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INSULIN-LIKE GROWTH FACTOR-I AND ITS BINDING PROTEINS IN CORD SERA: PHYSIOLOGICAL ROLES IN FETAL GROWTH

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To evaluate physiological significance of insulin-like growth factor-I (IGF-I) and its binding proteins (IGFBPs), IGF-I and IGFBP-1 in cord sera were measured by immunoradiometeric assay (IRMA) and enzyme-linked immunosorbent assay (ELISA), respectively. Furthermore, IGFBPs in cord sera were analyzed by Western ligand blot. The levels of IGF-I in cord sera increased along with gestation and correlated with birth weight. In contrast, the levels of IGFBP-1 decreased with gestation and inversely correlated with birth weight. Western ligand blot revealed molecular forms of 42/39 kDa doublet, 34 kDa, 30 kDa and 24 kDa IGFBPs corresponding to IGFBP-3, IGFBP-2, IGFBP-1, and IGFBP-4, respectively. Term fetuses with light-for-gestational-age (LGA) revealed marked decrease in IGFBP-3 band with increase in IGFBP-2 band compared to those in fetuses with appropriate-for-gestational-age (AGA). In contrast, term fetuses with heavy-for-gestational-age (HGA) from mothers with diabetes mellitus showed increased IGFBP-3 with decreased IGFBP-2 and IGFBP-1. In preterm fetuses, IGFBP-2 was markedly increased especially in fetuses with LGA compared to AGA at preterm. These results suggest that IGF-I and IGFBPs reflect to growth and developmental stages of fetuses and may have important roles in fetal development.

Several lines of evidence indicate important roles for maternal insulin-like growth factor-I in fetal growth[1]. Fetal IGF-I also has been postulated to be involved in fetal growth[2], although fetal serum concentrations of IGF-I are reduced compared to the concentrations in adults[3]. Both insulin-like growth factors (IGF-I and IGF-II) are associated with binding proteins (IGFBPs) in the circulation and these binding proteins are known to modify the actions of IGFs. At present, seven distinct IGFBPs have been characterized based on their complete primary structure obtained by molecular cloning[4]. IGFBP-1 is the most studied IGFBPs in relation to fetal growth. Maternal IGFBP-1 levels have been found to be elevated during pregnancy and inversely correlated with birth weights[5]. Since IGFBP-1 has been shown to inhibit actions of IGF-I in many cell culture systems[6][7], it has been postulated that IGFBP-1 influences fetal growth by inhibiting IGF-I action in the placenta. In contrast to low levels of IGF-I in the fetal circulation, levels of IGFBP-1 in the fetus are higher compared to these of adult levels[8].

Recently, serum IGFBPs were characterized by ligand blot[9] in which four different IGFBPs, IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-4 were identified. More recently, maternal IGFBPs through pregnancy were analyzed by using ligand blot[10]. It has been demonstrated that all IGFBPs except IGFBP-1 markedly reduced during pregnancy due to protease activity in the matermal circulation.

To elucidate physiological significance of IGFBPs in the fetus, we analyzed IGF-I and IGFBPs in cord sera from term as well as preterm delivery.

Materials and Methods

Cord sera

Cord sera were obtained from term and preterm infants at vaginal delivery or caesarean section. Since there was no difference in IGFBPs profiles between blood in umbilical artery and vein when analyzed by ligand blot, blood was taken from umbilical vein. Fetuses were divided into 3 groups based on their birth weights. Fetuses whose birth weights were between −1.5 SD and +1.5 SD of standard fetal growth curve for Japanese[11] were defined as appropriate-for-gestational-age (AGA) fetus. Similarly, fetuses whose birth weights below −1.5 SD and above +1.5 SD were defined as light-for-gestational-age (LGA) fetus and heavy-for-gestational-age (HGA) fetus, respectively. Blood was allowed to clot at room temperature, then centrifuged for 1000 ×g for 20 min at 4°C . Obtained sera were kept at −20°C until analyzed. For measurement of IGF-I and IGFBP-1, 43 and 40 samples from fetuses at various gestational age were analyzed, respectively. For Western ligand blot, samples analyzed were from 6 term AGA fetuses, 3 term intrauterine growth retardation (IUGR) fetuses, 8 term HGA fetuses from mothers with diabetes mellitus, 4 preterm AGA fetuses and 4 preterm IUGR fetuses. Preterm cord sera mean the sample during 30~36 weeks of gestation.

