP-3 was measurable by RIA; (b) low levels of intact IGFBP-3 were detected by Western-ligand or Western-immunoblot analysis; (c) smaller molecular weight fragments, undetectable by ligand binding techniques, were only detected with immunological analysis; and (d) pure IGFBP-3 substrate was proteolysed by these biological fluids.

Clearly all techniques are important in the characterization of IGFBPs. While ligand-dependant techniques reveal the biologically active IGFBPs, RIA and Western-immunoblot analysis identify the immunoreactive forms of IGFBP-3. In addition, the IGFBP-3 protease assay has become invaluable in the final analysis, to demonstrate the presence of the confounding effect of proteolytic activity.

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