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Abstract

Our purpose was to investigate developmental alterations of human alpha-fetoprotein (AFP) oligosaccharides in maternal serum by lectin affinity electrophoresis and to compare the AFP glycoforms in maternal serum with those in umbilical cord serum and amniotic fluid. AFP glycoforms were separated by affinity electrophoresis with concanavalin A (Con A), lentil lectin (LCA), erythroagglutinating phytohemagglutinin (E-PHA) and Allomyrina dichotoma lectin (allo A) and detected by sensitive antibody affinity blotting. In maternal serum, increased proportions of Con A-nonreactive AFP (AFP-C1), LCA strongly-reactive AFP (AFP-L3) and E-PHA-reactive AFP (AFP-P4 and AFP-P5) decreased gradually during the early gestational weeks. Allo A-nonreactive AFP (AFP-A1 and asialo-AFP) were found only in amniotic fluids during early gestational weeks. The percentages of these glycoforms at full term were almost the same among those body fluids. Since the glycoforms of maternal serum AFP were close to those of umbilical cord serum AFP, lectin-affinity electrophoretic analysis of maternal serum AFP may be useful for evaluating the developmental state of fetus by examining the nature of AFP sugar chain.

KEYWORDS: alpha-fetoprotein, affinity electrophoresis, lectin, maternal alpha-fetoprotein

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Developmental Alterations in the α-Fetoprotein Sugar Chain in Maternal Serum Analyzed by Lectin Affinity Electrophoresis

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Our purpose was to investigate developmental alterations of human α-fetoprotein (AFP) oligosaccharides in maternal serum by lectin affinity electrophoresis and to compare the AFP glycoforms in maternal serum with those in umbilical cord serum and amniotic fluid. AFP glycoforms were separated by affinity electrophoresis with concanavalin A (Con A), lentil lectin (LCA), erythroagglutinating phytohemagglutinin (E-PHA) and *Allomyrina dichotoma* lectin (allo A) and detected by sensitive antibody affinity blotting. In maternal serum, increased proportions of Con A-nonreactive AFP (AFP-C1), LCA strongly-reactive AFP (AFP-L3) and E-PHA-reactive AFP (AFP-P4 and AFP-P5) decreased gradually during the early gestational weeks. Allo A-nonreactive AFP (AFP-A1 and asialo-AFP) were found only in amniotic fluids during early gestational weeks. The percentages of these glycoforms at full term were almost the same among those body fluids. Since the glycoforms of maternal serum AFP were close to those of umbilical cord serum AFP, lectin-affinity electrophoretic analysis of maternal serum AFP may be useful for evaluating the developmental state of fetus by examining the nature of AFP sugar chain.

Key words: α-fetoprotein, affinity electrophoresis, lectin, maternal α-fetoprotein

Human α-fetoprotein (AFP) is an oncofetal glycoprotein, and its carbohydrate structure consists principally of biantennary complex-type oligosaccharides linked to asparagine. The sugar chain heterogeneity of human AFP, or glycoforms, in fetal sera was first demonstrated by Smith and Kelleher (1) in 1973 using lectin affinity chromatography. Amniotic fluids have high levels of AFP and their sugar chain alterations have been extensively studied by Kelleher *et al.* (2), Mackiewicz *et al.* (3), Ishiguro *et al.* (4, 5), Nørgaard-Pedersen *et al.* (6) and Toftager-Larsen *et al.* (7-9). These researchers analyzed AFP glycoforms by column affinity chromatography (2).

In their studies, it was not necessarily easy to analyze the glycoforms of low levels of AFP in early gestational stages because of the limited sensitivity of their method in detecting separated AFP glycoforms. Taketa *et al.* (10, 11) developed a sensitive method of antibody-affinity blotting for the detection of AFP glycoforms separated by lectin affinity electrophoresis. In the present study, we adopted the sensitive method of antibody-affinity blotting coupled with immunoenzymatic amplification for the detection of separated AFP bands in samples with low levels of AFP, such as those of maternal serum in early stages of gestation. This allowed us to see the sugar chain alterations which were not studied by the above-mentioned authors. In addition to commonly used lectins, such as concanavalin A (Con A) or lentil lectin (LCA), we also used erythroaggluting phytohemagglutinin (E-PHA) and *Allomyrina dichotoma* lectin (allo A), which have different sugar specificities.

Materials and Methods

Maternal sera, amniotic fluids and umbilical cord sera were obtained at the time of dilatation and curettage, amniocentesis, caesarean section or vaginal delivery from normal pregnant women at between the 6th to the 42nd gestational weeks after informed consent was obtained. These samples were kept frozen at $-25^{\circ}C$ until analysis.

