Mouse fetal antigen 1 (mFA1), the circulating gene product of *mdlk*, *pref-1* and *SCP-1*: isolation, characterization and biology

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The mouse homologue to human fetal antigen 1 (hFA1) was purified from mouse amniotic fluid by cation exchange chromatography and immunospecific affinity chromatography. Mouse FA1 (mFA1) is a single chain glycoprotein with an M_r of 42–50 kDa (SDS–PAGE). The N-terminal amino acid sequence (39 residues) revealed 74% identity to hFA1 and 100% identity to the translated cDNAs referred to as mouse *dlk*, *pref-1* and *SCP-1*. mFA1 is the secreted processed molecule encoded by the mRNA defined by these identical mouse cDNAs. Monospecific rabbit anti-mFA1 antibodies, purified by ammonium sulfate precipitation and immunospecific affinity chromatography, were used for immunohistochemical and quantitative ELISA techniques. The indirect immunoperoxidase technique demonstrated mFA1 within the endocrine structures of adult mouse pancreas, whereas the exocrine tissue remained unstained. FA1-positive staining was also seen in the pituitary gland and the mouse adrenal gland. The concentration of mFA1 in a pool of amniotic fluid was assessed at $25 \mu\text{g ml}^{-1}$ and the serum concentration in normal nonpregnant adult mice ($n = 28$) was $11.3 \pm 5.0 \text{ ng ml}^{-1}$ (2 SD). During pregnancy the concentration of mFA1 in maternal serum increased above the nonpregnant reference value at midpregnancy, reaching a maximum concentration ($> 0.35 \mu\text{g ml}^{-1}$) 2 days prepartum. The maternal serum concentration was positively correlated with the number of fetuses. After delivery the rate of disappearance of mFA1 in maternal serum was very fast with a $t_{1/2} < 1 \text{ h}$. The concentration of mFA1 in newborn mice was about $15 \mu\text{g ml}^{-1}$ and did not reach normal adult values until the age of > 50 days.

Introduction

Human fetal antigen 1 (hFA1) was originally isolated from second trimester amniotic fluid (Fay *et al.*, 1988). The fetal origin of hFA1 was suggested after observations on the quantitative distribution in fetal and maternal compartments, together with immunohistochemical analysis (Fay *et al.*, 1988; Tornehave *et al.*, 1989). Radioimmunoassay revealed hFA1 concentrations in normal adult serum 1000 times lower than that seen in amniotic fluid (Jensen, 1992).

Immunohistochemical studies have demonstrated human FA1 in the fetal hepatocytes, fetal adrenal cortex, all glandular cells of the early pancreas primordium and around the blood islands of the yolk sac (Tornehave *et al.*, 1989, 1993; Palomino *et al.*, 1992; Jensen *et al.*, 1993, 1994). In adult tissue, however, the liver is FA1 negative, whereas FA1 in the pancreas is restricted to the insulin producing β -cells, and in the adult adrenal the most pronounced staining is seen in the cortex (Jensen *et al.*, 1993, 1994).

The primary structure of hFA1 (Jensen *et al.*, 1993, 1994) demonstrates the presence of six epidermal growth factor

(EGF)-like repeats, categorizing this single chain molecule of 32–38 kDa as a new circulating member of the EGF superfamily. There is a striking similarity between hFA1 and the Delta and Notch proteins in *Drosophila*, and to the mammalian homologues of these proteins (Artavanis-Tsakonas *et al.*, 1995) which are involved in the development of the embryonic ectoderm into epidermal and neural structures. Comparison of the hFA1 sequence to the published translated cDNA sequences reveals that hFA1 is the processed circulating form of the gene product of human *dlk* (Delta like), which may be a membrane protein synthesized in neuroendocrine tumours (Laborda *et al.*, 1993). Human *dlk* cDNA is identical to the cDNA *pG2* described as 'adrenal specific' when the latter sequence is corrected for sequence errors (Jensen *et al.*, 1993, 1994). Moreover, hFA1 has been demonstrated in neuroendocrine tumours of the lung (Jensen *et al.*, 1994) and in the somatroph cells of the pituitary gland (Larsen *et al.*, 1996). The mouse homologue to human *dlk* (*mdlk*) is identical to the cDNA from a mouse stromal cell line referred to as SCP-1 (Maruyama *et al.*, 1993) and a cDNA described as *pref-1* (Smas and Sul, 1993), which may be involved in the differentiation of 3T3-L1 fibroblasts to adipocytes. The gene product of *pref-1* may be a membrane protein and no secreted form has been described.

