Blood Coagulation Factors in Human Embryonic-Fetal Development: Preferential Expression of the FVII/Tissue Factor Pathway


The expression of a number of blood coagulation factors (F) (FX, FIX, FVIII, FVII, α-, β-, γ-fibrinogen chains, protein C, and antithrombin III [AT III]) was analyzed at RNA and protein level in 5- to 10-week-old human embryos and fetuses. FX, FIX, and FVII were also analyzed at protein level. Total and poly(A)+ RNA, extracted from embryonic-fetal (FL) and adult liver (AL), were analyzed by dot and Northern blot hybridization with specific cDNA probes. The results indicate that: (1) the size of the messenger RNAs of these factors is equivalent in FL and AL; (2) in the 5- to 10-week period, their abundance in FL increases from 30% to 50% of the adult level except for FIX (from 2% to 10%) and FX (always 100% of the adult value). Western blot analysis of FIX, FX, and FVII in 5- to 10-week soluble liver proteins and 6- to 8-week plasma showed a low level of FIX versus a higher concentration of both FVII and FX, when compared with corresponding adult values, ie, a liver protein level of 10% versus 100% and a plasma concentration level of 10% versus 40%. Although little is known so far on the activity and the functional role of the clotting factors in early human ontogenic development, these studies suggest an activation of FX via the FVII/tissue factor activity rather than the FIXa/FVIIIa phospholipid complex in human embryonic and early fetal life.

MATERIALS AND METHODS

Human embryos and fetuses. These were obtained virtually intact from legal curettage abortions and maintained in Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY) at 4°C until processing. The women had previously provided fully informed consent for research studies. The age of 5- to 8-week-old embryos was established by morphologic staging according to multiple criteria. Early fetuses (9 to 10 weeks) were staged on the basis of standard age/crown rump length plots. The dating error was as little as ± 2 days. Embryonic-fetal livers (FL) and other organs, dissected under an inverted microscope, were stored in liquid nitrogen.

Adult liver (AL) biopsies. These were obtained for diagnostic purposes from patients with suspected liver disease (eg, chronic hepatitis, liver carcinoma). Fully informed consent was obtained according to the Declaration of Helsinki as amended in Venice (1983). This study includes only samples from patients with normal liver histology and clotting factor levels.

cDNA probes. FX and protein C cDNAs were provided by E.W. Davie (University of Washington, Seattle); fibrinogen chain cDNAs by D.W. Chung (University of Washington, Seattle); a 4.7-kilobase (kb) EcoRI fragment of FVII cDNA by the Genetics Institute (Cambridge, MA); cVII-FIX cDNA by G.G. Brownlee (University of Oxford, UK); AT III and human albumin cDNAs by R. Cortese (EMBL, Heidelberg, FRG).

RNA analysis. Total RNA was extracted from fresh or frozen samples by the guanidinium thiocyanate technique and poly(A)+ selected by one passage on oligo(dT)-cellulose columns.

Northern blot. Total RNA, 10 to 15 μg, or 2 to 4 μg of poly(A)+ RNA were run on 1.0% agarose-formaldehyde gels, transferred to nitrocellulose by capillary blotting, and hybridized to 105 cpm of the cDNA probe labeled by nick translation to a specific activity of 3 x 108 disintegrations per minute/μg. After washing under stringent conditions (0.1 x 0.15 mol/L sodium chloride, 15 mmol/L sodium citrate [pH 7.0] SSC/0.1% sodium dodecyl sulfate [SDS] at 65°C) the blots were exposed to Kodak X-Omat film SO-282 for 1 to 4 days at ~ 70°C in an X-omat intensifying screen cassette. Probes were removed by washing in 50% formamide solution containing 0.1% SDS at 65°C and the filters rehybridized to a different cDNA probe.

Albunin was used as a hepatic marker. A human ribosomal probe (pHR 28A) was used for normalization of total RNA. Embryonic-fetal poly(A)+ RNA level was normalized using β-actin cDNA probe. Because β-actin is expressed at a higher level in fetal than adult samples, this probe was not used for normalization of adult specimens.

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**Dot blot.** Total RNA was dotted onto nitrocellulose filters in graded amounts (5.0 to 0.3 μg) and the baked filters were hybridized as described for Northern blots. The concentration of specific messenger RNA (mRNA) in total RNA was calculated by comparison with a standard curve, obtained with predetermined amounts of a denatured cDNA probe. The filters were scanned with a 2202 Ultrascan Laser Densitometer (LKB, Bromma, Sweden). The mean mRNA levels from 3 to 7 different experiments were evaluated. The data are expressed as percentage of normal adult values.

cDNA libraries. A λ phage gt10 library was prepared with the poly(A)+ RNA from 7-week-old human FL. The library, containing 1.4 × 106 independent recombinants, was screened with FIX and FX cDNA probes. Positive clones were subcloned in pGEM-4Z vector and sequenced using the dideoxy chain terminating method.