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Glycoforms of AFP in amniotic fluids and sera were separated by affinity electrophoresis with lectins and separated AFP bands were detected by antibody-affinity blotting followed by immuno-enzymatic amplification (10, 11). Briefly, AFP samples were diluted with barbital/barbital-Na buffer (ionic strength 0.025, pH 8.6) to give an AFP concentration of 100 ng/ml. Affinity electrophoresis was carried out by applying 4 ml of the diluted samples to troughs (5.0 × 0.8 × 1.0 mm) in 1.0 mm thick agarose gels (Agarose M; Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) containing 1.0 mg/ml ConA (Pharmacia Fine Chemicals, Uppsala), 0.2 mg/ml LCA (Seikagaku Corp., Tokyo), 0.3 mg/ml E-PHA (Seikagaku Corp.) or 0.3 mg/ml allo A (Cosmo Bio Corp., Tokyo). Electrophoresis was run at 10°C by giving a constant voltage of 15 V/cm until free bromophenol blue migrated 55 mm from the origin. Separated AFP bands were detected by antibody-affinity blotting as follows: the gels were overlaid with antibody-precoated nitrocellulose (NC) membranes and with filter paper pads to transfer the AFP bands by capillary blotting to the NC membranes. The antibody-precoated NC membranes were prepared by immersing NC membrane into a solution of mouse anti-human AFP monoclonal antibody (NB-011, Nippon Bio Test Laboratories, Tokyo) diluted in Tris-buffered saline (TBS: 20 mM Tris-HCl, pH 7.5, 500 mM NaCl) to yield a concentration of 100 mg/ml and by fixing with a vapor of 25 % glutaraldehyde for 30 min, followed by neutralization. The transferred AFP on NC membranes was visualized by treating the transfers with rabbit anti-human AFP antibodies (DAKO, Copenhagen, Denmark) diluted 1,000-fold with 1.0 % gelatin-containing TBS, followed by horseradish peroxidase-labeled goat antibodies against rabbit IgG (Bio-Rad Laboratories, Richmond, VA, USA) diluted 1,000-fold with 1.0 % gelatin-containing TBS. Membranes were washed twice each for 5 min for every step of the treatments. The peroxidase-labeled antibody-treated membranes were stained by the tetrazolium method (11) for the peroxidase reaction.

Intensities of stained AFP bands were quantitated by scanning with a densitometer Model 1650 (Bio-Rad Laboratories) after immersing the developed and dried NC membranes in decalin. The intensities of AFP bands were expressed as percentages of total AFP bands. The AFP bands were identified by the nomenclature system of Taketa *et al.* (12); namely, major AFP bands were numbered consecutively from the anode, giving the lowest Arabic numeral 1 to the most anodic band, and the numerals were suffixed to capitalized initial letters of the lectin used. Minor or infrequently appearing bands were identified by adding "s" for slow-migrating and "f" for fast-migrating bands relative to the major bands.

Results

Patterns of AFP bands separated by affinity electrophoresis with 4 lectins for maternal serum, amniotic fluid and umbilical cord serum are shown in Figs. 1–4. Con A separated AFP into 2 bands: Con A-nonreactive AFP (AFP-C1) and -reactive AFP (AFP-C2), the latter being slightly broader. LCA separated AFP into 3 bands: LCA-nonreactive AFP (AFP-L1), weakly-reactive AFP (AFP-L2) and strongly-reactive AFP (AFP-L3). E-PHA separated AFP into 5 sharper bands: AFP-P1, AFP-P2, AFP-P3f, AFP-P4 and AFP-P5 in the order of increased reactivity. Allo A separated AFP into 3 bands: allo A-nonreactive AFP (AFP-A1), intermediate AFP (AFP-A2), strongly-reactive AFP (AFP-A3) and AFP-A1s, which was migrating between AFP-A1 and AFP-A2.

The total AFP levels in maternal serum ranged from 12.8 ng/ml to 290.9 ng/ml. The total AFP levels in amniotic fluid ranged from 2.3 ng/ml to 14,560 ng/ml. And the total AFP levels in umbilical cord serum ranged from 68,600 ng/ml to 1,441,600 ng/ml. These AFP levels were essentially identical with those previously reported. The results of densitometric scanning of these AFP bands for maternal serum, amniotic fluids and umbilical cord serum of different gestational weeks are summarized in Figs. 5–9. The proportions of AFP-C1 are plotted against the gestational week in Fig. 5. The highest proportion of AFP-C1 band intensity was observed for amniotic fluid followed by maternal serum in early gestational weeks. The percentage of AFP-C1 in maternal serum decreased from 15 % in the 11th week to barely detectable levels after the 24th week. No AFP-C1 band was detected in umbilical cord serum when it was analyzed in the 15th gestational week.

