A Major Fraction of Fibronectin Present in the Extracellular Matrix of Tissues Is Plasma-derived*

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The origin of the fibronectin (FN) found in the extracellular matrix of tissues has not been defined experimentally. Previous studies suggest that there is contribution from both local tissue production and transfer from plasma, but the extent of this phenomenon has not been addressed. We have shown before that engineered mice constitutively expressing extra domain A-containing FN (EDA +/−FN) have a significant decrease of FN levels in plasma and most tissues. We showed that hepatocytes modified to produce EDA +/−FN have normal extracellular matrix-FN levels but secrete less soluble FN. We performed a liver-specific EDA-exon deletion in these animals, FN levels were restored both in plasma and tissues. Therefore, an important fraction of the tissue FN, approximately an equal amount of that produced by the tissue itself, is actually plasma-derived, suggesting that plasma is an important source of tissue FN. The present results have potential significance for understanding the contributions of plasma FN, and perhaps other plasma proteins, in the modulation of cellular activities and in the formation of the extracellular matrix of tissues.

Fibronectins (FN) are a family of multifunctional glycoproteins known to play key roles in fundamental processes related to adhesive and migratory behavior of cells, such as embryogenesis, malignancy, homeostasis, wound healing, and maintenance of tissue integrity (1). FN generates protein diversity as a consequence of alternative processing of a single primary transcript at three different sites, the extra domain A (EDA), the extra domain B (EDB), and the type III homologies connecting segment (IIICS) (2–4). Two major forms of FN exist, plasma FN (pFN) and cellular FN. pFN is a soluble dimeric form that is secreted into the bloodstream by hepatocytes (5, 6) and found at 300 and 580 μg/ml in plasma of humans and mice, respectively (1, 7). pFN lacks both the EDA and EDB domains, whereas cellular FN is locally produced and deposited as insoluble fibrils in the extracellular matrix of tissues and contains these domains at variable proportions (1, 8, 9). Previous studies suggested that circulating pFN contributes to the extracellular matrix of tissues (10, 11) but the extent of the phenomenon has not been addressed.

The levels of FN in plasma are critical for hemostasis, tissue repair, and susceptibility to infections. Depletion of pFN (liver-specific knockout of FN) results in increased brain injury after transient focal cerebral ischemia (12), a delay in thrombus formation and decreased thrombus stability (13), decreased angiogenesis (14), and increased susceptibility to bacterial infections (15). Heterozygous null FN mice appear healthy and fertile (7) but show delayed thrombus growth in injured arterioles (16). Regrettably, the levels of FN present in the tissues of heterozygous null FN and in the pFN null mice have not been reported.

We have previously shown that knock-in mice having constitutive inclusion of the EDA exon of the FN gene (EDA +/+/strain) had up to 70–80% reduction in the levels of plasma and tissue FN (17). Taking advantage of the “floxed” EDA exon present in those mice, we generated liver-specific EDA-null mice (EDA +/+/CRE) after crossing EDA +/+/ animals with a transgenic strain expressing the CRE recombinase only in hepatocytes (18). Consequently, hepatocytes of EDA +/+/CRE mice, without the EDA exon, were able to produce and secrete pFN at normal levels.

We show here that the levels of pFN were restored in those mice and also that the levels of tissue FN were similar to those observed in EDA WT/WT animals. These results showed a major flow of pFN into the extracellular matrix of tissues and suggest the importance of the pFN as an essential source of FN for the tissues. The presented results might have potential significance for understanding the contributions of pFN, and perhaps other plasma proteins, to cellular activities and in the formation of the extracellular matrix of tissues.

EXPERIMENTAL PROCEDURES

**Mice**—The generation and genetic background of the mice devoid of regulated splicing at the EDA exon have been previously described (17). EDA +/+/ mice were mated with the transgenic strain Tg Alf pCRE mice, which have CRE recombinase under the control of the albumin promoter and enhancer (18). EDA +/+/WT mice having the CRE recombinase were mated in order to obtain EDA +/+/ mice with the CRE transgene (EDA +/+/CRE). This strain expresses the CRE recombinase exclusively in hepatocytes (18). The genotype of mice was determined by PCR from tail biopsies.

