Detection of the immunoregulator p43-placental isoferritin in the human embryo and fetus

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Human placental isoferritin (PLF) is known to exert an immunosuppressive activity in vitro and is involved in the down-regulation of the maternal immune system during pregnancy. We have investigated the presence of PLF in the human embryo and early fetus and its secretion into amniotic fluid (AF) and fetal blood. Immunohistochemistry was performed on 25 normal embryos and fetuses, at 7–22 weeks gestation, using the CM-H9 monoclonal antibody (mAb), generated specifically against the human p43-PLF protein. The amount of p43 was measured in AF of 81 fetuses at 11–22 weeks and in the blood of 19 fetuses at 15–22 weeks by means of an enzyme-linked immunosorbent assay with the same mAb. Positive p43-PLF immunostaining was found from 7 weeks gestation in proximal tubules of the primitive nephron and macrophages of the liver sinusoids, blood vessels and mesenchymal tissue. In the AF samples, p43-PLF was first detected at week 15 gestation and thereafter steadily increased with advancing gestation whereas in fetal blood, p43-PLF was below or just above the lower limit of the assay. The gap between the first appearance of 43-PLF in embryonic tissue and its secretion into the amniotic fluid is probably linked to maturation of the renal function. The detection of the p43-PLF immunomodulator protein in macrophages at a very early stage of embryonic development and its very low concentration in fetal blood suggests that its immunoregulatory role is limited to the feto–maternal interface.

Key words: p43-isoferritin/pregnancy/amniotic fluid/fetal macrophages/fetal kidneys

Introduction

Isoferritin from placental tissue (Brown et al., 1979), exerts an immunosuppressive activity (Matzner et al., 1985), and is involved in the down-regulation of the maternal immune system during pregnancy (Sirota et al., 1989). The placental isoferritin (PLF) serum concentration has been evaluated using the monoclonal antibody (mAb), CM-H-9, which binds exclusively to the 43 kD subunit (p43) of PLF (Moroz et al., 1987a). The serum p43-PLF concentration is very low in normal individuals but elevated serum p43-PLF concentrations (>10 U/ml) have been measured in the serum of normal pregnant women (Moroz et al., 1987a;b; Maymon and Moroz, 1996) as well as in patients with malignancies (Rosen, 1996), and in newly diagnosed insulin dependent diabetes mellitus (Assa and Moroz, 1990). Serum p43-PLF concentrations are not correlated with serum ferritin concentrations, indicating that the production of p43-PLF by maternal or fetal tissues is not related to maternal iron stores (Maymon and Moroz, 1996). Elevation of serum p43-PLF commences at the very beginning of pregnancy reaching around 55 U/ml during the third trimester of normal pregnancy. Low maternal serum p43-PLF concentrations are found in various pathological pregnancies, in particular in those associated with a defect of placentation (Maymon and Moroz, 1996; Rosen et al., 1996; Bar et al., 1998; Jauniaux et al., 2000).

We have recently shown that p43-PLF is detected in the syncytiotrophoblast, Hofbauer cells and decidual macrophages of the first trimester placenta (Maymon et al., 2000). The placental p43-PLF immunostaining decreases during the second trimester of pregnancy and from 15 weeks it is lower than detection limits in the syncytiotrophoblast. However, it continues to be expressed in Hofbauer cells and decidual macrophages until term pregnancy suggesting that this protein is mainly a marker of placentation (Maymon et al., 2000). The clinical manifestations of miscarriage, pre-eclampsia, intrauterine growth retardation and even gestational diabetes, may all be linked to a defect in placentation. A deficit in p43 (PLF) which is involved in pregnancy-related immunotolerance around implantation or during placentation may play a role in the pathogenesis of these diseases.

Contrary to the extensive data accumulated on p43-PLF during pregnancy in the maternal compartments, the presence and pattern of p43-PLF in the fetal compartments have not been previously reported. Therefore, the aim of this study was to evaluate its presence in the embryonic and fetal tissues during the first half of pregnancy. Furthermore, we investigated the secretion of p43-PLF by the embryo or
fetus, measuring its concentration in the amniotic fluid (AF) and fetal blood.

**Material and methods**

**Samples**

Intact embryos and fetal organs were collected from 25 healthy pregnant women undergoing elective termination of pregnancy at 7–22 weeks gestation for psycho-social reasons. Intact embryos were collected using low-pressure suction aspiration and under ultrasound guidance (Figure 1A). They were immediately fixed in 4% buffered formalin solution, and subsequently intact embryos at 12 weeks \((n = 13)\) were embedded in paraffin blocks. Following uterine evacuation of fetuses at 14 weeks gestation onward \((n = 12)\), the following organs: brain, thymus, liver, gut and kidneys were separated using a dissecting microscope, immediately fixed and embedded as previously described. All pregnancies were scanned before uterine evacuation and the fetal heart rate was within the normal range. Gestational age was determined from the menstrual history and confirmed by ultrasound measurements as well.