The proportions of AFP-L2 and AFP-L3 are plotted against the gestational week in Fig. 6. In maternal serum, the AFP-L2 band was not detected. The proportion of AFP-L3 in maternal serum decreased from 70 % in the 11th week to nearly zero after the 24th gestational week. The proportion of AFP-L3 in umbilical cord serum decreased in a manner similar to that in maternal serum or amniotic fluid. The proportion of AFP-L3 in amniotic

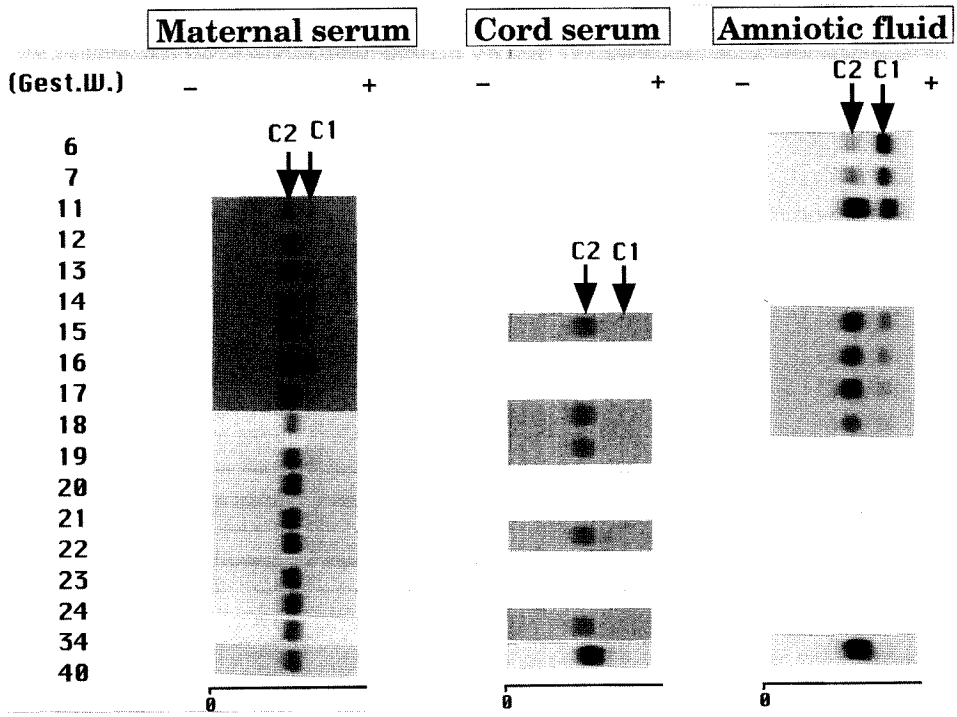

Fig. 1 Patterns of α-fetoprotein bands separated by affinity electrophoresis with concanavalin A and detected by antibody-affinity blotting for maternal sera, amniotic fluids and umbilical cord sera at different gestational weeks (Gest. W.).

