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Ectodysplasin A in Biological Fluids and Diagnosis of Ectodermal Dysplasia

J. Podzus[1*], C. Kowalczyk-Quintas[2*], S. Schuepbach-Mallepell[2], L. Willen[2], G. Staehlin[2], M. Vigolo[2], A. Tardivel[2], D. Headon[3], N. Kirby[4], M.L. Mikkola[5], H. Schneider[1], and P. Schneider[2]

Abstract

The tumor necrosis factor (TNF) family ligand ectodysplasin A (EDA) is produced as 2 full-length splice variants, EDA1 and EDA2, that bind to EDA receptor (EDAR) and X-linked EDA receptor (XEDAR/EDA2R), respectively. Inactivating mutations in *Eda* or *Edar* cause hypohidrotic ectodermal dysplasia (HED), a condition characterized by malformations of the teeth, hair and glands, with milder deficiencies affecting only the teeth. EDA acts early during the development of ectodermal appendages—as early as the embryonic placode stage—and plays a role in adult appendage function. In this study, the authors measured EDA in serum, saliva and dried blood spots. The authors detected 3- to 4-fold higher levels of circulating EDA in cord blood than in adult sera. A receptor binding-competent form of EDA1 was the main form of EDA but a minor fraction of EDA2 was also found in fetal bovine serum. Sera of EDA-deficient patients contained either background EDA levels or low levels of EDA that could not bind to recombinant EDAR. The serum of a patient with a V262F missense mutation in *Eda*, which caused a milder form of X-linked HED (XLHED), contained low levels of EDA capable of binding to EDAR. In 2 mildly affected carriers, intermediate levels of EDA were detected, whereas a severely affected carrier had no active EDA in the serum. Small amounts of EDA were also detectable in normal adult saliva. Finally, EDA could be measured in spots of wild-type adult or cord blood dried onto filter paper at levels significantly higher than that measured in EDA-deficient blood. Measurement of EDA levels combined with receptor-binding assays might be of relevance to aid in the diagnosis of total or partial EDA deficiencies.

Keywords: saliva, glycosylation, serum, genetic disease, X-linked, dried blood spot analysis

Introduction

Ectodysplasin A (EDA) is a tumor necrosis factor (TNF) family member important for the development of ectodermal appendages in vertebrates (Kere et al. 1996; Srivastava et al. 1997; Monreal et al. 1998; Harris et al. 2008). EDA deficiency in humans causes X-linked hypohidrotic ectodermal dysplasia (XLHED), a congenital disorder characterized by hypoplastic hair, teeth and sweat glands (Visinoni et al. 2009). The EDA protein consists of a short intracellular domain, a membrane spanning segment, a stalk region, a consensus furin cleavage site, a short proteoglycan-interacting sequence, an oligomerizing collagen domain and a C-terminal TNF homology domain that forms homotrimers and binds to receptors (Chen et al. 2001; Schneider et al. 2001a; Swee et al. 2009). Two EDA splice variants differing by only 2 amino acids (Yan et al. 2000) bind to distinct receptors: EDA1 binds to EDA receptor (EDAR), and EDA2 binds to X-linked EDAR (XEDAR/EDA2R) (Tucker et al. 2000; Yan et al. 2000). EDA1-EDAR interactions mediate the development of ectodermal appendages, whereas EDA2-XEDAR may serve as downstream effectors of the p53-induced anti-proliferative response in colon cancer (Tanikawa et al. 2010). Fully or partially inactivating mutations in *Eda* provoke XLHED and non-syndromic tooth agenesis, respectively (Mues et al. 2010). Remarkably, human, dog, cow and mouse EDA are 100% identical in their 145 amino acid-long receptor-binding domains, 98% identical to chicken EDA and 63% identical (82% similar) to a fish EDA (*Gasterosteus acuelatus*). During hair placode formation in mice, EDA is one of the most apical signaling molecules downstream of Wnt. EDA is in turn essential, via NF-κB activation, for sustained Wnt activity (Zhang et al. 2009). Primary hair placodes do not form in *Eda*-deficient mice, teeth are present but are small and abnormally shaped, sweat glands are

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[1]Department of Pediatrics, University Hospital Erlangen, Germany
[2]Department of Biochemistry, University of Lausanne, Epalinges, Switzerland
[3]Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, UK
[4]Edimer Pharmaceuticals, Cambridge, MA
[5]Developmental Biology Program, Institute of Biotechnology, University of Helsinki, Helsinki, Finland
*Authors contributing equally to this article.