AF samples were obtained by amniocentesis from 81 women with singleton pregnancies at 11–22 weeks gestation and pure fetal blood samples were obtained before uterine evacuation by cardiopuncture from 19 singleton pregnancies at 15–22 weeks. All pregnancies were karyotypically normal with an anatomically normal fetus. Ultrasound dating was available in all cases. AF and fetal serum samples were stored under similar conditions \((-80^\circ C)\) at University College London (UCL), the Cytogenetic Services Laboratories Ltd, London, UK and Cytogenetic Laboratory, Assaf Harofe Medical Center, Israel, until

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**Figure 1.** (A) Intact conceptus at 8 weeks gestation subject to immunohistochemical p43-PLF analysis. Note the fetus, yolk sack and the placenta. Immunohistochemical staining of p43-PLF protein using CM-H9 antibody. \(G = \) glomeruli; \(P = \) proximal tubule; \(B = \) Bowman’s capsule. (B) Kidney of a 8 week old embryo. A portion of the mesonephric kidney showing well developed glomeruli and proximal tubules. The cytoplasm of the epithelial cells of the proximal tubules are p43 immunopositive. (C) Kidney of a 12 week old fetus. Strong immunoreactivity for p43 is demonstrated in the epithelial cells of the proximal tubules in the vicinity of a fully developed nephron. Immunoreactivity is noticed at the cells of the urinary pole of the Bowman’s capsule. The subcapsular renal cortex shows a layer of developing nephrons (nephrogenic zone) which are immunonegative for p43 (arrow). (D) Higher magnification of the glomeruli. (E) Expression of p43 in macrophages (arrows) in liver sinusoids of 8 week old embryo. (F) Detection of p43 in macrophages (arrows) in blood vessel of 8 week old embryo. \((B \text{ and } C) \) bar = 450 µm. \((D) \) bar = 225 µm. \((E \text{ and } F) \) bar = 150 µm.
assayed. Written informed consent was obtained from each woman before the procedures. This study was approved by UCL Hospitals Committee on the Ethics of Human Research.

**Immunohistochemistry**

Consecutive longitudinal sections (3 µm) were performed and every fifth section was immunohistochemically stained for p43-PLF. The sections were stained with CM-H9 mAb, generated specifically against human p43-PLF protein as previously described (Moroz et al., 1985). The sections, mounted on Super Frost/Plus glass (Menzel Glaser, Braunschweig, Germany), were processed by the labelled-streptavidin–biotin (LAB-SA) method using Histostain™ Plus Kit (Zymed, San Francisco, CA, USA), according to the manufacturer’s instructions. The sections were treated with 3% H2O2 for 5 min, followed by incubation with normal human serum for 10 min and subsequently incubated for 1 h with 1:150 dilution of CM-H9 mAb (1 mg/ml). Consecutive sections were incubated with anti CD-68 antibody (Zymed, San Francisco, USA; prediluted for histology). This monoclonal antibody (clone: KP1) reacts with CD-68 antigen (T9 antigen), a glycoprotein (110 kDa) which is expressed in macrophages in a wide variety of tissues (Pulford et al., 1989; Muhlemann et al., 1992; Wetzka et al., 1997). In addition, consecutive sections were stained routinely using haematoxylin and eosin. Control incubations were performed by substituting non-immune serum for the primary antibody. The biotinylated second antibody was applied for 10 min, followed by incubation with horseradish peroxidase conjugated streptavidin (HRP-SA) for 10 min. After each incubation, the slides were washed thoroughly with Optimax wash buffer (Biogenix, San Ramon, CA, USA). The immunoreaction was demonstrated using a HRP based chromogen/substrate system, including DAB (brown) chromogen (Liquid DAB Substrate Kit; Zymed, San Francisco, USA)). The sections were then counterstained with Harris’ hematoxylin, dehydrated in ascending alcohol concentrations, cleared with xylene, mounted and examined by light microscopy.

**Enzyme-linked immunosorbent assay (ELISA)**

p43-PLF concentration was measured in AF and fetal serum samples using the anti-human PLF CM-H-9 mAb, as described (Moroz et al., 1987a). The amount of p43-PLF that bound 250 pg alkaline phosphatase conjugated CM-H9 was arbitrarily defined as 10 IU of p43-PLF (Moroz et al., 1987b). Values <10 U/ml similar to those measured in 95% of healthy non-pregnant women (Moroz et al., 1987b) were considered below the normal range in pregnancy (Maymon and Moroz, 1996). Results were expressed as U/ml of AF or fetal serum. This cut-off level was adopted from serum assays based on receiver-operating characteristics constructed by plotting sensitivity versus specificity (Rosen et al., 1995). The inter- and intra-assay coefficients of variation were <5% (Maymon et al., 1998a).