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RNA Preparation and Reverse Transcription (RT)-PCR Analysis—Total RNA was prepared from freshly extracted tissues and cells as described (19). The radioactive RT-PCR reactions were performed and quantified as previously described (20).

Protein Extracts and Western Blot Analysis—Mice were anesthetized with 2.5% Avertin (300 µl/20 g mouse), and organs were perfused with 25 ml of cold phosphate-buffered saline through the left ventricle of the heart. Organs were immediately dissected and snap-frozen in liquid nitrogen. Organs were homogenized, and protein content was determined by Bradford protein assay (Bio-Rad). Identical amounts of protein sample were run on a 6% SDS-PAGE and analyzed by Western blot with polyclonal rabbit anti-FN antibody (50 µg of protein extract, 1:1500; Sigma), anti-EDA 3E2 monoclonal antibody (100 µg of protein extract, 1:300; Sigma), or anti β-tubulin monoclonal antibody (20 µg of protein extract, E7, 1:3000; Developmental Studies Hybridoma Bank, University of Iowa) as described (17). Three animals per genotype were analyzed. To determine the efficiency of perfusion and elimination of plasma proteins in each of the organs analyzed, a Western blot analysis of 50 µg of protein extract was performed using an anti-mouse IgG antibody (1:2000; DAKO). Serial ECL expositions of the membranes were performed to determine the optimum linear range to quantify the signals. Films were scanned with the Versadoc (Bio-Rad) and quantified with the help of the Quantity One software package (Bio-Rad).

Bile was collected by holding the gallbladder with forceps. Two µl of each sample were mixed with protein loading buffer (0.125 Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.002% bromphenol blue) and boiled for 5 min. Bile FN was analyzed by Western blot as described above.

In Vivo Labeling of Hepatocytes—Hepatocytes were purified by the two-step collagenase perfusion method (21), using Liver Digest Medium (Invitrogen) as described by the manufacturers. Hepatocytes were plated in rat tail collagen for 1 h, washed, and then incubated for 24 h with Met-Cys-free medium supplemented with 300 µCi/ml of [35S]Met/Cys (ProMix; Amersham Biosciences). The supernatant was collected, and a fraction was affinity-purified with gelatin-Sepharose as described (22, 23).

Southern Blot Analysis of Tissues—DNA was extracted from tissues, and 15 µg were digested with HindIII. The DNA was then run in an agarose gel and blotted onto Z-Probe membrane. A probe corresponding to the exon downstream to the EDA exon was used as described (17).

Immunohistochemistry of Tissue Sections—Organs were fixed in 4% formaldehyde and paraffin-embedded. 4-µm sections of each tissue were cut and incubated with affinity-purified polyclonal rabbit anti-FN antibody (1:200; Sigma). Then sections were incubated with biotinylated goat anti-rabbit IgG (5 µg/ml; VectaStain) followed by avidin-biotin-peroxidase mixture (ABC Reagent; Vector Laboratories), 3,3′-diaminobenzidine substrate (Vector Laboratories), and Gill’s hematoxylin. An AS LMD Leica microscope was used to visualize and photograph the sections.