Measurement of IGF-I and IGFBP-1

Levels of IGF-I in cord sera were measured in duplicate by an immunoradiometric assay kit obtained from Diagnostic Systems Laboratories Inc (Webster, TX, USA) after ethanol extract. This assay is cross-react with IGF-II and insulin at the potency of less than 0.1%. The sensitivity of the assay was 0.8 ng/ml and itra- and inter-assay coefficients of variation were less than 5% respectively. Levels of IGFBP-1 in cord sera were measured in duplicate by an immunoenzymometric assay kit kindly provided by Medix Biochemica (Kauniainen, Finland). In this assay system, IGFBP-1 is sequentially bound to a monoclonal antibody (6305) that is immobilized on microtiter plates and another horse radish conjugated monoclonal antibody (6303). The sensitivity of the assay was 0.1 ng/ml and intra- and inter-assay coefficients of variation were less than 5% and 5~7%, respectively.

Western ligand blotting

Three to four μl of samples were electrophoresed on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel under non-reducing conditions[10] except protein Mr standards (Rainbow Marker, Amersham Japan, Tokyo). After electrophoresis, proteins were transferred onto nitrocellulose membranes (0.45 μM pore size) in a transblot cell (Bio-Rad Laboratories, Richmond, CA) overnight at 70 V in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol). The nitrocellulose was blocked successively with 3% Nonidet P-40, 1% BSA and 0.1% Tween-20 in Tris-saline (pH 7.4) and incubated with a mixture of 0.6×10^6 cpm [^{125}I]IGF-I and IGF-II (Amersham Japan, Tokyo) for 24 hr at 4°C. The membranes were washed and visualized by autoradiography according to the method of Hossenlopp et al[9]. Serum from non pregnant woman was also analyzed with cord sera by ligand blot as control.

Densitometric analysis

Autoradiographs of Western ligand blots scanned and analyzed using a computer program (NIH Image). The integrated area under the absorbance curves were measured for each band and relative amounts of each band were culculated among fetuses.

Data analysis

The results from IGF-I and IGFBP-1 assay were expressed as the mean ± SE. The correlations among IGF-I, IGFBP-1, gestational weeks and birth weight were determined after fitting data to four parameter logistic equation. Since dispersion in each group was equaled by Bartlett test, a Student's t test was used to deter-

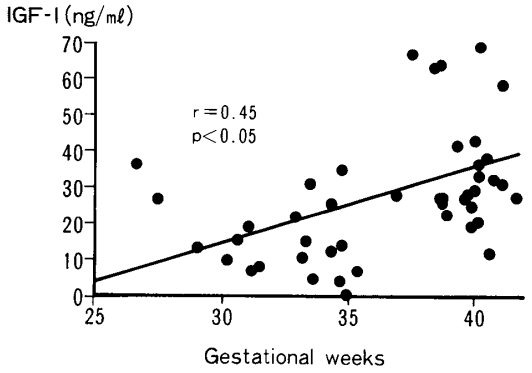

Fig. 1 Levels of IGF-I in cord sera during pregnancy

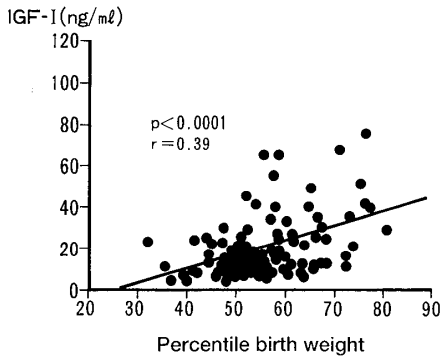

Fig. 2 Relationship between levels of IGF-I in cord sera and percentile birth weight

mine statistically significant differences as indicated in Fig. 3 and the Table.

Results

IGF-1 and IGFBP-1 in cord sera

Levels of IGF-I in cord sera increased along with gestational age (n=43, r=0.45, p<0.05) (Fig. 1) and correlated with percentile birth weight (n=43, r=0.39, p<0.0001) (Fig. 2). In contrast, levels of IGFBP-1 in cord sera decreased along with gestational age in which levels at 25~32 weeks, 33~36 weeks and 37~40 weeks were 552 ± 48 ng/ml (n=8), 387 ± 23 ng/ml (n=10, p<0.05 vs 25~32 weeks) and 118 ± 8 ng/ml (n=22, p<0.0001 vs 33~36 weeks), respectively (Fig. 3) and inversely correlated with percentile birth weight (n=40, r=−0.44, p<0.05) (Fig. 4).