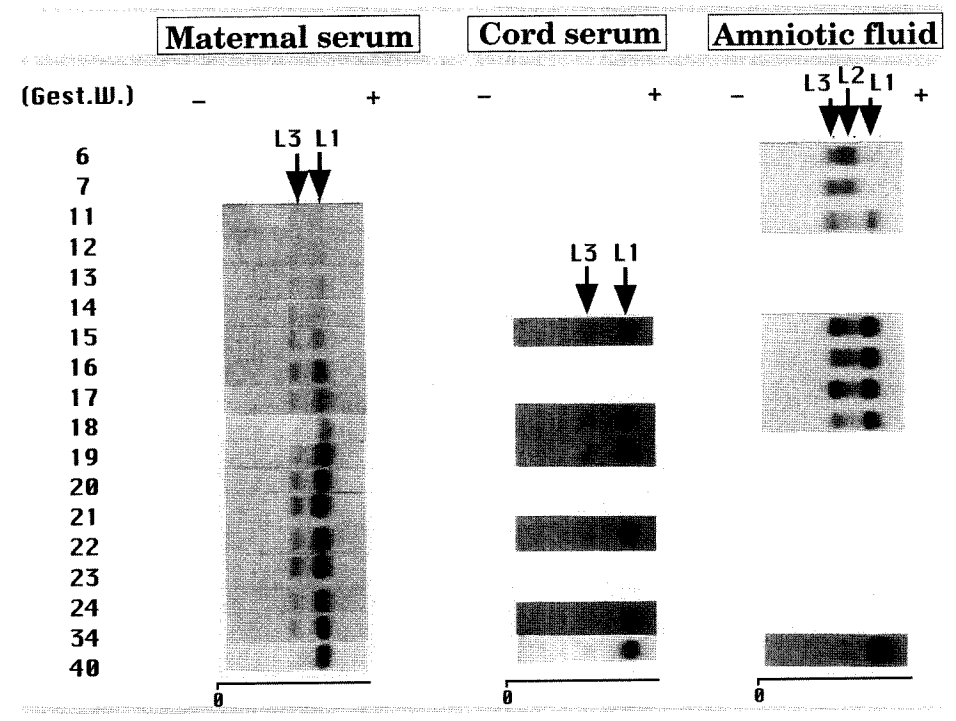

Fig. 2 Patterns of α-fetoprotein bands separated by affinity electrophoresis with lentil lectin and detected by antibody-affinity blotting for maternal sera, amniotic fluids and umbilical cord sera at different gestational weeks (Gest. W.).

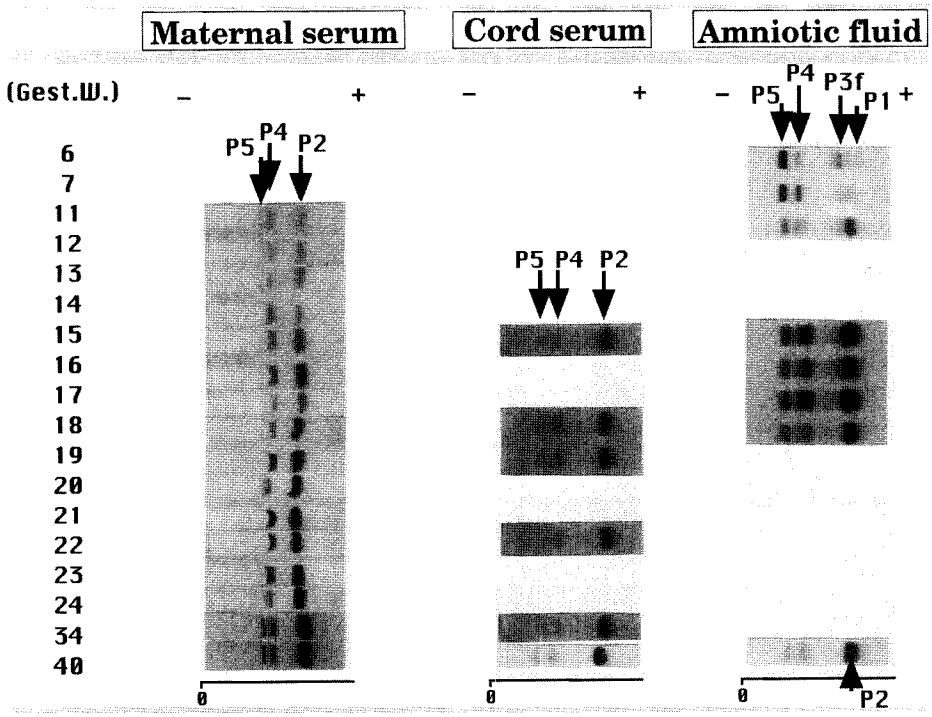

Fig. 3 Patterns of α-fetoprotein bands separated by affinity electrophoresis with erythroagglutinating phytohemagglutinin and detected by antibody-affinity blotting for maternal sera, amniotic fluids and umbilical cord sera at different gestational weeks (Gest. W.).