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The present study describes the isolation and characterization of circulating mouse FA1 (mFA1), production of antibodies, immunohistochemistry, quantitative methods and biology of mFA1, with the primary aim to establish a laboratory animal model for further analysis of the biological significance of FA1.

Materials and Methods

Antigen source

Mouse FA1 was purified from amniotic fluid obtained at days 16–19 of mouse pregnancy.

Physicochemical purification of mFA1

Amniotic fluid was dialysed against 50 mmol citrate-phosphate buffer l^{-1} , pH 4.5, centrifuged at 40 000 *g* for 30 min at 4°C and the supernatant was applied to a CM-Sepharose FF column (Pharmacia, Uppsala) equilibrated with citrate-phosphate buffer, pH 4.5. The column was washed with the citrate-phosphate buffer and the peak effluent was pooled, adjusted to pH 7.5, and applied to a concanavalin A column equilibrated with 20 mmol Tris l^{-1} containing 1 mmol $MgCl_2$ l^{-1} , 1 mmol $CaCl_2$ l^{-1} and 0.6 mol $NaCl$ l^{-1} . After washing, the column was eluted with 10% (w/v) α -D-mannoside and the eluted peak fractions were pooled and dialysed against 10 mmol Tris-HCl l^{-1} , pH 8.5 and applied to a Mono Q, H5/5 column, pre-equilibrated with the Tris buffer. The column was washed with ten bed volumes of Tris buffer and eluted with 10 mmol Tris-HCl l^{-1} , pH 8.5, 1 mol $NaCl$ l^{-1} .

Immunospecific purification of mFA1

The first affinity chromatography matrix was based on direct coupling of dissociated immune complexes to CNBr-activated Sepharose 4B (Pharmacia) essentially as described by Folkersen *et al.* (1980). The immune complexes were formed by mixing 3.5 ml mouse amniotic fluid with 10 ml antiserum against mFA1 purified based on physicochemical properties, and absorbed to monospecificity with normal mouse serum, followed by incubation at 37°C for 1 h and 4°C overnight. The immune complexes were isolated, washed, dissociated and coupled to CNBr-activated Sepharose as described by Jensen *et al.* (1993).

The matrix was packed on a column and connected to the fast performance liquid chromatography (FPLC) system (Pharmacia). Before application of sample, the column was washed with PBS + 1 mol $NaCl$ l^{-1} (washing buffer), eluted with 0.5% (w/v) citric acid, pH 2.3 and re-equilibrated with washing buffer.

After application of amniotic fluid, the column washed to baseline (Absorbance 280 nm) and mFA1 was eluted with citric acid. The eluted mFA1 was dialysed against PBS and subsequently used for immunization of new rabbits and for coupling to CNBr-activated Sepharose.

The next affinity matrix was produced by coupling of affinity purified rabbit anti-mFA1 Ig (see below) to

CNBr-activated Sepharose according to the manufacturer's recommendation. This column was run as described above and the eluate used for antibody production and analysis of the primary structure of FA1.

Production and preparation of rabbit anti-mFA1 antibodies

Purified mFA1 (30 μ g) was mixed with an equal volume of Freund's incomplete adjuvant and injected into rabbits at intervals of 28 days. The resulting antiserum was absorbed to monospecificity with serum from adult mice and defined as monospecific when one immunoprecipitate was seen using amniotic fluid as antigen and no precipitate was seen using adult mouse serum as antigen in crossed immunoelectrophoresis (50 μ l antiserum cm^{-2} agarose; 13 μ l undiluted antigen samples).