Western ligand blot analysis of cord sera

Ligand blot of cord sera from AGA fetuses at term showed molecular forms of 42, 39, 34, 30 and 24 kDa that were similar to IGFBPs profile observed in non pregnant woman (Fig. 5). The 42/39 kDa doublet, 34, 30 and 24 kDa bands correspond to IGFBP-3, the main serum carrier of IGFs in the adult serum, IGFBP-2, IGFBP-1, and IGFBP-4, respectively from previous report[10]. In sera from LGA fetuses at term, the IGFBP-3 was remarkably reduced by 65% of term AGA, while IGFBP-2 was elevated that of 186% of term AGA compared to that from AGA fetuses at term. HGA fetuses at term from mother with diabetes mellitus revealed increased IGFBP-3 (127% of term AGA) with reduced IGFBP-2 (73% of term AGA) and IGFBP-1 (53% of term AGA).

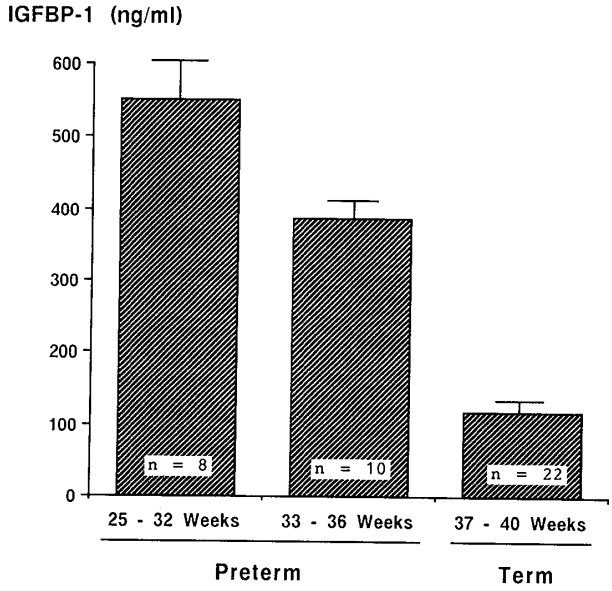

Fig. 3 Levels of IGFBP-1 in cord sera during pregnancy

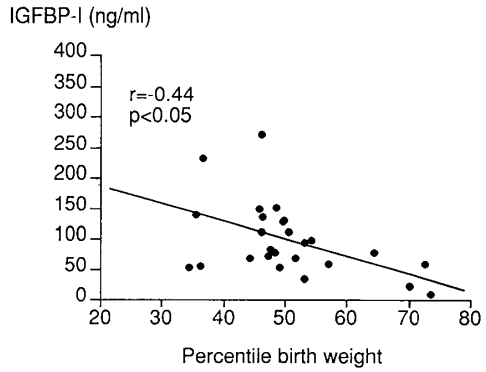

Fig. 4 Relationship between levels of IGFBP-1 in cord sera and percentile birth weight

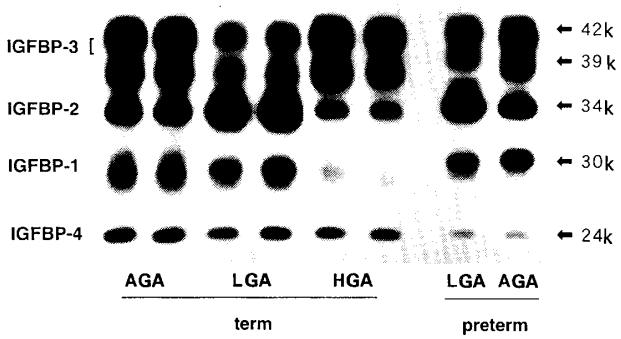

Fig. 5 Autoradiograph of IGFBPs in cord sera from fetuses at term and preterm analyzed by Western ligand blotting

In sera from LGA fetuses at preterm, the IGFBP-2 showed remarkable increase to 129% of preterm AGA, while other bands didn't show any difference between AGA and LGA fetuses at preterm (Table).