**Results**

**p43-PLF immunostaining**

A positive immunostaining for p43-PLF was found in the kidneys and macrophages of human fetuses. At 7–9 weeks, there was a strong immunoreactivity in the mesonephric structures (Figure 1B). From 10 weeks, fetal renal tissue exhibited positive p43-PLF immunostaining in the proximal tubules of the metanephric nephrons where it could be found until 22 weeks gestation. The immunohistochemical staining was confined to the cytoplasm of cells of the proximal tubules. No specific immunoreaction was found in distal tubular cells or in Wolfian epithelial duct. In the metanephric kidney, the immunoreactivity was occasionally detected in a few cells of the urinary pole of Bowman’s capsule (Figure 1C,D). The nephrogenic zone (peripheral developing nephrons) was always negative for p43-PLF (Figure 1C) and no immunostaining was found in the nephrons which had developed to the stage of nephrogenic vesicle or S-shaped body.

There was a strong immunoreactivity for p43-PLF in macrophages (pleomorphic cells with round fusiform or stellate appearance, numerous cytoplasmic vacuoles of different sizes, granulated eosinophilic cytoplasm, and a large and dense basophilic nucleus). These cells were further characterised by immunohistochemistry using a specific antigen (CD-68 macrophage associate antigen) on alternate slides (Figure 2). These macrophages were demonstrated, throughout the period of gestation investigated, in embryonic liver sinusoids (Figure 1E), blood vessels (Figure 1F) and mesenchymal tissue (data not shown). These p43-PLF positive macrophages were abundant in embryos, whereas they were rarer in fetal organs.

**Figure 2.** Immunohistochemical staining of CD68 antigen (macrophage marker). Histological section of an 8 week old embryo. Positively stained macrophages are indicated by an arrow. (A) Glomeruli of the developing kidney. (B) Blood vessel. (C) Liver sinusoids. Bar = 150 µm.
serum p43-PLF concentrations of 12 and 19 U/ml were presented in Figure 3. The mean p43-PLF concentration measured in 50 samples of AF at 15–22 weeks gestation was 24.4 ± 15 U/ml (range 6–81). The mean fetal blood concentration was 4 ± 5 U/ml (range 0–19). Fetal serum p43-PLF concentrations of 12 and 19 U/ml were measured in two samples at 16–21 weeks gestation respectively.

Discussion

The present data indicate that p43-PLF is only detected in renal tissue and macrophages of the human embryo and fetus and that it is excreted mainly into the amniotic cavity from 15 weeks gestation. The p43-PLF immunoactivity exhibited in placental tissue has been found to vary with gestational age (Maymon et al., 2000). At 5–12 weeks gestation, p43-PLF immunostaining was uniformly strong in the villous syncytiotrophoblast. It becomes weak and variable at 13–15 weeks and is below detection after 15 weeks. The suppressive effect of PLF in vitro has been evaluated using maternal and fetal mixed lymphocyte cultures (Sirota et al., 1989). This study has shown that PLF suppresses the immunoreactivity of maternal lymphocytes to fetal alloantigens. Furthermore, the immunosuppressive effect of PLF is more pronounced in lymphocytes obtained from pregnant women exhibiting T-cells with a PLF receptor (Moroz et al., 1989; Sirota et al., 1989). The factors affecting the down-regulation of p43-PLF expression in placental trophoblastic tissue during the second trimester of pregnancy are still unknown.

The fetal kidneys originate from the ‘nephrogenic mesoderm’, where three nephronic excretory structures develop in quick succession: the pronephros, the mesonephros and the metanephros (Bostwick and Eble, 1997). The metanephros, which is the permanent kidney, appears during the seventh week of gestation and, theoretically, it begins to function at the end of the first trimester (Taylor, 1992; Bernstein et al., 1997). We found an 8 week period between the first demonstration of p43-PLF in the embryonic kidneys (at week 7 of gestation) and its appearance in the amniotic fluid (at 15 weeks gestational age). There is a direct relationship between AF composition and fetal kidney maturation (Gulbis et al., 1996). AF electrolyte composition, protein patterns and acid-base balance change rapidly at the end of the first trimester (Gulbis et al., 1992; Jauniaux et al., 1994). The development of nephrons starts around the beginning of the third month of gestation and the metanephros or definitive kidney produces urine from 10 weeks onwards. This correlates strongly with an abrupt AF increase in β2-microglobulin concentrations and can be linked with the establishment of glomerular filtration in the definitive fetal kidneys at a time when the tubular function is still limited (Gulbis et al., 1996). However, the cells of the proximal tubules may have the ability to reabsorb proteins even during early embryonic life. Lauriola et al. (1986) have demonstrated the capacity of human meso- and metanephric proximal tubule cells to absorb and filter various serum proteins such as endogenous lysozyme, α-1-antitrypsin and ferritin, which were detected in the mesonephric and metanephric proximal tubules, starting from the eighth week of gestation (Lauriola et al., 1986). This suggests that p43-PLF protein is progressively reabsorbed by these cells and accumulates inside them rather than being produced by the embryonic or fetal renal tissue itself.