The monospecific rabbit anti-mFA1 antiserum was prepared in two ways: (1) For immunohistochemistry and electroimmunoassay, the IgG fraction of the antiserum was isolated by ammonium sulfate precipitation (Harboe and Ingild, 1973) and dissolved in PBS. (2) For the affinity purification of rabbit anti-mFA1 immunoglobulin, an affinity matrix was coupled with purified mFA1. The purification procedure was identical to that described for immunospecific purification of mFA1.

Electroimmunoassays

Crossed immunoelectrophoresis and rocket immunoelectrophoresis were performed essentially as described by Jensen *et al.* (1993) and Fay *et al.* (1988). Rocket immunoelectrophoresis was used to quantify mFA1 during purification using a twofold dilution series of amniotic fluid as calibrator.

SDS-PAGE

SDS-PAGE was carried out under reducing conditions on precast 8–20% polyacrylamide gradient gels on a Phast system (Pharmacia) followed by silverstaining according to the manufacturer's recommendations.

N-terminal amino acid sequencing

N-terminal sequencing was performed as described by Jensen *et al.* (1993). Before sequencing, mFA1 was reduced and alkylated using 4-vinylpyridine and purified on a Vydac C4 reversed-phased column (4 mm $\times$ 250 mm) running a gradient of 10–60% 2-propanol in 0.1% (v/v) trifluoroacetic acid (TFA) water. The N-terminal sequence was determined on an automated Knauer 910 protein sequencer with identification of phenylthiohydantion derivatives by online reversed-phase high performance liquid chromatography (HPLC).

Immunohistochemistry

Indirect immunoperoxidase staining was performed on 2–4 μ m 4% (w/v) formalin fixed air-dried sections of pancreas from adult mice, essentially as described by Jensen *et al.* (1993). The sections were incubated with ammonium sulfate precipitated purified rabbit anti-mFA1 IgG, diluted 1:300 in PBS

Fig. 1. Flowchart showing the sequence in preparation, purification and applications of mouse fetal antigen 1 (mFA1) (—) and rabbit antibodies against mFA1 (---). IC: immune complexes.

containing 1% (w/v) BSA. After washing, the section was reincubated with peroxidase conjugated swine anti-rabbit IgG (DAKO, Copenhagen) diluted 1:75 in PBS containing 10% (v/v) mouse serum. The specimens were developed with H₂O₂ and 3-amino-9-ethyl carbazole and counterstained with haematoxylin.

Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed as antibody capture assays using affinity-purified rabbit anti-mFA1 as capture antibody. The plates were incubated overnight with test samples and

calibrator (mouse amniotic fluid in twofold dilution series). Biotinylated affinity-purified rabbit anti-mFA1 was used as detector antibody and peroxidase-conjugated streptavidin, H₂O₂ and O-phenyldiamine were used to develop the reaction.

Results

Production of immunoreactants

The sequence of purification of mouse FA1 and the preparation of rabbit anti-mFA1 antibodies is shown (Fig. 1). Before

Fig. 2. Comparison of mouse fetal antigen 1 (mFA1) (lane a) with human FA1 (lane b) by SDS-PAGE under reducing conditions. The positions of the M_r markers are shown to the left. Both lanes were loaded with 0.9 μ g FA1 protein.

the generation of antibodies, FA1 was identified by SDS-PAGE analysis as a characteristic broad, diffuse band similar to that seen for human FA1 (Jensen *et al.*, 1993). In contrast to human FA1 (32–38 kDa), mouse FA1 protein migrated corresponding to an M_r of 42–50 kDa (Fig. 2).

The final purification procedure for FA1 (cation exchange chromatography followed by immunospecific affinity chromatography; Fig. 1) resulted in a recovery of 85% and a purity that allowed direct sequencing of the 39 N-terminal amino acid residues.

