

Prostaglandin D₂ synthase: a component of human amniotic fluid and its association with fetal abnormalities

DIMITRIOS N. MELEGOS, HE YU, and ELEFTHERIOS P. DIAMANDIS*

Prostaglandin D₂ (PGD₂) synthase is responsible for PGD₂ production in the brain. Western blot analysis of human amniotic fluid and probing with a polyclonal antibody against prostate-specific antigen (PSA) revealed a strong immunoreactive band with a molecular mass of 25 kDa. The immunoreactive species, which does not react with monoclonal anti-PSA antibodies, was purified to homogeneity from 1 L of amniotic fluid through successive cycles of gel filtration and ion-exchange chromatography. Amino acid sequence analysis (15 cycles) revealed that the protein was highly homologous or identical to PGD₂ synthase. On semiquantitative analysis, PGD₂ synthase concentration appears to increase dramatically during gestational weeks 12-25 and then declines slowly until term. PGD₂ synthase concentration in amniotic fluid was altered in many abnormal pregnancies, most notably its decrease in trisomic fetuses and fetuses with renal abnormalities.

INDEXING TERMS: prostaglandin D₂ • fetal development • trisomy • prenatal diagnosis • lipocalins

Prenatal screening for fetal abnormalities is now achieved by measuring fetally derived proteins or steroids in the mother's circulation during the second trimester of pregnancy. Such screening markers include α -fetoprotein, choriogonadotropin, and estriol. An abnormal screening test is usually accompanied by amniocentesis and analysis of amniotic fluid components or cells. Recently, we described the presence in amniotic fluid of prostate-specific antigen (PSA)¹, a 33-kDa serine protease thought to be exclusively produced by prostatic epithelial cells

[1]. PSA also circulates in maternal serum, and its concentration increases in amniotic fluid from gestational weeks 11 to 22 and then stabilizes until delivery. An apparent association exists between PSA concentrations in amniotic fluid and certain fetal abnormalities (Melegos DN, et al.; ms. submitted). The role of PSA in amniotic fluid is currently unknown, but recent data support the view that PSA may be a growth factor or a growth factor or cytokine regulator [2].

Prostaglandins are arachidonic acid metabolites with many biological functions [3]. Prostaglandin D₂ (PGD₂), a potent inhibitor of platelet aggregation in vitro, is involved in smooth muscle contraction and relaxation [4]. PGD₂ has been implicated in a variety of central nervous system functions including synaptic transmission [5], hypothalamic control of temperature [6], recovery from seizures [7, 8], and release of lutropin [9]. PGD₂ is also implicated in sedation and sleep induction [10, 11]; Hayaishi et al. [12-14] have provided strong evidence that PGD₂ is involved in sleep induction in rats and monkeys.

Glutathione-independent PGD₂ synthase (EC 5.3.99.2) catalyzes the conversion of PGH₂ to PGD₂ in the presence of various sulfhydryl compounds [15]. This enzyme is responsible for the biosynthesis of PGD₂ in the brain and is localized in the central nervous system [16]. Postnatally, the cellular localization of the enzyme changes and now the enzyme is thought to play important roles in both maturation and maintenance of the central nervous system.

Previously, Nagata et al. [17], White et al. [18], and Urade et al. [19] cloned the human and rat PGD₂ synthase gene and showed that PGD₂ synthase belongs to a superfamily of secretory proteins called lipocalins. Lipocalins share a common feature for binding and transport of small hydrophobic molecules [20]. Among the 16 members of this superfamily, PGD₂ synthase shows the greatest homology with α_1 -microglobulin, suggesting a close evolutionary relation between these two proteins [17].

We report here the isolation and identification of PGD₂ synthase in human amniotic fluid. It appeared in amniotic fluid as a 25-kDa protein that reacted with polyclonal but not monoclonal anti-PSA antibodies on Western blots. Extensive purification of the protein from amniotic fluids and sequencing

Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, ON M5G 1X5, and Department of Clinical Biochemistry, University of Toronto, Toronto, ON M5G 1L5, Canada.

*Address correspondence to this author at: Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Ave., Toronto, ON M5G 1X5, Canada. Fax 416-586-8628.

¹ Nonstandard abbreviations: PSA, prostate-specific antigen; PGD₂, prostaglandin D₂; and ACT, α_1 -antichymotrypsin.

Received December 4, 1995; accepted March 4, 1996.

of 15 amino acids has shown almost complete homology with PGD₂ synthase between amino acids 30 and 44. Apparently, PGD₂ synthase in amniotic fluid is cleaved, with removal of 7 amino acids from the amino-terminal end; native secreted PGD₂ synthase is thought to be released after cleavage of only the first 22 amino acids [17]. Semiquantitative assessment of PGD₂ synthase concentrations in amniotic fluids from fetuses with abnormal phenotypic or genotypic characteristics suggests that some abnormalities are associated with overexpression or underexpression of PGD₂ synthase.

Materials and Methods

CLINICAL SPECIMENS

Amniotic fluids of various gestational ages, remaining from routine testing for fetal abnormalities, were obtained from the Department of Clinical Biochemistry, The Toronto Hospital and stored at -20 °C until analysis. Our study was approved by the ethics committee of The Toronto Hospital.

PSA AND PGD₂ SYNTHASE PREPARATIONS

Highly purified preparations of PSA and PSA- α_1 -antichymotrypsin complex (PSA-ACT) were provided by Tom Stamey, Stanford University, Stanford, CA. Purified PGD₂ synthase, isolated from human brain, was a gift from O. Hayaishi, Osaka Bioscience Centre, Osaka, Japan.

MEASUREMENT OF PSA

The concentration of amniotic fluid PSA was measured with an immunofluorometric procedure described in detail elsewhere [21]. This assay was based on a mouse monoclonal capture antibody, a biotinylated rabbit polyclonal antibody, alkaline phosphatase-conjugated streptavidin, and the fluorogenic substrate 5'-fluorosalicyl phosphate. The delayed fluorescence generated by 5'-fluorosalicylate, Tb³⁺, and EDTA was measured with time-resolved fluorometry [22]. The PSA assay can quantify PSA at concentrations of 0.01 μ g/L or higher. Other versions of the PSA immunofluorometric procedure, used to identify PGD₂ synthase, are described later in the text. These assays are based on the general principles described for the PSA immunofluorometric procedure [21].