RESULTS

Hepatocytes of EDA+/+ Mice Have Normal Levels of Extracellular Matrix-FN but Do Not Secrete pFN—We have previously observed that mice having constitutive inclusion of the EDA exon of the FN gene (Fig. 1A) had a significant decrease of FN in plasma and in most tissues (17, 20). Further characterization of pFN levels (embryo, young, and adult mice) from EDA+/+ mice showed very low amounts compared with EDA+/− mice showed a significant decrease of FN (Fig. 1, B and C). Embryos had 60% of the pFN levels in the control sample, whereas young and adult EDA+/+ mice showed a higher decrease in pFN levels. The decrease in pFN was due neither to lower levels in mRNA in tissues of EDA+/+ mice (17) nor to a reduced FN production by EDA+/+ tissues, as FN secreted by EDA+/+ embryonic fibroblasts or adult heart fibroblasts was similar to that produced by EDA+/− embryonic fibroblasts.
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The specific degradation of FN in the EDA+/+ mice by proteases was ruled out by a series of experiments: 1) Metalloprotease activity levels in plasma and tissues by gelatin zymography analysis were similar among EDAWT/WT, EDA−/−, and EDA+/− mice (supplemental Fig. S1, A and B); 2) [35S]Met-labeled fragments of FN containing or not containing the EDA exon were not differentially degraded when incubated with EDAWT/WT or EDA+/+ tissue extracts or plasma in the absence of protease inhibitors (supplemental Fig. S1, C and D); 3) no increase in FN degradation rate was observed after mixing protein extracts from EDA+/+ or EDAWT/WT liver with those originating from different organs or plasma from EDA+/+ mice in the absence of protease inhibitors (data not shown); 4) Western blot analysis of tissue extracts done with different sets of anti-FN polyclonal antibodies, run on 5–17% gradient gel, did not show any specific degradation products in EDA+/+ mice (data not shown).

Because the hepatocytes are the source of pFN (5, 6), we performed metabolic labeling of hepatocyte primary cultures from EDAWT/WT, EDA+/+, and EDA−/− livers followed by FN affinity purification of the conditioned medium to analyze pFN production. We observed a decreased secretion of pFN by the EDA+/+ hepatocytes, suggesting that the reduced levels in pFN in the EDA+/+ mice were the consequence of a defect in hepatocytes (Fig. 2, A and B). However, the FN amounts detected by Western blot in hepatocyte cell extracts were similar (Fig. 2C). Additionally, the decrease of pFN in EDA+/+ embryos (Fig. 1B) but not in embryonic tissues (17) confirmed that the deficiency in FN secretion was limited only to EDA+/+ hepatocytes. These results showed that EDA+/+ hepatocytes were unable to secrete pFN in normal amounts. We hypothesized that the reduced levels of tissue FN could be due to the decreased supply of FN from plasma to tissues.

Generation of Liver-specific EDA Null Mice—To determine the extent of FN flow into tissues we restored the capacity of hepatocytes to produce pFN not containing the EDA exon by cross-breeding EDA+/+ mice with a transgenic strain expressing the CRE recombinase only in hepatocytes (18). The aim was to perform a tissue-specific deletion of the EDA exon without modifying the EDA+ allele in other cell types and tissues (Fig. 3, A and B). Southern blot analysis of different tissues from the EDA+/+ mice carrying the liver-specific CRE recombinase (EDA+/+CRE mice) showed CRE-mediated recombination only in the liver (Fig. 3C). The percentage

FIGURE 2. Hepatocytes of EDA+/+ mice do not secrete soluble EDA+ fibronectin. A, hepatocytes from EDAWT/WT, EDA+/+, and EDA−/− animals were isolated, plated, and metabolically labeled with [35S]Met/Cys for 24 h. Identical aliquots of the conditioned medium were run in a 6% SDS-PAGE and autoradiographed. B, equal quantities of the samples prepared in panel A were affinity-purified with a gelatin-agarose resin, eluted, separated in an SDS-PAGE, and autoradiographed. C, total cell extracts prepared from equal number of hepatocytes from EDAWT/WT, EDA+/+, and EDA−/− mice cultured in non-radioactive medium were collected after 24 h and analyzed by Western blot with a polyclonal anti-FN antibody. D, total cell extracts from the hepatocytes used in panel A were collected, and identical aliquots were run in a 6% SDS-PAGE and autoradiographed. Molecular weight markers are indicated.