The specificity of the ammonium sulfate-purified IgG fraction of the first generation antibodies was analysed in crossed immunoelectrophoresis (Fig. 3). The anti-mFA1 IgG met the criteria set for monospecificity.

The anti-mFA1 antibodies showed pronounced crossreaction to rat FA1 but no apparent crossreaction to human FA1 when tested in ELISA with application of 2.5 μ g FA1. Analysis of mouse amniotic fluid and mouse serum in the hFA1 ELISA showed no crossreaction ($< 1\%$).

N-terminal amino acid sequence

The N-terminal sequence (39 residues) of the purified mFA1 showed a 74% identity to the N-terminal sequence of human FA1 (Jensen *et al.*, 1993) and 100% identity to the translated sequences of the cDNAs described as *mdlk* (Laborda *et al.*, 1993), *pref-1* (Smas and Sul, 1993) and *SCP-1* (Maruyama *et al.*, 1995) (Fig. 4).

Immunohistochemistry

One of the characteristic observations for hFA1 is the localization of this antigen within the islets of Langerhans (Torrehave *et al.*, 1993; Jensen *et al.*, 1994). The anti-mFA1 antibodies were applied by an indirect immunoperoxidase technique on sections of pancreas from adult mice to verify that the localization of mFA1 is similar to that of hFA1. A pronounced staining reaction of the endocrine tissue (islets of

Langerhans) was seen, whereas the exocrine tissue remained unstained (Fig. 5).

Quantification of mFA1 in serum

Quantitative amino acid analysis of purified mFA1 and quantification in ELISA using mouse amniotic fluid (days 17–19 of pregnancy) as calibrator revealed a concentration of 25 μ g mFA1 ml^{-1} in amniotic fluid. The ELISA technique for quantification of mFA1 using mouse amniotic fluid as calibrator had a detection threshold of 0.1 ng ml^{-1} .

The normal serum concentration of 28 nonpregnant mice, age > 60 days was $11.3 \text{ ng ml}^{-1} \pm 5.0$ (2 SD). No significant difference was observed between male ($n = 14$) and female ($n = 14$) mice sera (unpaired *t* test).

During pregnancy the serum concentrations did not rise above the normal nonpregnant values until 8–9 days before delivery, and the maximal concentration was reached 2 days prepartum (Fig. 6a). Postpartum, the serum concentration decreased rapidly to nonpregnant values within 12 h. In one mouse (Fig. 6a), the exact time of birth was recorded and a tenfold decrease in the serum mFA1 3 h postpartum was observed.

In contrast to the rapid decrease of mFA1 postpartum in adult mice, a much slower decrease was seen in newborn mice (Fig. 6b). The normal range ($11.3 \pm 5.0 \text{ ng ml}^{-1}$) was not reached until the age of > 50 days.

The fetal serum concentration of mFA1 at day 16–19 was 1400 times that of the normal nonpregnant range and similar to that seen in amniotic fluid (25 μ g ml^{-1}). The correlation between the maternal serum concentration of mFA1 (day 17–19 of pregnancy) and the number of fetuses at the same time is shown (Fig. 6c).

Discussion

The isolation and characterization of the mouse homologue to human FA1 which is a new member of the epidermal growth factor superfamily (Jensen *et al.*, 1993, 1994) is described. After a physicochemical purification procedure related to that applied for hFA1, the purified mFA1 showed a broad band upon SDS-PAGE analysis. The purified mFA1 was used for generation of monospecific antibodies which made more efficient purification procedures possible and allowed further characterization of mFA1.