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Corresponding Author:
P. Schneider, Department of Biochemistry, University of Lausanne, Boveresses 155, CH-1066 Epalinges, Switzerland.
Email: pascal.schneider@unil.ch

missing, and a number of other glands are either absent or reduced (Grüneberg 1971).

Transgenic expression of EDA1 in the skin of wild-type mice induces supernumerary tooth and mammary gland formation, longer hair and nails, and enlarged sebaceous glands (Mustonen et al. 2003). In *Eda*-deficient mice, the transgene stimulates the formation of guard hairs, sweat glands and induces sebaceous gland hypertrophy (Cui et al. 2003). Repression of transgenic EDA1 in adult mice normalizes sebaceous glands, indicating a limiting role of EDA1 also in some adult structures (Cui et al. 2003). The prenatal or perinatal, but not adult, administration of recombinant EDA1 or agonist anti-EDAR antibodies to *Eda*-deficient mice or dogs can ameliorate the EDA-deficient phenotype, paving the way for protein replacement therapy in XLHED (Gaide and Schneider 2003; Casal et al. 2007; Kowalczyk et al. 2011). Thus, early diagnosis of EDA-deficiency in humans is warranted, particularly since an EDA replacement drug has been under investigation for use in newborn infants with XLHED (www.clinicaltrials.gov NCT01775462 and NCT01992289). However, early diagnosis of XLHED is not trivial. If family history suggests a risk, and if this is known to the doctor, diagnosis can sometimes be established prenatally when ultrasound examination reveals a significant deficit of tooth germ formation (Wunsche et al. 2015), or at birth, when an array of clinical features, such as an absence of sweat gland openings in the epidermis, skin dryness or specific craniofacial abnormalities is observed. Sometimes, diagnosis is only made in the second year of life upon failure of tooth eruption. Definitive evidence of XLHED as opposed to other forms of ectodermal dysplasia or tooth agenesis is provided by identification of the causative mutation in *Eda* (Schneider et al. 2011; Burger et al. 2014).

In this study, we measured EDA in serum, saliva and dried blood spots. We report the detection of circulating EDA, which is capable of binding to receptors and contains the collagen domain, and a predominance of the EDA1 over the EDA2 isoform. EDA levels are high in fetuses and newborns, lower in adults, and very low to absent in XLHED patients, in line with the known roles of EDA during development, adulthood and disease. These results indicate the possibility of diagnosing XLHED by measuring EDA and its receptor-binding capacity in biological fluids.

Materials and Methods

Animals

K14-*Eda-A1* transgenic mice, *Edar*-deficient OVE1B mice, *Eda*-deficient *Tabby* mice and their wild-type controls were as described (Mustonen et al. 2003; Kowalczyk et al. 2011; Kowalczyk-Quintas et al. 2014b). Mice were handled according to guidelines and under the authorization of the Swiss Federal Food Safety and Veterinary Office (authorization 1370.6 to PS) or in accordance with the guidelines and with approval from the Finnish National Board of Animal Experimentation.

Human Samples

Unstimulated saliva and serum samples were obtained from adult patients affected by XLHED, carriers of *Eda* mutations or non-affected controls (age range, 21 y to 52 y for all groups). Sera were also prepared from cord blood of neonates or from cord blood of pre-term babies. In some cases, blood was applied directly onto filter paper cards for blood sampling (Perkin-Elmer). Samples were obtained with informed consent at the University Hospital of Erlangen. All samples were stored at -70°C. Analyses were performed in Lausanne under the approval of the Commission cantonale d’éthique de la recherche sur l’être humain, Lausanne.