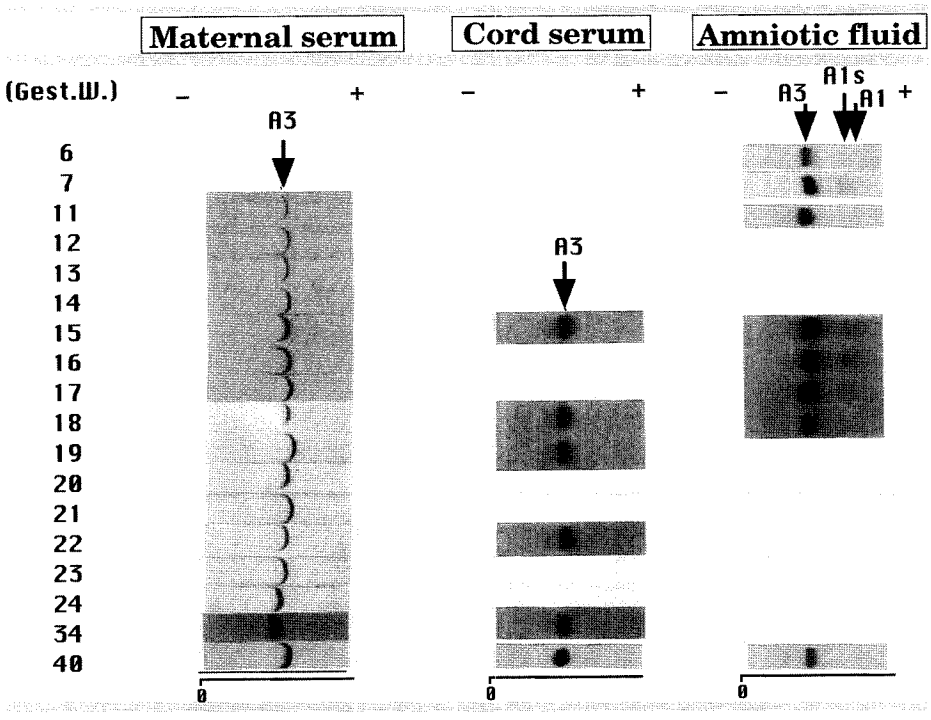

Fig. 4 Patterns of α-fetoprotein bands separated by affinity electrophoresis with *Allomyrina dichotoma* lectin and detected by antibody-affinity blotting for maternal sera, amniotic fluids and umbilical cord sera at different gestational weeks (Gest. W.).

fluid was 98 % in the 6th gestational week and disappeared around the 18th gestational week and the proportion of AFP-L2 in amniotic fluid reached a maximum of 45 % in the 7th–11th gestational weeks and then decreased with advancing gestation.

The proportions of AFP-P3f, AFP-P4 and AFP-P5 are plotted against the gestational week in Figs. 7 and 8. AFP-P3f was observed only in the amniotic fluid from the 6th (22.5 %) to the 11th (7.6 %) weeks. The proportion of AFP-P4 in amniotic fluid decreased from 32 % to 9 % with increasing gestational weeks with a peak observed around the 7th gestational week. The proportion of AFP-P5 in amniotic fluid was as high as 60 % in the 6th week and gradually decreased to 5 % near term. The proportion of AFP-P4 and AFP-P5 in umbilical cord serum increased transiently reaching a maximum of 25 % for AFP-P4 in the 22th week and a maximum of 14 % for AFP-P5 in the 19th week. In maternal serum, the

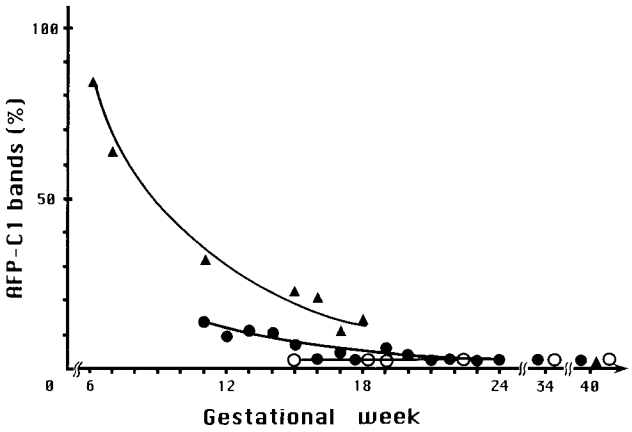

Fig. 5 Percentages of Con A-nonreactive α-fetoprotein (AFP-C1) band of maternal sera (●), amniotic fluids (▲) and umbilical cord sera (○) against gestational week of pregnancy.