The N-terminal amino acid sequence (39 residues) revealed 74% identity to the corresponding amino acid sequence of hFA1. Human FA1 is the processed and secreted gene product of the cDNA referred to as 'adrenal specific mRNA pG2' and human *dlk*, both of which have been isolated in the context of neuroendocrine tumours (Helman *et al.*, 1990; Jensen, 1992; Jensen *et al.*, 1993; Laborda *et al.*, 1993). The amino acid sequence of mFA1 could be aligned to a translated cDNA sequence of mouse *dlk* and *SCP-1* from a mouse stromal cell line (Maruyama *et al.*, 1995) with 100% identity. Recently, Smas and Sul (1993) isolated a cDNA (*pref-1*) from mouse preadipocytes (3T3-L1) and their results indicated that the gene product of *pref-1* was involved in the differentiation of preadipocytes to adipocytes. Like *dlk* and *SCP-1*, the translated

Fig. 3. Crossed immunoelectrophoretic analysis of the rabbit anti-mouse fetal antigen 1 (mFA1) antiserum after absorption with serum from nonpregnant adult mice. The wells of the first dimensional electrophoresis received 15 μ l mAF (a and c) or 15 μ l normal mouse serum (b and d). The second dimensional gels contained 50 μ l IgG cm^{-2} agarose (a and b) or 10 μ l IgG cm^{-2} (c and d).

		Identity
Mouse FA1	AECDPPCDPQYGFCEADNVCRCHVGFEGPLCDKCVTAPG ..	
Mouse dlk/SCP-1/pref-1 .. leader ..	AECDPPCDPQYGFCEADNVCRCHVGFEGPLCDKCVTAPG ..	100%
Human FA1	AECFPACNPQNGFCEDDNVCRCPGFQGPLCDQCVTSPG ..	74%

Fig. 4. N-terminal sequence (39 residues) of purified mouse fetal antigen 1 (mFA1) aligned to human FA1 (Jensen *et al.*, 1993) and the translated cDNA sequences of *dlk* (Laborda *et al.*, 1993), *pref-1* (Smas and Sul, 1993) and *SCP-1* (Maruyama *et al.*, 1993).

sequence of *pref-1* revealed 100% identity to the N-terminal amino acid sequence of mFA1. These data suggest that mFA1 is the processed, secreted gene product of *mdlk*, which in turn is identical to *pref-1* and *SCP-1*, and the protein isolated from mouse amniotic fluid is the mouse homologue to human FA1. The homology to human FA1 was further substantiated by the results of the immunohistochemical analysis of mFA1 in the pancreas, where the staining reaction was restricted to the endocrine cells of the islets of Langerhans, similar to the

staining pattern observed for hFA1 on adult human pancreas (Jensen *et al.*, 1993). Moreover, positive immunostaining was observed in the adrenal and pituitary glands of adult mice (results not shown) in agreement with our findings on hFA1 (Jensen *et al.*, 1993, 1994; Larsen *et al.*, 1996).

The general biology of FA1 remains to be elucidated but the extensive tissue distribution in the fetus and the more restricted tissue distribution in adults, together with the observed presence in certain cell lines, clearly points to involvement in

Fig. 5. Immunohistochemical analysis of mouse fetal antigen I (mFA1) in the pancreas from an adult mouse using rabbit anti-mFA1 IgG obtained by ammonium sulfate precipitation of absorbed rabbit anti-mFA1 anti-serum. Scale bar represents 40 μm .

differentiation. The secreted cleaved form of mFA1 is the most obvious source to exert this effect and human FA1 has been shown to be a heterogeneous glycoprotein with a molecular mass of 32–38 kDa; the heterogeneity is a result of a ragged C-terminal part of the molecule and glycosylation (Jensen *et al.*, 1994). A similar broad band observed in SDS-PAGE analysis of mFA1 (42–50 kDa) suggests that a similar post-translational modification of mFA1 takes place. The difference in size between the two molecules could be due to differences in glycosylation, position of C-terminal truncation, unfolding of the compact molecule upon reduction in SDS-PAGE analysis or to a difference in amino acid sequence.

The 39 N-terminal amino acids of mFA1 revealed 74% identity to hFA1 but a < 1% immunological crossreaction was produced when hFA1 was applied to the mFA1 ELISA and vice versa. This apparent lack of crossreactivity is most likely due to shared epitopes between rabbit, human and mouse FA1 resulting in the rabbit recognizing those epitopes as 'self'. Only epitopes that are different in human and mice and not shared by the rabbit can result in crossreactive immunoresponse.