Antibodies, Recombinant Proteins and Plasmids

Anti-EDA antibodies EctoD2 and biotinylated EctoD3, EDAR-Fc, XEDAR-Fc and BMCA-Fc were as previously described (Schneider et al. 2001a; Schneider et al. 2001b; Kowalczyk-Quintas et al. 2014b). Fc-EDA1 (EDI200) was provided by Edimer Pharmaceuticals. Anti-EDA monoclonal antibody Renzo-2 is commercially available (Enzo Life Sciences, ALX-522-038). Sequences of proteins encoded by plasmids used for this study are listed in Appendix Table 1.

Affinity Purification of EDA

EctoD2 and EctoD3 coupled at 5 mg/mL to NHS-activated Sepharose (GE Healthcare) were used to capture EDA in 500 mL of fetal calf serum. The complex was washed extensively with PBS and eluted with 50 mM citrate-NaOH, pH 2.7. The eluate was neutralized with 1 M Tris-HCl, pH 9, concentrated, and the buffer was exchanged for PBS with a 30-kDa cutoff centrifugal concentrator device.

Deglycosylation

Denatured EDA samples were digested with peptide N-glycanase F according to manufacturer’s instructions (New England Bio Labs).

Immunoprecipitations and Western Blot

EDA was immunoprecipitated for 16 h at 4°C with 10 µL of Protein A-Sepharose beads and 1 µg of EDAR-Fc or of XEDAR-Fc in 500 µL of PBS. Beads were collected in mini-columns (Schneider et al. 2014), washed with PBS, and eluted with 15 µL of 100 mM citrate-NaOH pH 3. The eluate was neutralized with 5 µL of 1 M Tris-HCl, pH 9. EDA was also incubated for 16 h at 4°C with 10 µL of heparin-Sepharose beads in 500 µL of PBS. Beads were washed with PBS and eluted with PBS containing 0.8 M NaCl. Western blotting was performed according to standard protocols. Samples were heated in denaturing and reducing conditions for 5 min at 70°C because EDA tends to aggregate when boiled. Western blots were revealed with Renzo-2 at 1 µg/mL, followed by