WESTERN BLOT ANALYSIS

All reagents and equipment for Western blot analysis were purchased from Novex (San Diego, CA). The manufacturer's protocols were followed throughout. Samples for Western blot analysis were electrophoresed under reducing (with mercaptoethanol) and nonreducing (no mercaptoethanol) conditions on 8–16% gradient polyacrylamide minigels, and proteins were electrotransferred to Hybond-ECL nitrocellulose membranes (Amersham International, Arlington Heights, IL). Membranes were blocked for 1 h and processed further as recommended by the manufacturer of the ECL-Western blot detection kit (Amersham). Briefly, the membranes were probed with either a rabbit polyclonal anti-PSA antibody (Medix Biotech, Foster City, CA; 1 g/L, diluted 1000-fold in the blocking buffer) or a mouse monoclonal anti-PSA antibody [clone 2E1(F5), 1 g/L, diluted 2000-fold in the blocking buffer; a gift from T. Stamey].

Similar experiments were performed with nonimmune rabbit serum and a rabbit antibody against human brain PGD₂ synthase (a gift from O. Hayaishi). The membranes were then washed and reacted with a horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibody. The enzyme activity on the membranes was revealed by chemiluminescence captured on x-ray film. Biotinylated molecular mass markers were visualized by reacting with a streptavidin-horseradish peroxidase conjugate added simultaneously with the anti-rabbit antibody.

HPLC

Characterization of the PSA immunoreactivity and purification of PGD₂ synthase were achieved with HPLC. The gel filtration chromatography was performed on a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan), run isocratically with a mobile phase of 0.1 mol/L Na₂SO₄-0.1 mol/L NaH₂PO₄, pH 6.80. Flow rate was 0.5 mL/min. We used a Bio-Sil SEC-250, 600 \times 7.5 mm (Bio-Rad Labs., Richmond, CA) column calibrated with a molecular mass calibrator solution from Bio-Rad. Fractions of 0.5 mL were analyzed for PSA by the immunofluorometric procedure or analyzed by Western blot to detect PGD₂ synthase. For preparative purposes, the fractions were collected, pooled, and preconcentrated. Preconcentration was achieved by ultrafiltration on Centriprep 10 preconcentration modules from Amicon (Amicon Division, W. R. Grace and Co., Beverly, MA). These disposable devices were used as recommended by the manufacturer. Amniotic fluid was preconcentrated by the same method.

PGD₂ synthase was further purified by a second HPLC procedure based on ion-exchange chromatography. This gradient method involves two mobile phases: solution A, a 0.02 mol/L Tris buffer, pH 9.20; and solution B, 0.02 mol/L Tris buffer, pH 7.50, containing 1 mol/L NaCl. The gradient elution program was as follows: 0–10 min, solution A; 10–60 min, linear gradient from 100% A to 100% B. The flow rate was 0.5 mL/min. The column used was a Mono Q fast-protein liquid chromatography anion-exchange column (Pharmacia, Piscataway, NJ). Fractions were collected, pooled, and preconcentrated as necessary with Amicon Centriprep 10 modules.

PROTEIN MICROSEQUENCING

The purified protein was microsequenced with a Porton Model 2090 gas-phase microsequencer with on-line phenylthiohydantoin amino acid analysis. After 15 sequencing cycles, the resultant sequence was compared with sequences deposited in NBRF-PIR databases. Homology searching was done with DNASIS software.

Results

The PSA concentration in amniotic fluid is <0.005 μ g/L at gestational weeks \leq 12 but rises quickly to concentrations of \sim 1 μ g/L during gestational weeks 14–22 (Melegos DN et al., ms. submitted). PSA concentrations fluctuate at \sim 0.3–0.6 μ g/L until delivery. Two molecular forms of PSA exist in amniotic fluid (Fig. 1): a minor form (molecular mass \sim 100 kDa)

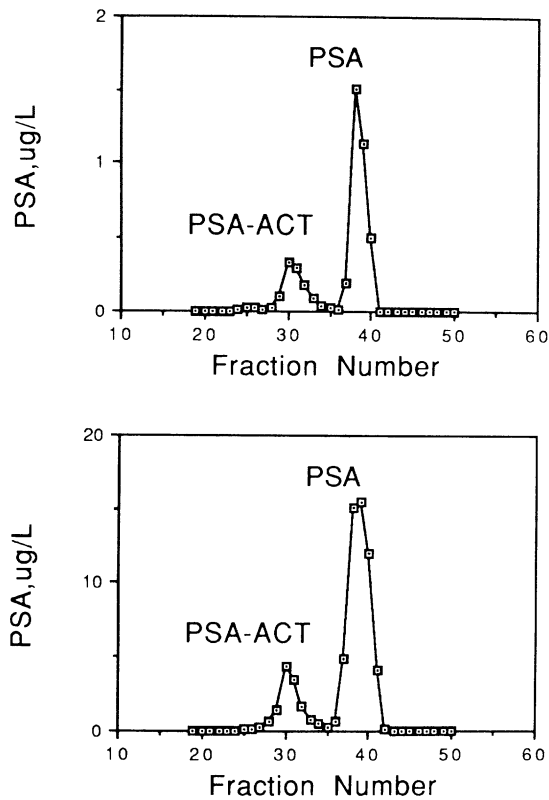


Fig. 1. HPLC of two amniotic fluids and assay of the HPLC fractions with a PSA immunoassay [22].

Details of the HPLC procedure and the PSA assay are described under *Materials and Methods*. Amniotic fluids contained $\sim 14 \mu\text{g/L}$ (upper panel) and $\sim 500 \mu\text{g/L}$ (lower panel) PSA. PSA immunoreactivity elutes in two peaks at fractions 30 and 39. The first peak, corresponding to a molecular mass of $\sim 100 \text{ kDa}$, represents PSA-ACT. The second peak, corresponding to a molecular mass of $\sim 33 \text{ kDa}$, represents free PSA. In HPLC, PGD₂ synthase coelutes with free PSA at fraction 39 ± 1 .

represents PSA-ACT; the major form (molecular mass 33 kDa) represents free PSA.