FIGURE 3. Liver-specific deletion of the EDA exon. A, scheme of the Alf P-Cre transgene. The CRE recombinase (dotted box) is expressed under the control of the mouse albumin enhancer and promoter (dashed box) and the mouse α-fetoprotein enhancer (gray box). Correct polyadenylation was directed by the hGH fragment (empty box). B, partial map of the EDA+ and EDA− FN alleles. The EDA exon is indicated as a dashed box and the lox- P sites as black triangles. HindIII sites (H), the probe used in panel C (black line below +1 exon), the flanking exons (+1 and +1), and the expected size of HindIII-digested fragments are as indicated. C, EDA+/+ mice were crossed with a transgenic strain expressing the CRE recombinase in hepatocytes. Southern blot analysis of the different tissues from EDA+/+CRE mice was performed, and specific recombination was observed only in the liver. EDA+ and EDA− bands are indicated. Liver DNA samples from three mice are shown. Tissues are as indicated: K, kidney; H, heart; L, lung; T, testis; M, skeletal muscle; I, intestine; Ta, tail; B, brain; M, molecular weight markers. D, RT-PCR analysis showing the inclusion/exclusion of the EDA exon in total RNA prepared from liver and purified hepatocytes from the different genotypes. The position of the EDA+ and EDA− bands is indicated.
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FIGURE 4. Plasma and tissues from EDA$^{+/+}$CRE mice have normal FN levels. A, plasma and protein extracts were prepared from EDAWT/WT, EDA$^{+/+}$, and EDA$^{+/+}$CRE mice (3 month-old), and total FN levels were detected by Western blot analysis. The protein load was controlled by detecting $\beta$-tubulin in the same extracts. B, the intensity of the signals in panel A was quantified with the help of the Quantity One software. The ratio between FN and $\beta$-tubulin signals was used for normalization, and results are shown in the bar graph. The ratio obtained in the EDAWT/WT samples was considered 100%. The mean ± S.D. of three independent experiments is shown.

FIGURE 5. A, plasma from EDA$^{+/+}$CRE mice has no EDA-containing FN. Plasma and liver protein extracts prepared from EDAWT/WT, EDA$^{+/+}$, and EDA$^{+/+}$CRE mice were analyzed for the presence of the EDA domain with the 3E2 anti-EDA monoclonal antibody. B, similar levels of EDA$^+$ FN were seen in tissues from all three genotypes. The same protein extracts from testis used in Fig. 4 were analyzed by Western blot with the 3E2 anti-EDA monoclonal antibody. C, the ratio between EDA$^+$ FN and $\beta$-tubulin signals was used for normalization, and results are shown in the bar graph. The ratio obtained in the EDAWT/WT samples was considered 100%. Data are presented as the mean ± S.D. of three independent animals/genotype.
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FIGURE 6. Immunohistochemical analysis of liver, brain, and testis. Tissue sections of liver (A–C), brain (D–F), and testis (G–I) from EDAWT/WT (A, D, and G), EDA+/− (B, E, and H), and EDA+/-CRE mice (C, F, and I) were stained with an affinity-purified polyclonal anti-FN antibody. The black arrows indicate FN in the extracellular matrix of EDAWT/WT and EDA+/-CRE mice from different tissues and EDA+/- liver. White arrows indicate the decrease in levels of FN from the same areas of brain and testis only in EDA+/- mice. S, sinusoids; BM, basement membrane; and IR, interstitial region, are indicated by black triangles. The black bars correspond to 50 μm.

differences of FN levels among the different strains were not due to residual contamination of pFN.