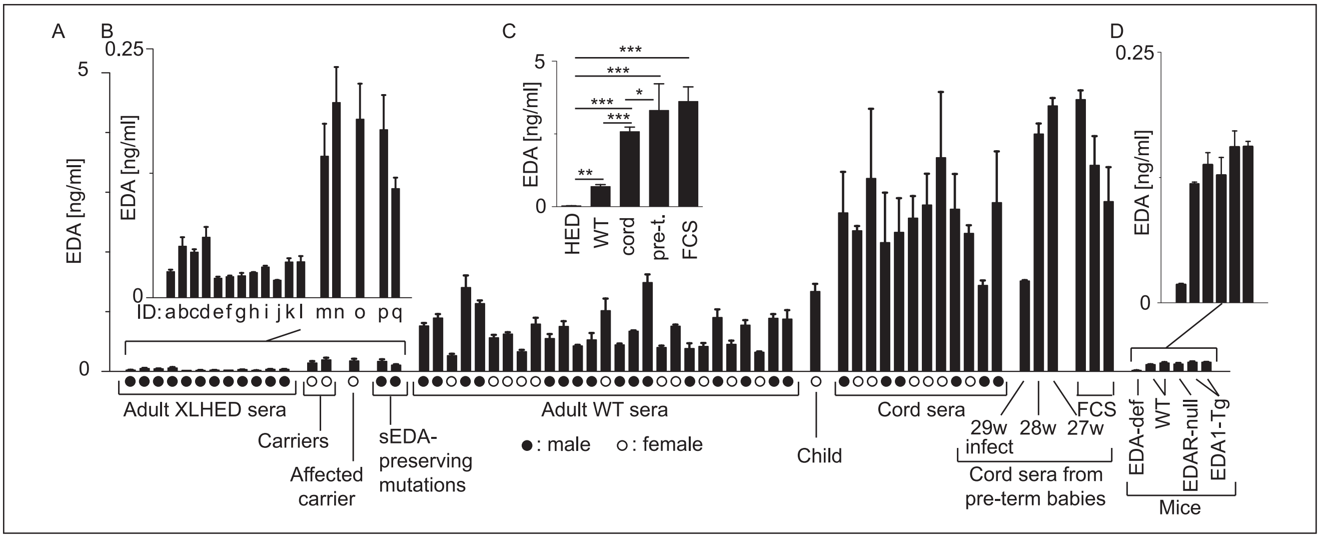

Figure 1. Human EDA levels are higher in cord blood than in the adult circulation. (**A**) EDA was measured in human sera, with Fc-EDA1 diluted in EDA-deficient human serum used as a standard. EDA was also measured in fetal calf sera, and in sera of adult mice of the indicated genotypes. The pre-term baby at 29 wk suffered from a generalized infection (infect). The child was 7 y old. Mean of duplicates ± SEM. Black circles: males. White circles: females. (**B**) Enlargement of the graph with XLHED and related sera. Letters refer to sample IDs in Table 1. (**C**) Comparison of the mean ± SEM of XLHED (HED; $n = 12$), wild-type adult (WT; $n = 27$), cord blood ($n = 12$), pre-term ($n = 3$) and fetal calf ($n = 3$) sera. A one-way ANOVA with Bonferroni's multiple comparison tests was performed. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (**D**) Enlargement of the graph with mouse sera. def: deficient. Tg: transgenic. This experiment was performed twice with comparable results.

horseradish peroxidase-coupled anti-mouse secondary reagent and ECL. When required, the membrane was probed again with horseradish peroxidase-coupled anti-human IgG.

AlphaLISA

Samples of 5 µL (e.g., serum-diluted 1/2 or undiluted saliva with or without pre-depletion on ELISA plates coated with BCMA-Fc, EDAR-Fc or EctoD2) were mixed with biotinylated EctoD3 at 15 ng/mL and 0.5 µg of EctoD2 acceptor beads, and the signal was recorded with an Enspire plate reader (Perkin-Elmer). Standard curves were generated with Fc-EDA1 diluted into buffer or into XLHED serum or saliva, as indicated. For the measurement of EDA in dry blood spots, blood was eluted from filters papers in PBS, then immunoprecipitated with 0.2 µL of EctoD2 acceptor beads for 1 h at room temperature prior to measurement by AlphaLISA (PerkinElmer). Further details are available in the online Appendix.

Gel Permeation Chromatography

Serum (300 µL) from human cord blood with high EDA content, or from normal human serum with low EDA content, were loaded onto a Superdex 200 column equilibrated and eluted in PBS. Fractions of 1 mL were collected and EDA was measured in these fractions by AlphaLISA.

Statistics

Statistics were performed by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison tests using Prism. P values lower than 0.05 were considered significant.

Results

Specific Detection of EDA in Serum Reveals Higher Circulating EDA Levels in the Fetal and Newborn Stages Compared with Adult

ELISA experiments using a previously characterized pair of mouse IgG1 anti-EDA antibodies (EctoD2 and EctoD3) (Kowalczyk-Quintas et al. 2014b) indicated the feasibility of detecting endogenous EDA in adult serum (Appendix Fig. 1). When used in a homogenous AlphaLISA assay (Eglen et al. 2008), this pair of antibodies detected a standard of Fc-EDA1 diluted in EDA-deficient serum with a sensitivity of about 0.1 ng/mL in as little as 2.5 µL of serum. Signals obtained in a panel of XLHED sera were consistently very low (Fig. 1A, B). In this cohort of XLHED patients, the majority is not expected to produce any soluble EDA protein as a consequence of a frameshift mutation, exon deletion or duplication, or splice site mutation or other mutations affecting the furin cleavage site (Table 1). In 2 patients with point mutations in the extracellular domain that may preserve the EDA protein, EDA levels were higher (0.15 ng/mL) and similar to those found in 3 carriers, one of which was severely affected by XLHED. These levels remained lower than the average EDA level in adult sera (0.7 ng/mL) or in cord blood sera (2.5 ng/mL) (Fig. 1B). We found no gender differences for EDA levels in wild-type humans, neither at birth nor in adults. EDA levels reached 4 ng/mL in cord blood sera of 2 pre-term babies, similar to that measured in fetal calf serum. EDA was present at only 1.5 ng/mL in another pre-term baby whose pre-term delivery was due to a generalized infection. Taken together, these results indicate that circulating EDA levels are significantly higher in premature

Table. Genotypes of Subjects with an EDA Mutation.