Western blot analysis of mercaptoethanol-reduced amniotic fluids and probing of the electrotransferred proteins with a polyclonal rabbit anti-PSA antibody reveals the presence of intense immunoreactive bands with a molecular mass of $\sim 25 \text{ kDa}$. The 25-kDa bands are distinct from the bands corresponding to PSA that appear at molecular mass $\sim 33 \text{ kDa}$ (Fig. 2). In the same experiment without mercaptoethanol reduction, the 25- and 33-kDa bands overlap on the Western blot (Fig. 3). When the blot was probed with a monoclonal anti-PSA antibody, only the 33-kDa PSA molecule was detected (Figs. 2C and 3C). When the blot was probed with nonimmune rabbit serum, no bands were detected in the region of 25–33 kDa (Figs. 2B and 3B). These data suggest that the immunoreactive bands appearing on Western blots at 25 kDa with the polyclonal anti-PSA antibodies represent non-PSA cross-reacting proteins or fragmented PSA that are recognized by the polyclonal but not by the monoclonal anti-PSA antibody. Figs. 2 and 3 (panel A in both blots) also show that the 25-kDa immunoreactive bands, recognized only by the PSA rabbit polyclonal antibody, are very weak at 11 weeks of gestation (lane 4); they progressively increase in

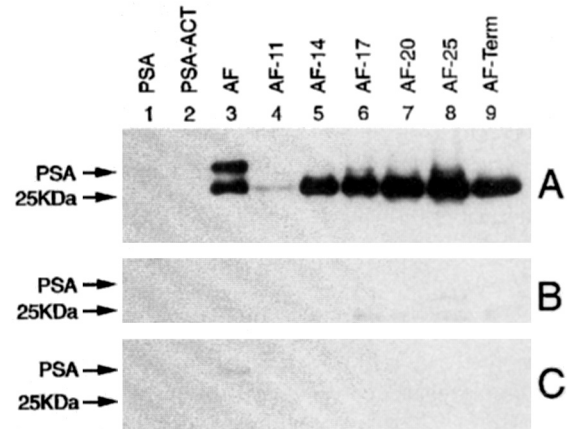


Fig. 2. Western blot analysis.

Samples were electrophoresed on 8–16% gradient polyacrylamide minigels with mercaptoethanol reduction, electrotransferred to nitrocellulose membranes, and probed with a rabbit polyclonal anti-PSA antibody (A), rabbit nonimmune serum (B), or a mouse monoclonal anti-PSA antibody (C). Detection was achieved by a technique involving horseradish peroxidase-conjugated secondary anti-rabbit (A, B) or anti-mouse antibody (C) and chemiluminescence. Lane 1, purified seminal plasma PSA (barely visible in lane 1 of A only). Lane 2, PSA-ACT. This complex is destroyed and becomes undetectable when the sample is reduced with mercaptoethanol. Lane 3, one amniotic fluid (AF) containing high concentrations of PSA. Lanes 4–9, amniotic fluids at gestational weeks 11, 14, 17, 20, 25, and 40 (term), respectively. PSA was loaded at 300 μg (lane 1), 220 μg (lane 2), and 2000 μg (lane 3) per lane. The amount of PSA loaded in lanes 4–9, $< 10 \text{ pg/lane}$, could not be detected by Western blot. Under reducing conditions, the detectability of PSA and PSA-ACT on Western blots is reduced (lanes 1 and 2). For data discussion, see text. Arrows indicate position of PSA and the 25-kDa band. Note that all amniotic fluids (lanes 3–9) contain prominent 25-kDa bands. These bands are not visualized with either nonimmune rabbit serum (B) or a monoclonal anti-PSA antibody (C). PSA in lane 3 can be detected with the monoclonal antibody.

concentration until gestational weeks 20–25 and then decline slightly at term.

Figs. 2 and 3 demonstrate that (a) the bands recognized at molecular mass 25 kDa in amniotic fluids are caused by interactions of amniotic fluid protein(s) with antibodies present in the polyclonal PSA antiserum and not with nonimmune rabbit IgG, since these proteins were not visualized by nonimmune rabbit IgG (panels B); (b) these protein(s) are not recognized by monoclonal anti-PSA antibodies (panels C), suggesting that they do not represent true PSA; (c) the concentration of these proteins changes in amniotic fluid during the progression of pregnancy.

To exclude the possibility that the immunoreactive bands at 25 kDa represent fragmented human IgG interacting with rabbit IgG, we analyzed by Western blot human IgG under conditions similar to those of Figs. 2 and 3. These data showed that human IgG interacts somewhat with rabbit IgG, but the immunoreactive bands detected on Western blots without mercaptoethanol reduction appear at a molecular mass of $\sim 180 \text{ kDa}$ (the molecular mass of IgG). We also found these bands after probing the membranes with nonimmune rabbit IgG (data not shown). Thus we concluded that human IgG does not contribute to the appearance of the 25-kDa bands on the Western blots shown in Figs. 2 and 3.

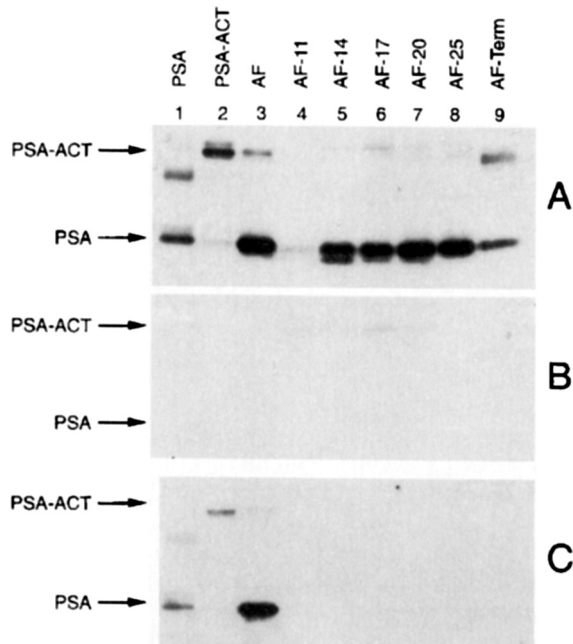


Fig. 3. Western blot analysis.

Samples were electrophoresed on 8–16% gradient polyacrylamide minigels without mercaptoethanol reduction, electrotransferred to nitocellulose membranes, and probed with a rabbit polyclonal anti-PSA antibody (A), rabbit nonimmune serum (B), or a mouse monoclonal anti-PSA antibody (C). Detection was achieved by a technique involving horseradish peroxidase-conjugated secondary anti-rabbit (A, B) or anti-mouse (C) antibody and chemiluminescence. Lane 1, purified seminal plasma PSA; lane 2, PSA-ACT (molecular mass ~ 100 kDa); lane 3, one amniotic fluid (AF) containing high concentrations of free PSA and low concentrations of PSA-ACT; lanes 4–9, amniotic fluids at gestational weeks 11, 14, 17, 20, 25, and 40 (term), respectively. PSA was loaded at 300 μ g (lane 1), 220 μ g (lane 2), and 2000 μ g (lane 3) per lane. The PSA loaded in lanes 4–9, <10 μ g/lane, was not detectable with the Western blot technique. For data discussion, see text. Upper arrow shows position of the PSA-ACT complex; lower arrow shows the position of PSA and the 25-kDa bands, which overlap under nonreducing conditions. Note that the monoclonal anti-PSA antibody recognizes PSA in lanes 1 and 3 and PSA-ACT in lane 2. No PSA immunoreactivity is seen in lanes 4–9 with the monoclonal antibody.