Application of the ELISA technique on serum from nonpregnant adult mice revealed a normal range for mFA1 of $11.3 \text{ ng ml}^{-1} \pm 5.0$ (2 SD). The most likely sources for this circulating pool of mFA1 are the adrenal and pituitary glands, and the endocrine cells of the pancreas. However, in the fetal compartment (the mouse amniotic fluid and fetal serum) the concentration of mFA1 was around 1400 times greater than the concentration seen in adult mouse serum, a difference similar to the 1000-fold difference observed between hFA1 in second trimester fluid and normal human serum (Jensen, 1992). During pregnancy the maternal serum concentration of mFA1 increased after mid-pregnancy to a serum concentration around 30-fold greater than that seen in nonpregnant mice. The increased concentration of mFA1 during pregnancy probably originates from the abundant fetal synthesis and secretion of mFA1. This hypothesis is supported by the positive correlation between maternal serum concentration and the number of fetuses. After delivery mFA1 is cleared very rapidly from the

Fig. 6. The concentration of mouse fetal antigen I (mFA1) measured by ELISA. (a) Serum concentration during and after pregnancy. (b) Serum concentration of mFA1 in relation to age (days) of mice. Area between dotted lines indicates normal range ($11.3 \pm 5.0 \text{ ng ml}^{-1}$) for mFA1 in adult mouse serum. (c) Maternal serum concentrations at day 18 of gestation in relation to the number of fetuses.

maternal circulation and in one mouse where the exact time of delivery was recorded we observed a tenfold decrease in mFA1 concentration within 3 h, indicating a disappearance rate with a $t_{1/2} < 1 \text{ h}$. By contrast, the decrease in serum mFA1 in newborn mice was slow and normal adult amounts were not reached until the mice were > 50 days old, suggesting an ongoing role of mFA1 in growth and differentiation.

The extensive distribution of FA1 in the fetus, the presence of mRNA encoding hFA1 in neuroendocrine tumours of the lung (Laborda *et al.*, 1993), and the identification of FA1 in the cytoplasm of small cell lung carcinoma and carcinoid tumours (Jensen *et al.*, 1994) suggest a role of FA1 in regulation of cell differentiation and growth. The presence of FA1 in the adrenal gland (Jensen *et al.*, 1993), pheochromocytoma (Helman *et al.*, 1990), in the pituitary gland and hormone-producing adenomas (Larsen *et al.*, 1996), suggests that FA1 synthesis is secondary to a signal from cells of a neural origin or cells with neuroendocrine potential (Jensen *et al.*, 1993). Moreover, the

extensive distribution of hFA1 in the glandular cells of fetal pancreas compared with adult pancreas, where hFA1 is restricted to the islets of Langerhans, associates hFA1 with the development of the endocrine structures of pancreas (Jensen *et al.*, 1993, 1994; Tornehave *et al.*, 1993).

This possible role of FA1 in differentiation and growth is further substantiated by the similarity between FA1 and the Notch and Delta proteins in *Drosophila* and their mammalian homologues. Notch and Delta proteins are involved in the differentiation of embryonic ectoderm into neural and epidermal structures (Campos-Ortega, 1993; Artavanis-Tsakonas *et al.*, 1995). Ligands for Notch proteins, like Delta and Serrate, contain a characteristic DSL (Delta-Serrate-Lag 2) motif (Tax *et al.*, 1994). Although FA1 bears a close similarity to the Delta protein, this motif is absent from the FA1 amino acid sequence (Jensen *et al.*, 1994), which makes it unlikely that FA1 is a ligand for mammalian Notch homologues.

In conclusion, the present work describes a mouse model for further studies of the biological significance of FA1 and demonstrates that mFA1 is the processed secreted gene product of *mdlk* (Laborda *et al.*, 1993), *pref-1* (Smas and Sul, 1993) and *SCP-1* (Maruyama *et al.*, 1995).

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