Phenotype	Fluid	ID	Mutation in Coding DNA of EDA1	Mutation in EDA1 Protein
XLHED	Serum	a	64_71dupAGCCAGGG	C25AfsX35
XLHED	Serum	b	467G>A	R156H
XLHED	Serum	c	4G>T	G2C
XLHED	Serum	d	707-2A>T	Splice site mutation
XLHED	Serum	e	821G>A	W274X
XLHED	Serum	f	Deletion of exons 4–8	
XLHED	Serum	g	Deletion of exon 2	
XLHED	Serum	h	457C>T	R153C
XLHED	Serum	i	457C>T	R153C
XLHED	Serum	j	Deletion of exon 1	
XLHED	Serum	k	457C>T	R153C
XLHED	Serum	l	Duplication of exon 2	
Carrier	Serum	m	81T>A	C27X (+WT)
Carrier	Serum	n	871G>A	G291R (+WT)
Affected carrier	Serum	o	449_456del8	E150AfsX6 (+WT)
XLHED	Serum	p	1091T>G	M364R
XLHED (mild)	Serum	q	784G>T	V262F
XLHED	Saliva	r	467_468del2	R156QfsX2
XLHED	Blood	r	467_468del2	R156QfsX2

and newborn babies than in adults, and very low or at background levels in EDA-deficient XLHED patients (Fig. 2C).

Low Levels of Circulating Murine EDA

EDA levels in wild-type adult mouse serum were low (0.13 ng/mL) but higher than those in an EDA-deficient serum. Curiously, circulating EDA levels in EDAR-deficient or in EDA1-transgenic mice, where the transgene is expressed in the skin under a keratin-14 promoter, were not different from that of wild-type (Fig. 1D).

A Fraction of Circulating Wild-Type EDA Is Capable of Binding to EDAR

Antibodies used in this study recognize and inhibit native forms of both EDA1 and EDA2 and therefore cannot distinguish between the protein products of these splice variants (Kowalczyk-Quintas et al. 2014b). EDAR, however, specifically binds to EDA1, as does its recombinant protein EDAR-Fc (Yan et al. 2000; Schneider et al. 2001a). Using a set of 3 different pre-depletions, including one on EDAR-Fc, it was possible to estimate that about three-quarters (52% to 94%) of wild-type EDA in the circulation can bind to EDAR. We have not been able to further characterize the fraction of EDA that does not bind to EDAR-Fc: this could be inactive EDA, EDA in complex with endogenous soluble receptors, or heteromers of EDA1 and EDA2. Pre-depletion with EDAR-Fc was also successful in 2 carriers and in a patient with the V262F point mutation (Fig. 2A–C). The later XLHED patient was rather mildly affected, with oligodontia and moderately reduced ability to sweat. Binding to EDAR was, however, defective in a severely affected carrier, in a patient with the M364R point mutation and in a few XLHED patients with EDA levels slightly above background, suggesting that these circulating EDA molecules are not functional (Fig. 2B, C). The patient with the M364R mutation is severely affected, with only 5 teeth and very little ability to sweat. In conclusion, the quantification of serum EDA protein concentration is in itself a valuable biomarker of EDA function; however, combined with the measurement of EDAR-binding activity, this measurement can further distinguish between hypomorphic and fully inactivating mutations.