The p43-PLF positively stained macrophages were found mainly in embryonic and fetal liver, blood vessels and mesenchymal tissue. The staining pattern was similar to that found in placental tissue where Hofbauer cells were found to be strongly positive for p43-PLF throughout gestation (Maymon et al., 2000). Hofbauer cells, which are considered to be placental macrophages, are numerous during the first half of pregnancy, then decrease in number and size as pregnancy progresses (Castelucci and Kaufmann, 1990). These cells are believed to have a capacity for movement along the villous cores of chorionic villi and thus function as a substitute for the lymphatic system allowing them to exert their host defence function (Castelucci and Kaufmann, 1990). An important indication of their fetal origin was drawn from the observation by Wyn (1967), which was based on sex chromatin staining. It is now accepted that Hofbauer cells are of chorionic mesenchymal origin during the early stages of pregnancy, before the fetal circulation is established. Later, the Hofbauer cells may originate from fetal bone marrow (Van Furth, 1982; Castelucci and Kaufmann, 1990) and they can be found inside the lumen of embryonic capillaries. Thus these cells may have different origins throughout gestation, representing a heterogeneous group of cells (Castelucci and Kaufmann, 1990). The detection of p43 (PLF) in fetal macrophages and placental Hofbauer cells may indicate a cross transfer possibility of these cells through the feto-maternal interface. This hypothesis is further supported by the morphological and immunohistochemical similarities between fetal macrophages and placental Hofbauer cells. Both cells are pleomorphic with round fusiform or stellate appearance, containing numerous cytoplasm vacuoles, and their nucleus is large and basophilic. They both express CD-68 macrophage-associated antigen (Pulford et al., 1989; Muhlemann et al., 1992; Wetzka et al., 1990).
1997), as well as p43-PLF, as demonstrated in the current study. Beside its function in immunotolerance, PLF may act as a growth regulator (Reinerova et al., 1993) or may affect cell differentiation (Sedlak et al., 1995), both of which are import for development of the conceptus.

During the second and third month of pregnancy the embryo and subsequently the fetus is surrounded by the amniotic cavity which is surrounded by the exocoelomic cavity containing the secondary yolk sac. During the first trimester, the amniotic membrane floats freely between the embryonic cavities. Despite its apparent anatomical simplicity (Jones and Jauniaux, 1995), direct transfer from the exocoelomic to the amniotic cavity via the amniotic membrane is limited and the AF contains very low concentrations of proteins (Gulbis et al., 1992, Jauniaux et al., 1993). The total AF protein concentration is respectively 50 and 900 times lower than in coelomic fluid and maternal serum. Almost all individual proteins, except α-fetoprotein, are in very low concentrations in the AF. The concentration of p43-PLF in AF was always lower in comparison with that in maternal serum from both normal and abnormal pregnancies (Moroz et al., 1987b; Maymon and Moroz, 1996; Maymon et al., 1998a). We have recently shown that the concentration of PLF is extremely low in the majority of coelomic fluid samples (Maymon et al., 1998b). We postulated that this is due to a lack of secretion of p43-PLF into the exocoelomic cavity or that this protein is rapidly degraded or coupled, thus rendering it nearly undetectable by the assay (Maymon et al., 1998b). The very low concentration of p43-PLF found in fetal serum, in the present study, using the same assay, supports the former hypothesis. This further suggests that p43-PLF is produced in very small concentrations by the fetal macrophages and is mainly eliminated from the fetal circulation via the kidneys without accumulating in any other fetal organs.

The early appearance of p43-PLF and its association with both embryonic/fetal macrophages and placental Hofbauer cells suggest that this protein may have an immunomodulatory function across the feto–maternal interface, starting at a very early stage of development. The very low p43-PLF concentration found in the exocoelomic cavity and in fetal serum and the very strong p43-PLF immunostaining of the syncytiotrophoblast during the first trimester suggest that the activity of this protein is directed towards the maternal compartment and is predominant during the early phases of placentation.

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