**In situ hybridization.** Six- to eight-week-old human embryos were fixed in phosphate-buffered saline (PBS)-parafomaldehyde 4%, coated in PBS-5% sucrose, embedded in O.C.T. compound (Miles Scientific, Naperville, IL) and serially cut into 8-μm sections. The sections were permeabilized by soaking in HCl 0.1N and 0.1 mol/L Tris-HCl (pH 8) containing 50 mmol/mL EDTA and 1 μg/mL proteinase K, then treated with 0.25% (vol/vol) acetic anhydride containing 0.1% mol/L triethanolamine, and prehybridized for 2 hours at 50°C in 50% formamide.

32P- and 35P-labeled FIX and FX single-strand RNA probes (Transcription System, Promega Biotech, Madison, WI) were hydrolyzed to obtain a 50 to 100-base pair length probe. Each slide was hybridized to 106 cpm of the riboprobe, labeled to a specific activity of 1 × 5 × 106 cpm/μg at 50°C for 16 hours in prehybridization buffer containing 10% dextran sulphate and 0.1% SDS (when 35S was used, 20 mmol/L β-mercaptoethanol was also added). After hybridization, the slides were washed in 50% formamide, 1 × SSC at 50°C, and 0.5 × SSC at room temperature, treated with RNase A (20 μg/mL), and then 0.1 × SSC at 60°C for 2 hours. The slides were then dehydrated in scalar ethanol solution and air-dried. 35P-labeled slides were autoradiographed with X-Omat SO-282 Kodak film. Slides labeled with 32S were dipped in emulsion (Kodak) and exposed at 4°C for 3 to 4 weeks in a light-proof box.

**Protein analysis.** Protein samples were extracted from fresh or frozen liver using a buffer solution of 10 mmol/L Tris-HCl pH 7.8, 2.5 mmol/L MgCl2, 0.5 mmol/L dithiothreitol, 1 mmol/L EGTA, 0.5 mmol/L phenyl methylsulfonyl fluoride, 1,000 KIU/mL Trasylol, and 1 mg/mL soybean trypsin inhibitor. Tissues were homogenized at medium speed in a Potter-Elvjeheim grinder for 30 to 60 seconds at 4°C. Sheared homogenates were centrifuged twice at 2,000 rpm for 20 minutes at 4°C.

Fetal cord or heart blood was collected in 3.2% sodium citrate and centrifuged at 10,000 rpm for 10 minutes. The plasma was stored at −70°C until used.

Soluble cytoplasmic fraction and protein levels were measured by a standard assay (Bio-Rad, Richmond, CA). The same amount of protein was fractionated on 10% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose filters in Tris-glycine buffer containing 20% methanol and 0.05% SDS. Filters were blocked before detection procedures by nitrocellulose incubation in 5% nonfat dry milk. Immune reactive proteins were detected by sequential nitrocellulose incubations with commercially available rabbit anti-human antibodies (ASSERA VII, 100 U/mL rabbit serum anti-FVII:Ag; ASSERA IX 20 U/mL rabbit serum anti-FIX:Ag; ASSERA × 10 U/mL rabbit serum anti-FX:Ag; Diagnostica Stago, Asnières, France) and with 125I-protein A (Amersham, UK). The autoradiograms were washed twice with 50 mmol/L Tris-saline (pH 7.4), once with Tris-saline containing 0.05% Triton X-100 and three times with Tris-saline alone. After the final wash, filters were air-dried and autoradiographed using X-Omat SO-282 Kodak film. The filters were scanned with a 2202 Ultrascan Laser Densitometer (LKB).

**RESULTS**

**FIX and FX RNA expression in embryonic-fetal liver.** FIX expression was analyzed by Northern blot of total or poly(A)+ RNA from 5- to 10-week-old FL. The results indicate that this factor is expressed as a single transcript of ~3.0 kb in both FL and AL (Fig 1).

The hybridization signal in FL was always weaker than in AL (Fig 1). However, the level of FIX mRNA slowly increased during the 5- to 10-week period, from 2% to 10% of the adult value (Fig 1). In AL, FIX mRNA represents 0.07% of total mRNA (30 pg/μg).