Detection of EDA in Saliva and in Dry Blood Spots

Low levels of EDA, which could be depleted on EDAR-Fc, were detected in wild-type but not in XLHED adult saliva (Appendix Fig. 2). Interestingly, signals obtained with recombinant Fc-EDA1 were 4- to 5-fold lower in EDA-deficient serum as compared with saliva or assay buffer (Fig. 2D). Thus, as exemplified for fetal calf serum, EDA in serum is underestimated about 5-fold (Appendix Fig. 2B) unless the standard curve is also measured in (EDA-deficient) serum (Fig. 1A). Moreover, EDA could be detected in dried blood samples. For this purpose, dried blood was eluted from filter paper in PBS, then immunoprecipitated with EctoD2-coupled AlphaLISA acceptor beads to circumvent the quenching effect of concentrated hemoglobin, and measured for EDA by AlphaLISA. In this procedure, the yield of Fc-EDA immunoprecipitation was about 50%, recovery of dry Fc-EDA from filter paper was about 33%, and the combined yield of both procedures approached 20% (Appendix Fig. 2). With this protocol, signals were obtained in wild-type adult blood and wild-type cord blood eluted and immunoprecipitated from 6-mm diameter filter paper punches, and only background signals were found

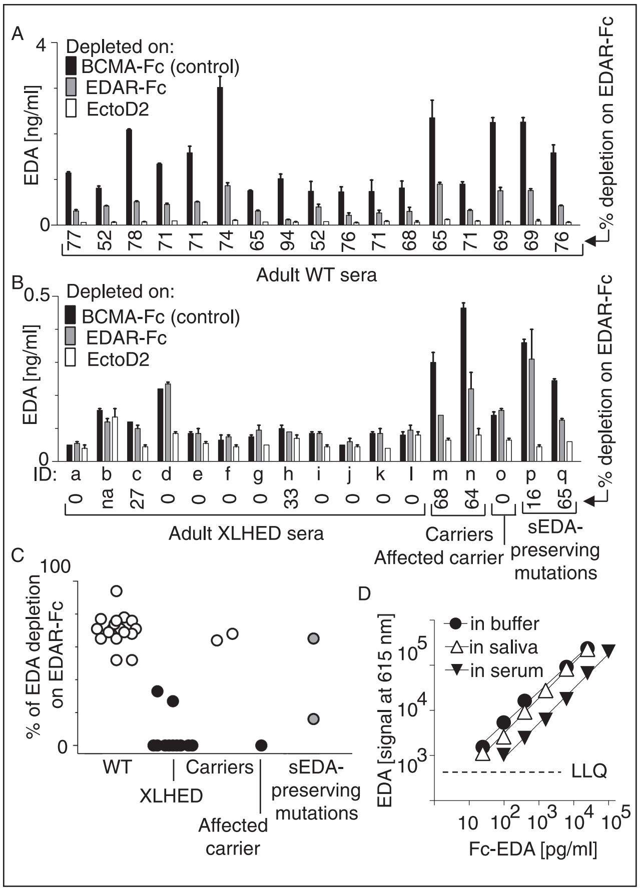

Figure 2. Wild-type, but not mutated, EDA in serum can bind to EDAR. (**A**) EDA levels in normal adult sera after pre-depletion on a control protein (BCMA-Fc), on EDAR-Fc, or on EctoD2. Percentages of depletion with EDAR-Fc are indicated at the bottom. Mean of duplicates ± SEM. (**B**) Same as panel A but for adult XLHED and related sera. Letters refer to sample IDs in Table 1. Mean of duplicates ± SEM. Percentage depletion was not calculated for patient "b", because the lack of depletion on EctoD2 questioned the origin of this signal. (**C**) Scatter plot representation of the percentage of EDA depletion on EDAR-Fc for the indicated groups of persons. (**D**) AlphaLISA was used to measure Fc-EDA1 at different concentrations is assay buffer (black circles), or in saliva of an XLHED patient (opened triangles), or in serum of an XLHED patient (black inverted triangles). The lower limit of quantification (LLQ) is shown by a dotted line. Experiments in panels A–D were performed once in the format presented. Similar results were obtained with the analysis of subsets of samples. Mean of duplicates ± SEM.