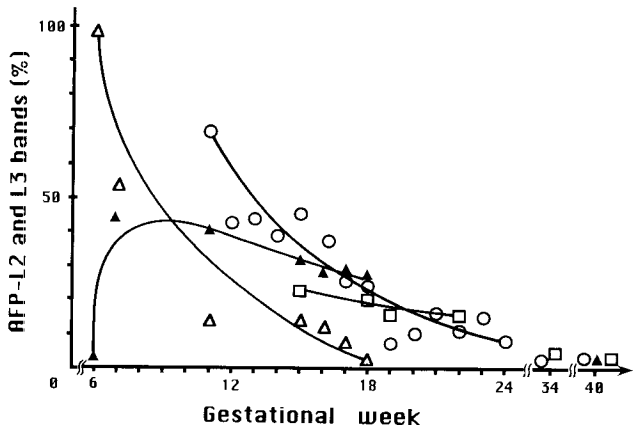

Fig. 6 Percentages of lentil lectin (LCA) strongly-reactive α-fetoprotein (AFP-L3) bands of maternal sera (○), amniotic fluids (△) and umbilical cord sera (□) and of LCA weakly-reactive AFP (AFP-L2) in amniotic fluid (▲) against gestational week of pregnancy.

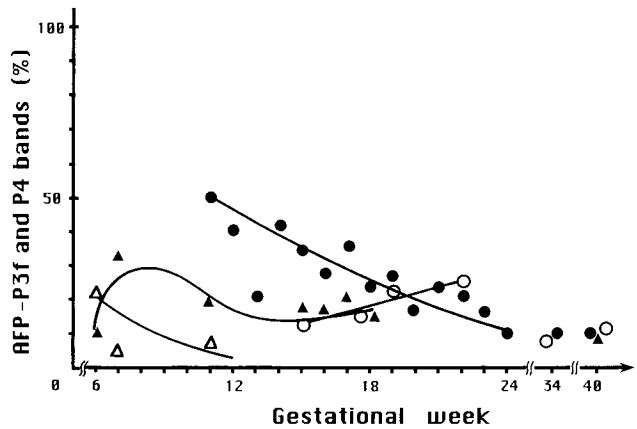

Fig. 7 Percentages of erythroagglutinating (AFP-P4) bands of maternal sera (●), am-niotic fluids (▲) and umbilical cord sera (○) and of AFP-P3f in amniotic fluid (△) against gestational week of pregnancy.

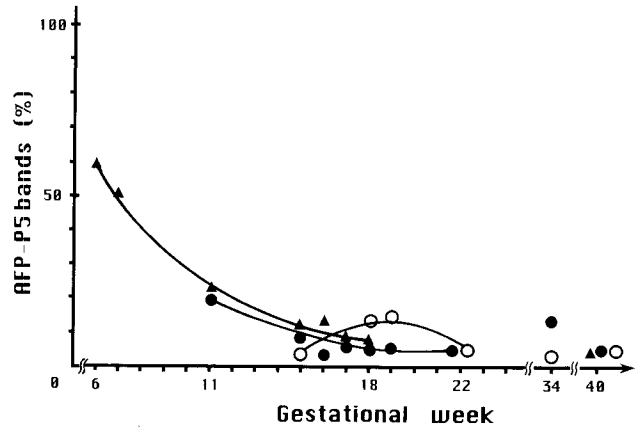

Fig. 8 Percentages of phytohemagglutinin-reactive α-fetoprotein (AFP-P5) bands of maternal sera (●), amniotic fluids (▲) and umbilical cord serum (○) against gestational week of pregnancy.

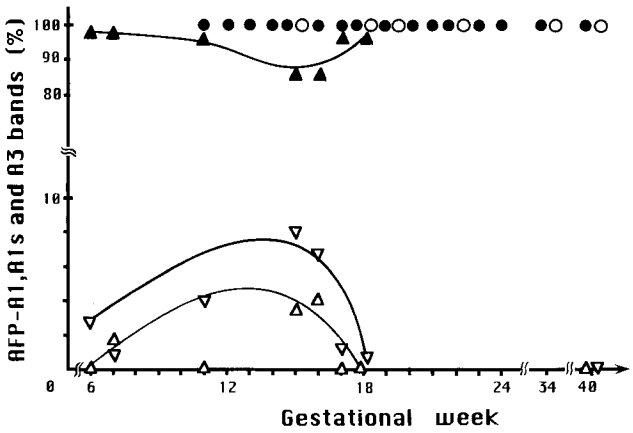

Fig. 9 Percentages of allo A strongly-reactive α-fetoprotein (AFP-A3) bands of maternal sera (●), amniotic fluids (▲) and umbilical cord sera (○) and of allo A-nonreactive AFP (AFP-A1) and A1s bands in amniotic fluid against gestational week of pregnancy. ●: AFP-A3 of maternal serum; ▽: AFP-A1 in amniotic fluid; △: AFP-A1s in amniotic fluid.

proportion of AFP-P4 decreased from 50 % in the 11th gestational week to 10 % at full term. The proportion of AFP-P5 decreased from 20 % in the 11th gestational week to 8 % at full term.