We also considered the possibility that the 25-kDa band appearing on Western blots may represent PSA, a PSA fragment, or a cross-reacting protein noncovalently bound to amniotic fluid components. The bound moiety may be released during the polyacrylamide gel electrophoresis step, thus becoming detectable only on Western blot analysis. We considered this because the 25-kDa band is not measurable by the immunofluorometric ELISA-type PSA assay. Many amniotic fluids with prominent 25-kDa bands on Western blots were negative for PSA as measured by the immunofluorometric procedure. As a check for the possibility that the 25-kDa band represents PSA, a PSA fragment, or a non-PSA cross-reactive protein bound to amniotic fluid components, we separated amniotic fluids on a gel filtration column under native conditions by HPLC, collected the HPLC fractions, and then subjected the fractions to Western blot analysis. We hypothesized that if the 25-kDa band represented a protein bound to amniotic fluid components, it would elute from the HPLC column as a higher-molecular-mass protein recognized on the Western blot as a 25-kDa band. These experiments have clearly shown that the 25-kDa band

elutes from the HPLC column as a protein with a molecular mass of 25-kDa. These data exclude the possibility that the protein was bound to amniotic fluid components before the Western blot analysis (data not shown) and are further confirmed by Fig. 3, which shows that the band of interest appears at a molecular mass of 25–30 kDa on Western blots performed under nonreducing conditions. Under these conditions, and in the HPLC procedure (Fig. 1), PSA-ACT, a well known complexed form of PSA, elutes as a protein with an apparent molecular mass of ~ 100 kDa.

Our finding that the 25-kDa band reacted with the polyclonal anti-PSA antibody on Western blots but not with the monoclonal anti-PSA antibody and that the same band was not recognized by our immunofluorometric PSA assay (monoclonal coating antibody; polyclonal detection antibody identical to the one used for Western blot) prompted us to check the possibility that if the 25-kDa band represented a PSA fragment, this fragment could be recognized by immunofluorometric procedures developed either with monoclonal coating antibodies recognizing different epitopes or with methods based exclusively on polyclonal coating and detection antibodies. Such assays were developed with three monoclonal antibodies for coating, three polyclonal antibodies for detection, or combinations of only polyclonal antibodies for coating and detection. In all assays, detection antibodies were biotinylated, and the final step involved streptavidin conjugated to alkaline phosphatase as described in the published PSA procedure [21]. Although we were able to set up operational assays that could measure efficiently free PSA and PSA-ACT complexes, none of the assays was able to recognize the amniotic fluid 25-kDa protein (data not shown).

The 25-kDa band does not seem to be uniquely present in amniotic fluids. We have previously identified PSA in milk of lactating women [23], breast cyst fluid [24], normal breast tissue extracts [25], and breast tumors [26–28]. Western blot analysis of these biological fluids and extracts gave the results seen in Fig. 4. The highest concentrations were in amniotic fluid, milk, and cyst fluid, preliminarily suggesting that this protein is also secreted by breast epithelial cells.

Our strategy to purify and sequence the 25-kDa band present in amniotic fluids is as follows: One liter of starting material (pooled amniotic fluid leftover samples from women at gestational weeks 20–25 who underwent amniocentesis for bilirubin monitoring) was centrifuged, filtered through a 0.45- μ m (pore size) filter, and then pre-concentrated fivefold with a Centricon ultrafiltration module. Experiments have shown that when ultrafiltration membranes with a 10-kDa cutoff were used, the 25-kDa protein was detected by Western blot only in the retained material. No protein penetrated the filter (data not shown). The pre-concentrated amniotic fluid (~ 200 mL, PSA concentration ~ 4 μ g/L) was then separated on a gel-filtration HPLC column (injected 2 mL at a time), and the fractions were collected. Fractions from each injection were pooled and analyzed by the immunofluorometric procedure (to localize the elution of true PSA) and by Western blot (to localize the elution of the 25-kDa band). Both PSA and the 25-kDa band eluted near each other, and the fractions containing these proteins were

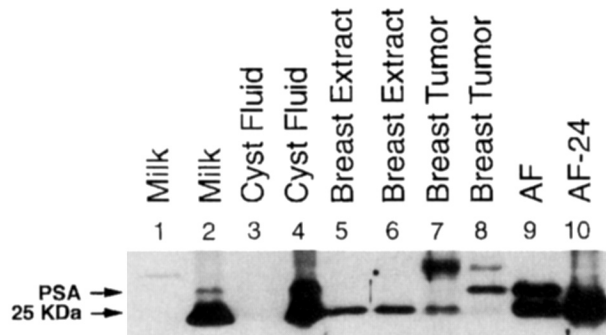


Fig. 4. Western blot analysis.

Samples were electrophoresed on 8–16% gradient polyacrylamide minigels with mercaptoethanol reduction, electrotransferred to nitrocellulose membranes, and probed with a rabbit polyclonal anti-PSA antibody. Detection was achieved by a technique involving a horseradish peroxidase-conjugated secondary anti-rabbit antibody and chemiluminescence. *Lane 1*, human milk from a lactating woman, negative for PSA; *lane 2*, human milk positive for PSA (8.7 $\mu\text{g/L}$); *lane 3*, breast cyst fluid negative for PSA; *lane 4*, breast cyst fluid positive for PSA (110 $\mu\text{g/L}$); *lanes 5 and 6*, two normal female breast extracts negative for PSA; *lane 7*, breast tumor extract negative for PSA; *lane 8*, breast tumor extract positive for PSA (18 $\mu\text{g/L}$); *lane 9*, amniotic fluid (AF) containing both free PSA (500 $\mu\text{g/L}$) and the 25-kDa band; *lane 10*, amniotic fluid at 24 gestational weeks (AF-24) negative for PSA. PSA-positive bands appear in *lanes 2, 4, 8, and 9*; the 25-kDa band appears in *lanes 2, and 4-10*. The two breast tumors contain additional bands of unknown nature with molecular masses of ~ 37 kDa.

pooled, preconcentrated with Centricon filters, and rechromatographed on the same column. Three successive cycles of the same procedure gave a solution containing amniotic fluid proteins with molecular masses of 30 ± 10 kDa. This solution was highly enriched in the 25-kDa protein, as judged by Western blot analysis.

Further purification of the 25-kDa protein was achieved with ion-exchange gradient chromatography on a Mono Q column. The protein mixture from the previous chromatography was injected, the proteins were eluted with a pH and salt gradient, and fractions were collected and analyzed by Western blot to localize the 25-kDa band. A typical ion-exchange chromatogram is shown in Fig. 5. The 25-kDa protein elutes at fraction 18 ± 1 . Under these conditions, PSA elutes at fraction 21. Successive rechromatography of fractions 18 ± 1 gave a final solution containing only one protein on HPLC separation that does not contain any PSA immunoreactivity.