Discussion

Levels of IGF-I increased along with gestation and correlated with birth weight[12] although levels are quite lower[13] compared to those in the maternal circulation. It suggests that fetal IGF-I is involved in fetal growth as well maternal IGF-I. We confirmed the result of previous report that fetal IGFBP-1 levels were negatively correlated with birth weight[14]. Since IGFBP-1 inhibits IGF-I action in many cell culture systems[6][7][15] by inhibiting IGF-I binding to its receptor[16], IGFBP-1 is presumed to inhibit IGF-I action in fetus thereby inhibiting fetal growth.

Western ligand blot revealed four IGFBPs in cord sera as previously reported[17]. Profiles of IGFBPs in fetal sera are quite different from those in maternal sera, since there is no protease activity in cord sera while all IGFBPs except IGFBP-1 are proteolyzed in maternal sera[18]. Levels of IGFBP-1 in cord sera decreased along with gestational weeks[19]. Both levels of IGFBP-1 in cord sera and in maternal sera are found to be negatively correlated with fetal growth[5] suggesting that IGFBP-1 has inhibitory effect on fetal growth. In a animal experiment, fetal rats with IUGR by maternal starvation express higher levels of IGFBP-1 mRNA in liver, major production site of IGFBP-1 in fetus[19]. Therefore, the increase in IGFBP-1 observed in fetus with IUGR is controlled by transcriptional level. Similarly, intrauterine growth retarded rat fetus produced by the ligation of uterine artery or administration of dexamethason to maternal rat also revealed increased levels of IGFBP-1 in fetal rat, it suggests that the increased IGFBP-1 levels are universal event in growth retarded fetus as observed in human fetus and that suggesting the existence of fetal down regulation of growth by IGFBP-1. In contrast to low levels of IGF-I in the fetal circulatin, levels of IGFBP-1 in the fetus are high compared to adult levels[5][20]. Fetus shows remarkable growth in utero. However, low levels of IGF-I and high levels of IGFBP-1 in the fetal circulation suggest that growth promoting activity of IGF-I in fetus is limited. Recently, phosphorylated isoforms of IGFBP-1 were found in various biological fluids[21]. One non-phosphorylated and four to five phosphorylated IGFBP-1 are identified by non-denaturing gel electrophoresis and anion exchange chromatography[22] by which phosphoisoforms are separated based on the degree of phosphorylation.

Table Relative amounts of each IGFBP

		IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	
Term AFD	(n=6)	19.2 ±0.9	21.6±0.9	53.0±1.5	5.6±0.8	%
Term LFD	(n=3)	18.15±0.6	40.2±1.4*	34.4±0.7*	7.3±0.2	
Term HFD	(n=8)	10.1±0.9*	15.8±1.1*	67.1±0.9*	7.2±0.7	
Preterm AFD	(n=4)	22.7±1.6	21.3±0.4	50.0±2.7	4.0±0.3	
Preterm LFD	(n=4)	22.4±0.4	27.5±0.7**	46.2±5.1	4.1±1.1	

*$p<0.01$ vs. term AFD, **$p<0.05$ vs. preterm AFD

Our laboratory recently reported that the levels of non-phosphorylated IGFBP-1 were similar between AGA and LGA fetus at term. However, phosphorylated isoforms of IGFBP-1 were increased in LGA fetus compared to those of AGA fetus, and the proportion of non-phosphorylated IGFBP-1 to total IGFBP-1 was lower in LGA fetus than those in AGA fetus[23]. Furthermore, phosphorylated IGFBP-1 inhibits while non-phosphorylated IGFBP-1 enhances IGF-I action in terms of amino acid uptake by cultured trophoblast cells[24]. It is therefore possible that the fetus can show remarkable growth in a low IGF-I environment due to the presence of predominantly a non-phosphorylated form of IGFBP-1 and that IGFBP-1 has more suppressive effect on IGF-I action in LGA fetus. Under the circumstances of low IGF-I levels and "suppressive" IGFBP-1, IGF-I activity is presumed to be suppressed in these fetuses.

The recent studies suggest that the existence of glycosylated IGFBP-4 that is observed as the 28 kDa band in Western ligand blot[25]. The possibility of elevated levels of IGFBP-4 can not be excluded in this study although there is no evidence that the cord sera contain glycosylated IGFBP-4.

As observed in this study, serum levels of IGFBP-2 are also elevated in the fetus with IUGR as well as IGFBP-1 in both preterm and term fetuses. Similar IGFBP-2 levels were reported in term fetus with IUGR[17]. In addition, we reported in this study similarly elevated IGFBP-2 levels in preterm fetus with IUGR for the first time. IGFBP-2 was shown to inhibit IGF-I action in several cultured cell systems[26] and may have similar inhibitory effect on fetal growth as seen in phosphorylated IGFBP-1 in these fetuses. However, there is no report concerning phosphoisoforms of IGFBP-2. Further studies are required to elucidate the physiological role of IGFBP-2 in fetal growth.