The proportions of AFP-A1, AFP-A1s and AFP-A3 are plotted against the gestational week in Fig. 9. In amniotic fluid, AFP-A1 was observed until the 16th week (0.9–8.3 %) and AFP-A1s was present in the 7th (1.9 %), 15th (3.3 %) and 16th (4.2 %) weeks. In maternal and umbilical cord sera, only AFP-A3 was detected.

Discussion

AFP is a fetal glycoprotein and is produced by several fetal organs, primary among which are the liver and yolk sac. The increased levels of AFP in maternal serum during pregnancy are considered to derive from the fetus by passing through the placental barrier. Thus, the direct source of maternal AFP would be the AFP in umbilical cord serum. Consequently, umbilical cord serum and maternal serum AFPs should have similar glycosylation, assuming that there is no preferential passage of AFPs with different sugar chains. This hypothesis has been tested with a less sensitive technique, providing no concrete evidence or definite conclusion.

In the present study, direct comparison of AFP glycoforms in maternal and umbilical cord sera were made possible by means of a sensitive detection method of antibody-affinity blotting of AFP glycoforms separated by affinity electrophoresis with lectins. The gestational week-dependent decrease in Con A-nonreactive AFP-C1 in amniotic fluid was as previously reported. Interestingly, maternal serum AFP-C1 showed a slightly higher proportion in comparison with that of cord serum AFP-C1 during the 15th–20th gestational weeks. However, there was no significant difference. There was a similar small difference in AFP-L3 between maternal and umbilical cord sera. On the other hand, AFP-L2 present in amniotic fluid never appeared in maternal or umbilical cord serum, indicating that there is no direct transfer of amniotic fluid AFP into the maternal circulation. However, umbilical cord serum AFP-P4 and -P5 showed entirely different pattern during the 15th–24th gestational weeks. The maternal serum had higher proportions of AFP-P4 and -P5 during early stages of gestation before about the 19th weeks. Here again, amniotic fluid-specific AFP-P3f was not detected in maternal or umbilical cord sera, supporting the contention that amniotic AFP does not directly diffuse into umbilical cord blood or maternal circulation. These results could be interpreted as indicating that AFP with different sugar chains may pass at different rates through the placental and maternal vessel barriers or may have different half-lives in maternal and fetal circulating blood.

Lectin-reactive profiles of AFP in malignancy (12) have been studied extensively without referring to the actual sugar chain alterations. Recent studies have revealed the correspondence between lectin reactivities and oligosaccharide structures of AFP (13–15).

Based on these results, it may be inferred that amniotic AFP has branched chain oligosaccharides or bisecting GlcNAc as shown by increased proportions of AFP-C1 (16) and AFP-L2 (17). Sialic acid α2–3 linked to the galactose of the mannose α1–6 arm of the biantennary structure seems present in amniotic fluid as inferred from the increased AFP-P5 (18). Fetal cord blood AFP in early gestational weeks (AFP-L3, AFP-P4 and AFP-P5) is characterized by increased proportions of fucosylated core GlcNAc and exposed galactose of the mannose α1–6 arm and α2–3 sialylated galactose of the mannose α1–6 arm of the biantennary sugar chain. Allo A-nonreactive AFP has not yet been characterized well for its oligosaccharide structure except that it has no α2–6 sialylated galactose of the mannose α1–3 arm (19).

Isoforms of human serum AFP and their corresponding sugar chain structures are shown in Fig. 10. AFP-P1 may be a carbohydrate-deficient or incompletely glycosylated AFP and AFP-P3f a monosialylated AFP with exposed N-acetylglucosamine of the mannose α1-3 arm (20). AFP-A1 has not yet been characterized well but probably lacks terminal galactose residues but retains negative charges.