Results of microsequencing (15 cycles) of the purified protein are shown in Fig. 6. The isolated protein was highly homologous to the human PGD₂ synthase, the sequence of which has been published on the basis of analysis of cDNAs isolated from human and rat brain [17–19].

Further confirmation that the immunoreactive 25-kDa band is indeed PGD₂ synthase came from the following experiments: (a) We raised a polyclonal rabbit antibody against a highly purified 25-kDa band from amniotic fluids; (b) we have obtained highly purified PGD₂ synthase isolated from human brain, and polyclonal antibodies against brain PGD₂ synthase from O. Hayaishi; (c) we have performed Western blot analysis using amniotic fluids and brain-type PGD₂ synthase as samples and probed with a PSA polyclonal antibody, the newly developed antibody, and the antibody against brain PGD₂ synthase. The

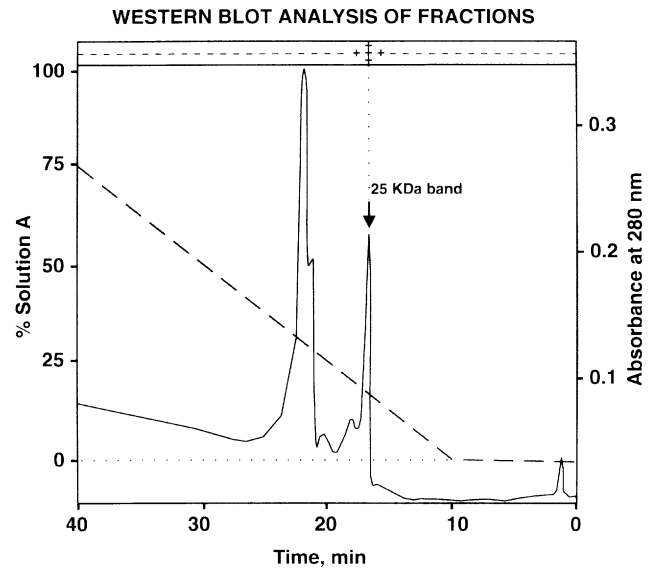


Fig. 5. Final purification of the 25-kDa protein with ion-exchange chromatography.

The gradient elution profile is shown by broken lines. Solutions A and B used for gradient elution are described under *Materials and Methods*. All HPLC fractions were Western-blotted; results are summarized on the top of the figure. Rechromatography of fraction 18, which contained the 25-kDa band, resulted in the elution of only one peak. The purified 25-kDa band was subjected to protein microsequencing. Under these chromatographic conditions, PSA elutes at fraction 21.

data (Fig. 7) show similar results for all three antibodies that recognized the 25-kDa bands in amniotic fluids as well as the purified brain PGD₂ synthase.

Preliminary analysis of human amniotic fluids for PGD₂ synthase enzymatic activity, with radiolabeled PGH₂ as substrate, showed that PGD₂ could be generated from PGH₂, consistent with the suggestion that PGD₂ synthase in amniotic fluid is enzymatically active (data not shown). We also showed that the newly developed antibody and the antibody against brain PGD₂ synthase did not cross-react with PSA on Western blots, even when PSA was tested at concentrations up to 423 000 000 $\mu\text{g/L}$. Further analysis confirmed the presence of PGD₂ synthase in seminal plasma.

The concentrations of PGD₂ synthase in amniotic fluids from normal pregnancies and pregnancies associated with various fetal abnormalities were assessed semiquantitatively by Western blot analysis with the polyclonal anti-PSA antibody. Preliminary results are presented in Fig. 8 and Table 1. Apparently, certain fetal abnormalities are associated with underexpression and overexpression of PGD₂ synthase in amniotic fluid.

Discussion

PGD₂ synthase, which regulates the concentrations of PGD₂, may play important roles in both maturation and maintenance of the central nervous system. It has recently been found there associated with tissues such as retina and cochlea; postnatal changes in the cellular localization of this enzyme were found in the brain and cochlea.

Various groups have isolated cDNAs coding for the PGD₂ synthase gene [17–19] from mRNA extracted from human or

Table 1. Fetal abnormalities and semiquantitative amniotic fluid concentrations of PGD₂ synthase.

Case no.	Fetal abnormality	Gest. week	PGD ₂ synthase ^a
1	Down syndrome	12	↔
2	Turner syndrome	12	↔
3	Down syndrome	13	↔
4	Down syndrome	14	↓
5	Demise of fetus; no positive fetal heart	14	↓
6	Moderate nuchal swelling, oligohydramnios, clubbed feet	15	↓
7	Omphalocele containing liver, gut, and part of stomach	15	↑
8	Large fetal cerebellum	16	↓
9	Arnold–Chiari malformation	16	↓
10	Two-vessel cord, large bilateral choroid plexus cysts	16	↓
12	Hydrocephalus	16	↓
13	Down syndrome	16	↓
14	AF-AFP screening states that it has abnormal ultrasound	16	↓
15	Trisomy 18	17	↓
16	Dandy–Walker syndrome	17	↑
17	Nuchal hygroma, anasarca, hydronephrosis	18	↓
18	Anencephaly	18	↔
19	Anencephaly	18	↓
20	Chromosomal abnormality in one of the twins	18	↔
20	Chromosomal abnormality, neural tube defect of lower twin	18	↔
21	Multicystic renal dysplasia, low-set ears, infraorbital folds	18	↓
22	Hydrops, horseshoe right kidney, 11 ribs on one side	18	↔
23	Large bilateral choroid plexus cysts (1 and 2 cm)	18	↔
25	Large echogenic kidneys, 11 pairs of ribs	18	↔
26	AF-AFP screening states that it has abnormal ultrasound	18	↔
27	Arnold–Chiari type of signs, with neural tube defects	18	↓
28	Cystic mass with large diverticulum, clubbed feet	19	↑
28	Cystic mass with large diverticulum, clubbed feet	21	↓
29	Osteogenesis imperfecta	19	↔
30	Nuchal thickening, small stomach, palatal and labial clefts	19	↔
31	Turner syndrome	19	↔
33	Anencephaly	19	↔
34	Borderline thickening of nuchal fold	20	↔
35	Cystic hygroma in the posterior nuchal region	20	↔
36	Midline defect in cerebellum, suspicion of Dandy–Walker syndrome	20	↓
37	Down syndrome	21	↔
38	AF-AFP report indicated ultrasound abnormality, stillbirth	21	↔
39	Amniotic band syndrome; exencephaly	21	↔
40	Arnold–Chiari malformation, spina bifida	21	↔
41	Semilobular holoprosencephalus, neural tube defect, cord	22	↔
42	Borderline of nuchal fold increase, suspicion of trisomy 21	22	↑
43	Hydrocephalus, polyhydramnios, atrial septal defect	22	↔
44	Arnold–Chiari malformation; lemon-shaped head	22	↔
45	Chiari malformation, ventriculomegaly, clubbed feet	23	↔
46	Anencephaly	23	↔
47	Lemon-shaped head, open neural tube defect, clubbed feet	23	↑

^a Concentration of AF PGD₂ synthase reported as increased (↑), decreased (↓), or unaltered (↔) in comparison with control amniotic fluids from normal pregnancies of the same gestational week.