**Immunostaining of Tissue Sections Confirmed the Decrease of FN Levels in EDA+/- in Tissues and the Recovery in Tissues of EDA+/-CRE Mice**—The above results were confirmed by immunohistochemical analysis of tissue sections. Similar levels of FN-specific signal were detected in the sinusoids of liver samples prepared from all three genotypes (Fig. 6, A–C, black arrows). In the brain sections of EDAWT/WT and EDA+/-CRE mice a stronger FN-specific signal, probably associated with the cell surface of glial cells (25–27), was observed when compared with the EDA+/- mice (Fig. 6E, white arrows). Similarly, in testis tissue sections, a stronger FN-specific signal was observed in the basement membrane of seminiferous tubules and interstitial regions of EDAWT/WT and EDA+/-CRE mice compared with EDA+/- samples (Fig. 6H, white arrows). However, we did not observe intracellular accumulation of FN in the tissue sections of EDA+/- mice by immunohistochemical analysis (Fig. 6, B, E, and H) or in primary culture of hepatocytes either by FN immunofluorescence (supplemental Fig. 3) or by metabolic labeling (Fig. 2D).

Gross histology of tissue samples from all three genotypes was similar, suggesting that the FN synthesized locally is sufficient to maintain the normal tissue architecture. These results indicate that there was a flow of FN from plasma to the extracellular matrix of tissues and plasma is an important source of tissue FN.

**DISCUSSION**

Our data demonstrate an important and novel role for plasma proteins, in particular that of fibronectin, in the formation of the extracellular matrix of tissues and, probably, in the modulation of cellular activities in tissues. The concept that there is FN flow from plasma into tissues or extracellular matrix of cells has been known for a long time. Addition or injection of soluble FN into the culture medium of cells or into the plasma of mice, respectively, resulted in the incorporation of FN into the extracellular matrix (10, 11, 28–30). However, the magnitude of the contribution of pFN to the extracellular matrix was not possible to address with either model. In the present report we have shown that in some tissues up to 60% of the fibronectin present in the extracellular matrix could be plasma-derived.

Sakai et al. (12) have recently shown that pFN supports neuronal survival and reduces brain injury following transient focal cerebral ischemia, suggesting the incorporation of pFN into the injured brain. Our results confirmed and extended their observations to non-injured tissues as we showed pFN incorporation into most normal organs, including brain. In fact, we are also demonstrating that this is a general mechanism that occurs in most normal tissues, and we suggest that other plasma proteins could also become incorporated into the extracellular matrix of tissues, modulating cellular activities.

Because FN is found both in blood and bile fluids, secretion of FN by hepatocytes seems not to be polarized as proposed for endothelial cells (31). Furthermore, we also observed a decrease in FN levels in the bile fluid of EDA+/- mice (data not shown), suggesting the absence of an EDA-dependent polarization of FN secretion in hepatocytes, as postulated for airway epithelial cells (32). The low levels of soluble FN in the plasma of EDA+/- mice and intermediate levels in EDAWT/WT and EDA+/- mice (data not shown) point toward the existence of a mechanism, analogous to that observed for the secretion of the IIICS variants (33), that detects the EDA domain during the secretory pathway of pFN from hepatocytes and prevents the release of EDA+FN into the bloodstream. The "defective" EDA+/EDA- pFN dimers might be formed but their transit through the pFN secretory pathway might be slower compared with EDA-EDA- dimers. However, immunostaining of tissue sections, immunofluorescence of primary culture of hepatocytes, or metabolic labeling of hepatocytes did not reveal any intracellular accumulation of FN in the liver of EDA+/- mice, suggesting that the putative "misfolded" dimers are quickly degraded. We observed that the amount of pFN supplied to the extracellular matrix of tissues varied among the different organs analyzed, suggesting that there might be equilibrium between the amount of FN locally produced by the tissues and the availability of cellular receptors to incorporate FN from the plasma pool.
To conclude, the presented results might have potential significance for understanding the contributions of pFN, and perhaps other plasma proteins, to cellular activities and in the formation of the extracellular matrix of tissues, as we have shown that a major fraction of tissue FN is plasma-derived. Because plasma provides approximately an equal amount of FN as the tissue itself, we suggest that plasma should be considered an important source of FN for tissues. Furthermore, hepatocytes have normal levels of FN in the extracellular matrix but do not secrete soluble EDA FN, suggesting the existence of separate secretory pathways for soluble and fibrillar FN.

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