Since the lectin reactivities of AFP in early gestational stages can also be seen in several malignant diseases, such as hepatocellular carcinoma, yolk sac tumors or gastrointestinal tumors, AFP can be regarded as an oncofetal glycoprotein not only with respect to its peptide but also to its carbohydrate moiety. This concept has

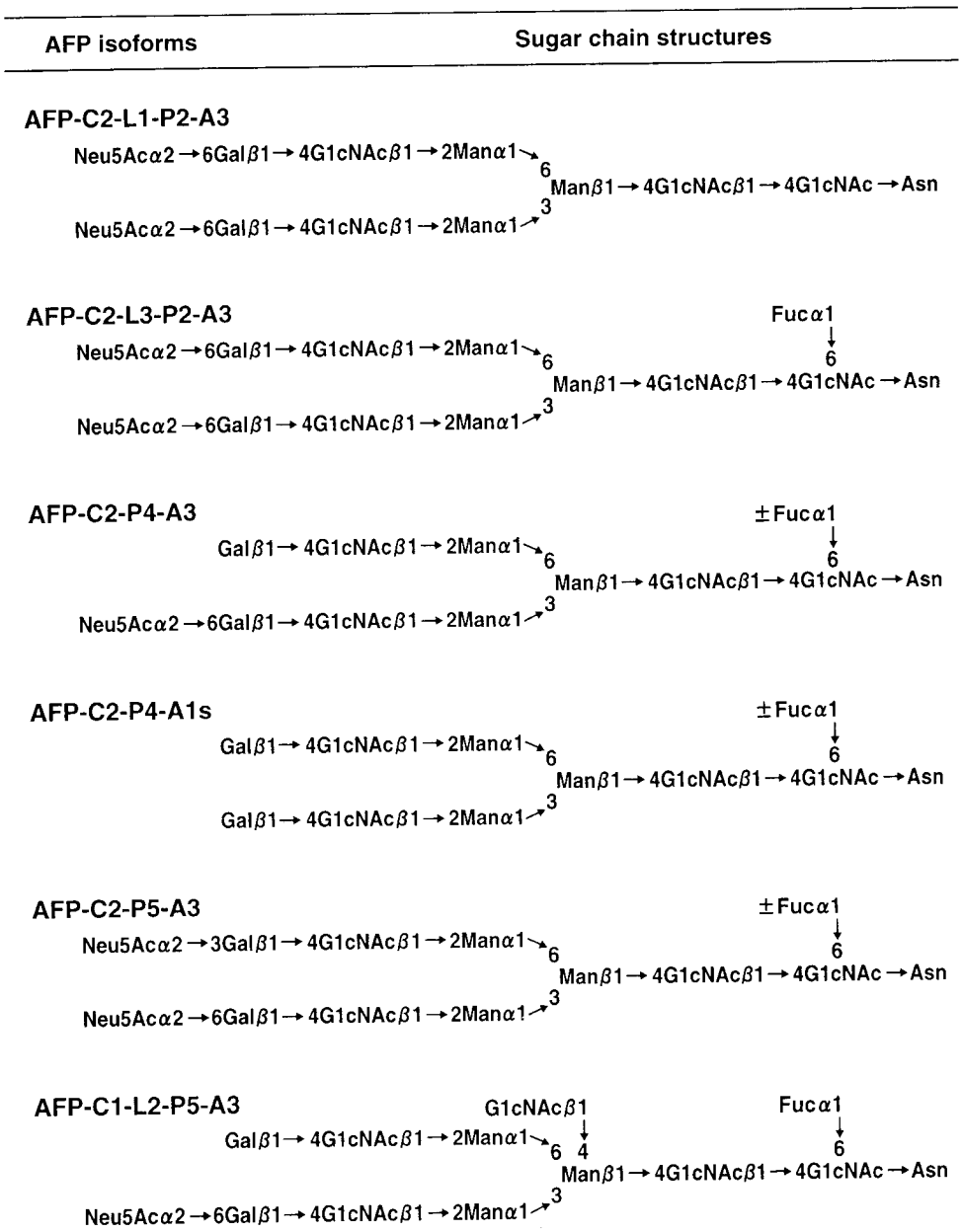

Fig. 10 Isoforms of human serum α-fetoprotein (AFP) and their corresponding sugar chain structures.

been proposed already for the appearance of AFP-L3 in terms of the differentiation of the liver (15). This can be now applied to other glycoforms of AFP originating from the yolk sac.

Recently, fetal organ AFP and maternal serum AFP have been analyzed (21, 22). The clinical significance of the present study may reside in the fact that the extent of fetal development with respect to AFP sugar chain can be monitored by analyzing the maternal serum for the lectin reactivity of AFP during pregnancy. Since the lectin-reactive patterns of AFP in the maternal serum are close to those of umbilical cord serum AFP, the maternal serum AFP glycoform may represent the maturation of the fetus or, more specifically, the differentiation of the liver (15). Accordingly, the analysis of maternal serum AFP glycoforms is potentially useful for the detection of malformation and other birth defects without having to perform amniocentesis.

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