AF, amniotic fluid; AFP, α -fetoprotein.

animal brain. The deduced amino acid sequence consists of 190 amino acids with a calculated molecular mass of 21 016 Da [17]. However, the molecular mass of the mature secreted protein may be higher, since there are two potential N-glycosylation sites. PGD₂ synthase contains signal sequences that are cleaved off before secretion (Fig. 6). PGD₂ synthase belongs to a

superfamily of various 160–190-amino acid secretory proteins, sharing a common feature for binding and transport of small hydrophobic molecules (lipocalins). Among all lipocalins, PGD₂ synthase shares extensive homology with α_1 -microglobulin [17].

We previously identified PSA as a component of amniotic fluid [1], normal female breast [25], breast milk [25], breast

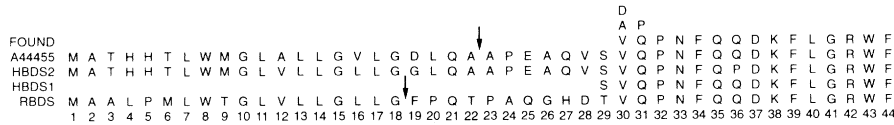


Fig. 6. Found amino acid sequence and its comparison with reported amino acid sequences of PGD₂ synthase.

Sequence A44455 was reported by White et al. for human PGD₂ synthase [18]. HBDS2 and HBDS1 are sequences reported by Nagata et al. for human PGD₂ synthase [17]. RBDS is a sequence of PGD₂ synthase from the rat as reported by Urade et al. [19]. The amino acid identity in the first two cycles of the sequencing was ambiguous as shown. Arrows indicate proteolytic sites for removal of the signal peptide, as suggested by Nagata et al. [17].

tumors [26–28], and breast cyst fluid [24]. During Western blot analysis of these extracts and fluids, we identified, especially in amniotic fluid, a highly intense immunoreactive band with a molecular mass of ~25 kDa. This band was seen only with polyclonal anti-PSA antibodies; monoclonal anti-PSA antibodies or nonimmune rabbit serum were unreactive.

We have extensively purified the 25-kDa band by gel filtra-

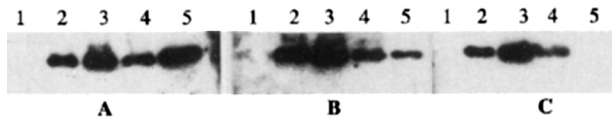


Fig. 7. Western blot analysis.

Samples were electrophoresed on 8–16% gradient polyacrylamide minigels with mercaptoethanol reduction, electrotransferred to nitrocellulose membranes, and probed with a rabbit anti-PSA antibody (A), a rabbit antibody raised against purified PGD₂ synthase from amniotic fluid (B), and a rabbit antibody raised against purified PGD₂ synthase from human brain. Lane 1, purified seminal plasma PSA (2 ng in A and 10 ng in B and C); lanes 2–4, human amniotic fluids of gestational ages 14, 20, and 40 weeks, respectively; lane 5, blots A and B, purified human brain PGD₂ synthase (1 µg). A faint PSA band is shown in lane 1A at ~33 kDa. In all cases the 25-kDa band, corresponding to PGD₂ synthase, is more intense at 20 weeks of gestation.

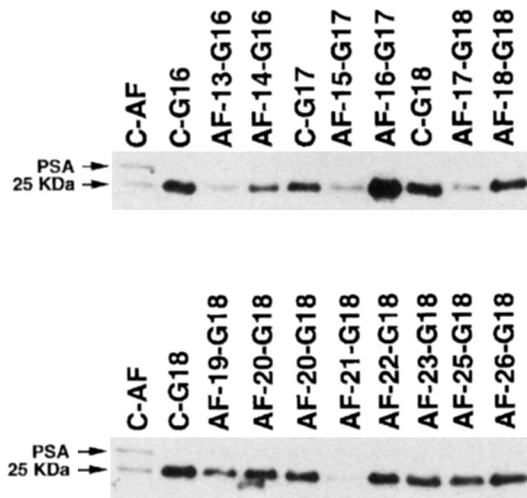


Fig. 8. Western blot analysis of amniotic fluids from normal and abnormal fetuses.

C-AF, control amniotic fluid containing both PSA and the 25-kDa band (positions shown by arrows). C-G16, C-G17, and C-G18 are amniotic fluids from normal pregnancies at gestational weeks 16, 17, and 18, respectively. AF-13-G16 to AF-26-G18 are amniotic fluids from abnormal pregnancies with a case number (e.g., 13) followed by the gestational weeks (e.g., G16). All cases studied are listed in Table 1, along with the pregnancy outcomes and semiquantitative concentrations of the 25-kDa protein.

tion and ion-exchange HPLC. Sequencing showed that the 25-kDa band shared extensive homology with PGD₂ synthase (Fig. 6). In our sequencing procedure, the first cycle yielded three possible amino acids, one of which is V in position 30. The second cycle yielded two possible amino acids, one of which is Q in position 31. All other cycles yielded unique amino acids identical to the amino acid sequence of PGD₂ synthase. These data, and the close agreement between the predicted and found molecular mass of PGD₂ synthase, strongly suggest that the 25-kDa band in amniotic fluids is indeed PGD₂ synthase, and are further confirmed by Western blot analysis of purified brain PGD₂ synthase with both newly developed and already established anti-PGD₂ synthase antibodies (Fig. 7). Nagata et al. [17] proposed that PGD₂ synthase is synthesized as a preprotein that is cleaved between amino acids 22 and 23 to remove the signal peptide. In our protein sequence, the first amino acid appears to be V in position 30. Apparently, the additional seven amino acids may be cleaved by an amniotic fluid protease between amino acids S and V.

A protein seemingly identical to PGD₂ synthase on Western blots appears to be present in other fluids including breast milk, breast cyst fluid, normal breast extracts, and breast tumor extracts. PGD₂ synthase concentration also appears to change during pregnancy from very low before gestational week 12 to a maximum at gestational week 25.