Levels of IGFBP-3 was demonstrated to be increased in HGA at term compared to these in AGA as LGA. This result coincides with the results of Giudice et al[27] although they analyzed HGA fetuses from mothers with non diabetes mellitus while we analyzed HGA fetuses from mothers with diabetes mallitus. We expected different IGFBPs profiles in fetus with HGA from mother with and without diabetes mellitus. Our results suggest that fetus with HGA reveales charasteristic IGFBPs profiles regardless of pathophysiology of HGA.

Levels of IGFBP-3 are the largest among other IGFBPs in the circulation and IGFBP-3 shows the highest affinity for IGFs than other IGFBPs[28]. Therefore, 80% of IGFs are bound to this IGFBP-3 in the circulation[29], and considered as storage site of IGFs in the circulation.

IGFBP-3 is positively controlled by growth hormone (GH) in adult[30]. Levels of GH are higher on the fetus at preterm compared to adult levels and the levels decrease with gestational age[31]. Since fetus dose not express receptor for GH before 26 weeks in fetal circulation[32], increase in IGFBP-3 levels may be controlled by GH. Although there is no evidence that fetal GH levels correlated with fetal growth, low levels of IGFBP-3 in LGA fetus at term and preterm can be explained by immature GH response system in these fetus.

Conclusion

Levels of IGF-I in cord sera increased along with gestational age, and correlated with percentile birth weight. In contrast, levels of IGFBP-1 in cord sera decreased along with gestational age, and inversely correlated with percentile birth weight.

We analyzed IGFBPs in the fetal sera by Western ligand blot. In term delivery, cord sera from AGA fetuses revealed two major bands of 42 kDa and 39 kDa corresponding to IGFBP-3 and three minor bands of 34 kDa (IGFBP-2), and 30 kDa (IGFBP-1) and 24 kDa (IGFBP-4). Sera from LGA fetuses showed diminished IGFBP-3 with increased IGFBP-2 and IGFBP-1 compared to those of AGA.

In contrast, increased IGFBP-3 with decreased IGFBP-2 and IGFBP-1 was observed in

HGA fetuses from diabetic mothers.

In preterm delivery, IGFBP-2 was markedly increased in fetuses with LGA compared to AGA at preterm. Thus, the profiles of IGFBPs in the fetus vary corresponding to fetal growth, suggesting that not only IGF-I but also these IGFBPs are involved in fetal growth by modulating IGF-I action.

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インシュリン様成長因子とその結合蛋白

—胎児発育に及ぼす生理学的役割について—

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IGF-I およびIGFBP-1の胎児発育に及ぼす生物学的特徴を検討するために，臍帯血中IGF-I およびIGFBP-1をIRMA およびELISA で測定した．臍帯血中IGF-I のレベルは妊娠週数とともに増加し，またpercentile birth weight とも正の相関を示したのに対し，IGFBP-1の臍帯血中濃度は妊娠週数とともに減少し，percentile birth weight とも逆相関した．これらのことより，IGFBP-1は，胎児発育に負に働いていると考えられた．IGFBP-1を含むIGF 結合蛋白の動態を更に臍帯血のWestern ligand blot を用いて分析した．正期産の正常発育児（appropriate-for-gestational-age（AGA）児）の臍帯血中には42/39，34，30，24kDa の4 つのIGF に対する結合活性が認められた．これらはそれぞれ，IGFBP-3，IGFBP-2，IGFBP-1，IGFBP-4と考えられた．これに対して，正期産の胎内発育遅延児（light-for-gestational-age（LGA）児）では，IGFBP-3の減少と，IGFBP-2の増加が認められた．逆に，糖尿病を合併した母体から生まれた正期産巨大児（heavy-for-gestational-age（HGA）児）ではIGFBP-3の増加とIGFGBP-2，IGFBP-1の減少を認めた．早産LGA 児の臍帯血中においては，早産児AGA と比較してIGFBP-2が著明に増加していた．これらの結果から，IGF-I だけではなく，IGFBP もIGF の活性を修飾することによって胎児発達に重要な役割を果たしていることが示唆された．