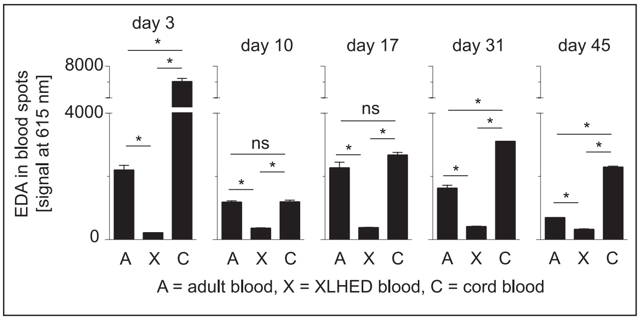

Figure 3. Detection of endogenous EDA in blood dried onto filter paper. Blood from a normal adult (A), blood from an adult XLHED patient (X), and normal cord blood (C) were dried onto filter paper. After 3, 10, 17, 31 and 45 d, blood was eluted from 6-mm diameter punches of blood-impregnated filter paper, immunoprecipitated with EctoD2-coupled AlphaLISA acceptor beads and tested for the presence of EDA. This experiment was performed once in this format. Mean of duplicates ± SEM.

with an adult XLHED blood processed in parallel (Fig. 3C). These measures, repeated at different time points after blood collection, always showed higher EDA signals in wild-type adult or cord blood than in the control XLHED blood (R156QfsX2) from 3 days to more than 1 mo after blood collection (Fig. 3C). EDA signals detected in cord blood were sometimes, but not always, higher than those of adult blood. The qualitative nature of these results perhaps reflects a suboptimally normalized procedure. In conclusion, these results demonstrate the feasibility of measuring EDA in dried blood spots and in saliva.

Circulating Bovine EDA is N-glycosylated, Contains the Collagen Domain and Exists Predominantly as the EDA1 Isoform with a Minority of the EDA2 Isoform

Circulating endogenous EDA was affinity-purified from fetal calf serum with anti-EDA antibodies and then immunoprecipitated with recombinant EDAR-Fc or XEDAR-Fc. Flag-tagged forms of EDA1 and EDA2 used as controls bound to EDAR-Fc and XEDAR-Fc, respectively (Fig. 4A). Endogenous EDA from 2 independent EDA preparations precipitated preferentially with EDAR-Fc and to a lesser extent with XEDAR-Fc (Fig. 4A). Endogenous EDA migrated with a molecular weight of about 35 kDa under denaturing conditions, larger than Flag-EDA1, which contains only the TNF homology domain but at the size of transfected, naturally processed full-length EDA1, suggesting that both the collagen and TNF homology domains are present in the circulating form. Deglycosylation with peptide N-glycanase F reduced the size of endogenous EDA from 35 to 29 kDa, and that of a minor fragment of 22 kDa to 18 kDa (Fig. 4B). This smaller fragment probably corresponds to the TNF homology domain. Recombinant EDA is not fully N-glycosylated, generating a characteristic doublet in the Western blot (Fig. 4A) (Schneider et al. 2001a), whereas endogenous EDA is almost fully N-glycosylated. The absence of intermediate glycosylation products between the 22 and 18 kDa bands in partially digested EDA further suggests that endogenous EDA, like transfected EDA expressed in cultured cells, carries a single N-linked glycan (Fig. 4B).

Endogenous EDA and naturally cleaved EDA both bound to heparin-Sepharose, whereas a mutant of the proteoglycan-binding domain showed minimal binding (Fig. 4C). Flag-tagged EDA1 and EDA2 lacking the proteoglycan-binding domain also did not bind to heparin-Sepharose (Fig. 4C). These data suggest that endogenous EDA has a functional proteoglycan-binding domain. Finally, the native size of human EDA in cord blood serum was estimated by size exclusion chromatography to be