Preliminary analysis of 44 amniotic fluids from abnormal pregnancies suggested that often the concentration of PGD₂ synthase seems to be lower or higher than that of amniotic fluids of the same gestational age and normal outcome. The most striking abnormalities shown in Fig. 8 include case 13 (Down syndrome), case 15 (trisomy 18), case 17 (hydronephrosis), and case 21 (multicystic renal dysplasia), all with low concentrations of PGD₂ synthase, and case 16 (Dandy–Walker syndrome), with high concentrations of PGD₂ synthase. We do not as yet have a quantitative assay for PGD₂ synthase in amniotic fluid, but semiquantitative assessment suggests concentrations up to 10 µg/L. On the basis of the molecular mass of PGD₂ synthase (lower than α -fetoprotein and PSA), we predicted the probable presence of PGD₂ synthase in maternal serum. Preliminary immunofluorometric assay data confirmed these suggestions (data not shown).

Homology analysis between amino acid sequences of PGD₂ synthase and PSA showed no relation between the two proteins. We thus postulated that the apparent cross-reactivity of anti-PSA polyclonal antibodies with PGD₂ synthase is due to contaminating anti-PGD₂ synthase antibodies, probably developed during immunization with PSA from seminal plasma,

which contained contaminating amounts of PGD₂ synthase. This is plausible because PGD₂ synthase is also present in seminal plasma (our findings) and its molecular mass is very similar to that of PSA. Another possibility is that PSA and PGD₂ synthase share a common conformational epitope that is recognized only by a subpopulation of polyclonal anti-PSA antibodies.

PGD₂ synthase has until now been localized in the brain. In cerebrospinal fluid, PGD₂ synthase is present in various glycosylated forms [29–31]. We show here that PGD₂ synthase is present in substantial amounts in amniotic fluid and probably in other fluids, tissue extracts, and breast secretions. The glycosylation pattern of PGD₂ synthase in amniotic fluid has not yet been studied. Although we do not yet know the role of PGD₂ synthase in amniotic fluid, we speculate that this protein, which appears in highest concentration during the explosive growth of the fetus, may be involved in PGD₂ regulation during this period. It remains to be seen whether PGD₂ synthase has a direct role as a growth factor or cytokine regulator during fetal development and whether monitoring its concentrations in amniotic fluid or maternal serum has any diagnostic value.

Previously, Minc-Golomb et al. found that PGE₂ and PGD₂ biosynthesis is severely reduced in trisomy 21 [32]. Our preliminary clinical data support this view; the concentrations of PGD₂ synthase, the enzyme responsible for PGD₂ biosynthesis, are reduced (Table 1). It remains to be seen whether a close connection exists between trisomy 21 and reduced concentrations of PGD₂ synthase and PGD₂ during embryonal life. Chaud et al. found hormonal regulation of PGD₂ by estrogens and progestins in rat uterine tissue, but the concentrations of the PGD₂ synthase were not evaluated [33].

Norwitz et al. studied PGD₂ production by term human decidua and concluded that bone marrow-derived cells (macrophages) are among the major sources of decidual PGD₂ at term [34]. Although the role of prostaglandins in the initiation of human parturition is not disputed, the specific role of PGD₂ in this process is still unclear.

Several investigations showed that PGD₂ can be produced *in vitro* by nonpregnant human endometrium and myometrium [35], by crude fractions of first-trimester decidua [36], and by whole-term placentas [37, 38]. These data support our view that the enzyme responsible for PGD₂ biosynthesis, PGD₂ synthase, is produced by placental cells and is then secreted into the amniotic fluid. PGD₂ synthase appears to be initially produced in substantial amounts in intrauterine life at about gestational week 11, and its concentration plateaus around gestational weeks 20–25. We do not yet know whether PGD₂ synthase is secreted as a protein starting at amino acid 30 (Fig. 6) or whether it is cleaved in amniotic fluid by as yet unknown proteases.

PGD₂ is suggested to help regulate cerebral capillary function [39]. It remains to be seen whether such a role exists for PGD₂ in the fetus during the explosive brain development at gestational weeks 11–22.

We anticipate that demonstration of the presence of PGD₂ synthase in human amniotic fluid and its possible association with certain fetal abnormalities will initiate more research

aiming to elucidate the role of this enzyme during embryonal life.

We thank Lynn Allen and Vicky Davies for providing us with amniotic fluids, and T.S. Chen and S. Joshi for protein sequencing and homology analysis. We also thank Tom Stamey and Tony Prestigiacomo for providing us with purified PSA and monoclonal anti-PSA antibodies, and Osamu Hayaishi for providing purified brain PGD₂ synthase and polyclonal antibodies. This work was supported by a grant from Nordion International, Kanata, Ontario, Canada.

References

1. Yu H, Diamandis EP. Prostate-specific antigen immunoreactivity in amniotic fluid. *Clin Chem* 1995;41:204–10.
2. Diamandis EP, Yu H. New biological functions of prostate specific antigen? *J Clin Endocrinol Metab* 1995;80:1515–7.
3. Smith WL. The eicosanoids and their biochemical mechanisms of action. *Biochem J* 1989;259:315–24.
4. Giles H, Leff P. The biology and pharmacology of PGD₂. *Prostaglandins* 1988;35:277–300.
5. Hayaishi O. Prostaglandin D₂: a neuromodulator. *Adv Prostaglandin Thromboxane Leukot Res* 1983;2:333–7.
6. Ueno R, Narumiya S, Ogorochi T, Nakayama T, Ishikawa Y, Hayaishi O. Role of prostaglandin D₂ in the hypothermia of rats caused by bacterial lipopolysaccharide. *Proc Natl Acad Sci U S A* 1982;79:6093–7.
7. Forstermann U, Heldt R, Knappen F, Hertting G. Potential anticonvulsive properties of endogenous prostaglandins formed in mouse brain. *Brain Res* 1982;240:303–10.
8. Forstermann U, Seregi A, Hertting G. Anticonvulsive effects of endogenous prostaglandins formed in brain of spontaneously convulsing gerbils. *Prostaglandins* 1984;27:913–23.
9. Kinoshita F, Nakai Y, Katakami H, Imura H, Shimizu T, Hayaishi O. Suppressive effect of prostaglandin (PG)D₂ on pulsatile luteinizing hormone release in conscious castrated rats. *Endocrinology* 1982;110:2207–9.
10. Laychock SG, Johnson DN, Harris LS. PGD₂ effects on rodent behavior and EEG patterns in cats. *Pharmacol Biochem Behav* 1980;12:747–54.
11. Pentreath VW, Rees K, Owolabi OA, Philip KA, Doua F. The somnogenic T lymphocyte suppressor prostaglandin D₂ is selectively elevated in cerebrospinal fluid of advanced sleeping sickness patients. *Trans R Soc Trop Med Hyg* 1990;84:795–9.
12. Onoe H, Ueno R, Fujita I, Nishino H, Oomura Y, Hayaishi O. Prostaglandin D₂, a cerebral sleep-inducing substance in monkeys. *Proc Natl Acad Sci U S A* 1988;85:4082–6.
13. Ueno R, Honda K, Inoue S, Hayaishi O. Prostaglandin D₂, a cerebral sleep-inducing substance in rats. *Proc Natl Acad Sci U S A* 1983;80:1735–7.
14. Hayaishi O. Molecular mechanisms of sleep-wake regulation: roles of prostaglandins D₂ and E₂. *FASEB J* 1991;5:2575–81.
15. Urade Y, Fujimoto N, Hayaishi O. Purification and characterization of rat brain prostaglandin D synthetase. *J Biol Chem* 1985;260:12410–5.
16. Ujihara M, Urade Y, Eguchi N, Hayashi H, Ikai K, Hayaishi O. Prostaglandin D₂ formation and characterization of its synthetases in various tissues of adult rats. *Arch Biochem Biophys* 1988;260:521–31.
17. Nagata A, Suzuki Y, Igarashi M, Eguchi N, Toh H, Urade Y, et al. Human brain prostaglandin D synthase has been evolutionarily