Filter rehybridization with the FX probe showed a single transcript of 1.7 kb, apparently identical to the corresponding AL mRNA (Fig 2). The intensity of the FL band was constant in the 5- to 10-week period, at a level comparable with that observed in AL (Fig 2). The FX transcript represents 0.01% of total mRNA (5 pg/μg).

Screening of 1.4 × 106 clones from a 7-week FL cDNA library with the FIX and FX probe showed 58 FX+ and two FIX− clones. The FIX+ clones and three FX+ clones were plaque-purified: the nucleotide sequences of the embryonic cDNAs were identical to that of their adult counterparts.

Results obtained by Northern and dot blot analyses were further confirmed by in situ hybridization on human FL sections. These were hybridized with 32P- or 35S-UTP-labeled RNA probes corresponding to the sense and antisense polarity of FIX and FX. The FX antisense probe provided a strong hybridization signal only in the liver (Fig 3, b, d, and e). Sections hybridized with the FX sense probe were negative (not shown). The FIX antisense probe produced a weak hybridization signal in the liver (Fig 3, c, f, and g).

**Fig 1.** Time course of FIX mRNA in total human hepatic RNA from 6 to 12 weeks postconception (lanes 1 through 6) and adult control (lane 8). Ten micrograms of total liver RNA was used for each lane. Ribosomal probe was used as loading control.
...plasma and liver. The size of embryonic-fetal transcripts is identical to that of corresponding adult mRNAs. The nucleotide sequence of FIX and FX mRNA in embryonic-fetal and adult life is also identical.

**DISCUSSION**

These studies shed light on the expression of blood coagulation factors during human ontogenic development.

We report that a variety of coagulation factors are transcribed in embryonic and early fetal human life, ie, at 5 to 10 weeks after fertilization (see Fig 8).

The size of embryonic-fetal transcripts is apparently identical to that of corresponding adult mRNAs. The nucleotide sequence of FIX and FX mRNA in embryonic-fetal and adult life is also identical.

Blood coagulation factors are expressed at lower level in FL than in AL. Detailed analysis of the RNA abundance at 5 to 10 weeks of age showed a similar pattern for the three fibrinogen chains, protein C, and AT III, which is characterized by a gradual increase from 30% to 50% of adult values. In contrast, a strikingly different pattern was observed for FIX and FX: FIX mRNA was expressed at very low level,
Fig 3. Expression of FX and FIX in human embryonic sections. (a) An 8-week-old human embryo Giemsa-stained section; (b and c) autoradiography of human embryo 8-μm sections hybridized in situ with 32P-labeled FX and FIX antisense riboprobes respectively; (d and f) section of a 6-week-old embryo showing bright field image of liver; and (e and g) dark field image of (d) and (f), respectively, after hybridization with 35S-labeled FX and FIX antisense riboprobes (original magnification × 100).
increasing from 2% to 10% of the adult value, whereas the abundance of FX transcripts was always comparable with the adult level. Accordingly, the screening of a 7-week FL cDNA library showed that FX mRNA is expressed ~30 times more frequently than FIX, although in AL the amount of FX is almost 10-fold lower than that of FIX. In situ hybridization of FL also confirmed these data: both FIX and FX are selectively synthesized in FL parenchymal cells, but the positive signal is more pronounced for FX than FIX.

Studies on FIX and FX protein in FL and plasma corroborate the results obtained by RNA analysis (Fig 8). FIX protein is synthesized at very low level in 7- to 8-week FL (less than 10%) and is barely detectable in 7- to 8-week cord plasma. FX is synthesized at higher levels in 7- to 8-week FL than in AL; however, the amount secreted in the 7- to 8-week embryonic plasma is reduced (20% to 40% of the adult value), thus indicating accumulation of FX in the hepatic cells. Similar results were obtained for FVII, with an FL concentration similar to that observed in AL. The correlation of FX and FVII level is not surprising, since the former factor is the main substrate of the latter.

Our results suggest that the expression of blood coagulation factors is modulated during ontogenic development. It is well-known that in adult life all blood coagulation factors are required for normal hemostasis, but little is known about the blood coagulation system in the prenatal period.

In embryonic-fetal life the sharply reduced level of FIX, as compared with higher concentrations of both FVII and FX, may indicate that the early activation of FX is directly mediated via the FVIIa/tissue factor mechanism rather than the FIXa and FVIIIa pathway. In this regard, FVII and
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Fig 8. Expression of blood coagulation factors during ontogenic development. (A) RNA level in the 5- to 10-week postconception period, and (B) protein level at 7 to 8 weeks in liver (□ bar) and plasma (□). Values are percentage of adult level.

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