- differentiated from lipophilic-ligand carrier proteins. *Proc Natl Acad Sci U S A* 1991;88:4020–4.
18. White DM, Mikol DD, Espinosa R, Weimer B, Le Beau MM, Stefansson K. Structure and chromosomal localization of the human gene for a brain form of prostaglandin D₂ synthase. *J Biol Chem* 1992;267:23202–8.
 19. Urade Y, Nagata A, Suzuki Y, Fuji Y, Hayaishi O. Primary structure of rat brain prostaglandin D synthetase deduced from cDNA sequence. *J Biol Chem* 1989;264:1041–5.
 20. Pervaiz S, Brew K. Homology and structure–function correlations between α_1 -acid glycoprotein and serum retinol-binding protein and its relatives. *FASEB J* 1987;1:209–14.
 21. Yu H, Diamandis EP. Ultrasensitive time-resolved immunofluorometric assay of prostate-specific antigen in serum and preliminary clinical studies. *Clin Chem* 1993;39:2108–14.
 22. Christopoulos TK, Diamandis EP. Enzymatically amplified time-resolved fluorescence immunoassay with terbium chelates. *Anal Chem* 1992;64:342–6.
 23. Yu H, Diamandis EP. Prostate-specific antigen in milk of lactating women. *Clin Chem* 1995;41:54–8.
 24. Diamandis EP, Yu H, Lopez-Otin C. Prostate specific antigen: a new constituent of breast cyst fluid. *Breast Cancer Res Treat* 1996;38:259–64.
 25. Yu H, Diamandis EP, Monne M, Croce CM. Oral contraceptive-induced expression of prostate specific antigen in the female breast. *J Biol Chem* 1995;270:6615–8.
 26. Yu H, Diamandis EP, Sutherland DJA. Immunoreactive prostate specific antigen levels in female and male breast tumors and its association with steroid hormone receptors and patient age. *Clin Biochem* 1994;27:75–9.
 27. Diamandis EP, Yu H, Sutherland DJA. Detection of prostate specific antigen immunoreactivity in breast tumors. *Breast Cancer Res Treat* 1994;32:301–10.
 28. Monne M, Croce CM, Yu H, Diamandis EP. Molecular characterization of prostate specific antigen mRNA expressed in breast tumors. *Cancer Res* 1994;54:6344–7.
 29. Hoffmann A, Conradt HS, Gerhard G, Nimtz M, Lottspeich F, Wurster U. Purification and chemical characterization of β -trace protein from human cerebrospinal fluid: its identification as prostaglandin D synthase. *J Neurochem* 1993;61:451–6.
 30. Hoffman A, Nimtz M, Getzlaff R, Conradt HS. “Brain-type” N-glycosylation of asialotransferrin from human cerebrospinal fluid. *FEBS Lett* 1995;359:164–8.
 31. Grabenhorst E, Hoffmann A, Nimtz M, Zettlemeissl G, Conradt H. Construction of stable BHK-21 cells coexpressing human secretory glycoproteins and human Gal(β 1–4)GlcNAc-R α 2,6-sialyltransferase α 2,6-linked NeuAc is preferentially attached to the Gal(β 1–4)GlcNAc((β 1–2)Man(β 1–3) branch of diantennary oligosaccharides from secreted recombinant β -trace protein. *Eur J Biochem* 1995;232:718–25.
 32. Minc-Golomb D, Knobler H, Groner Y. Gene dosage of CuZnSOD and Down’s syndrome: diminished prostaglandin synthesis in human trisomy 21, transfected cells and transgenic mice. *EMBO J* 1991;10:2119–24.
 33. Chaud M, Faletti A, Beron de Estrada M, Gimeno AL, Gimeno MAF. Synthesis and release of prostaglandins D₂ and E₂ by rat uterine tissue throughout the sex cycle: effects of 17- β -estradiol and progesterone. *Prostaglandins Leukot Essent Fatty Acids* 1994;51:47–50.
 34. Norwitz ER, Starkey PM, Lopez Bernal A. Prostaglandin D₂ production by term human decidua: cellular origins defined using flow cytometry. *Obstet Gynecol* 1992;80:440–5.
 35. Rees MCP, Kelly RW. Prostaglandin D₂ release by endometrium and myometrium. *Br J Obstet Gynaecol* 1986;93:1078–82.
 36. Asboth G, Gimes G, Herteleny F, Toth M. The relation between thromboxane and prostaglandin synthesis in human decidua tissue: a comparison of eicosanoid synthesis in minced tissue with that in a cell-free preparation. *Biochim Biophys Acta* 1989;1002:101–8.
 37. Mitchell MD, Kraemer DL, Strickland DM. The human placenta: a major source of prostaglandin D₂. *Prostaglandins Leukot Med* 1982;8:383–7.
 38. Harper MJK, Khodr GS, Valenzuela G. Prostaglandin production by human term placentas in vitro. *Prostaglandins Leukot Med* 1983;11:121–9.
 39. Spatz M, Stanimirovic D, Uematsu S, Roberts LJ. Prostaglandin D₂ and endothelin-1 induce the production of prostaglandin F_{2a}, 9 α , 11-prostaglandin F₂, prostaglandin E₂ and thromboxane in capillary endothelium of human brain. *Prostaglandins Leukot Essent Fatty Acids* 1993;49:789–93.