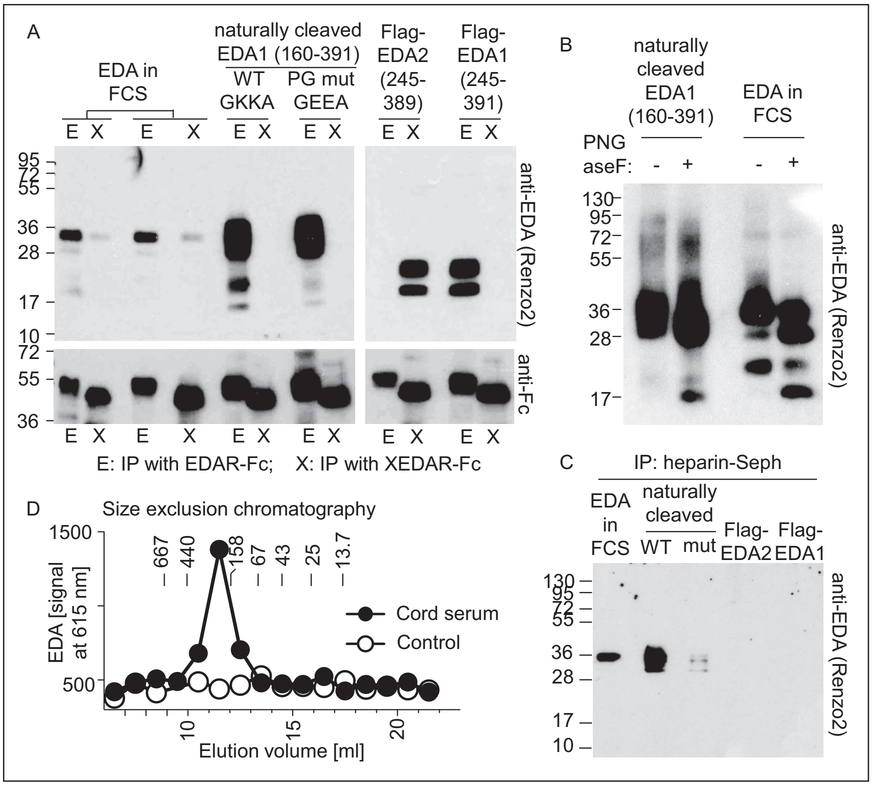

Figure 4. Biochemical characterization of endogenous EDA in human and bovine sera. (**A**) EDA from fetal calf serum was affinity-purified on immobilized EctoD2 and EctoD3, whereas recombinant EDA (naturally cleaved or Flag-tagged) expressed in 293T cells was collected in conditioned supernatant. EDA proteins were immunoprecipitated with EDAR-Fc (E) or XEDAR-Fc (X) and analyzed by Western blot with anti-EDA Renzo2 (top panels). EDAR-Fc and XEDAR-Fc were detected by re-probing the membrane with anti-Fc. Amino acid residues of EDA are indicated in brackets. PG mut: mutated proteoglycan-binding domain. GKKA, GEEA: relevant wild-type and mutated sequences in the proteoglycan-binding domain of EDA. (**B**) EDA proteins were (partially) digested with or without peptide N-glycanase F (PNGaseF) and analyzed by anti-EDA Western blot. (**C**) The indicated EDA proteins were captured on heparin-coupled beads, eluted with salt, and analyzed by anti-EDA Western blot. Mut: mutated proteoglycan-binding site. (**D**) Gel filtration elution profiles of human sera with high (cord serum) or low (adult serum) EDA content. EDA was detected in fractions by AlphaLISA (single measures). Elution positions of molecular weight standards (in kDa) are indicated. EDA concentration in a fraction of the elution of control serum was probably below the detection limit. Experiments of panels A–D were all performed 3 times with similar results.

~200 kDa (Fig. 4D). A calculated size ratio of 5.7 between the native and denatured EDA suggests that EDA circulates in blood as a hexamer or, if the collagen domain confers to the protein a rod-like shape, as a non-globular trimer. In summary, endogenous bovine EDA is a soluble glycoprotein containing the proteoglycan-binding, collagen and TNF homology domains, with a predominance of the EDA1 over the EDA2 isoform. Its posttranslational modifications appear to be more homogeneous that those of transfected EDA expressed in cultured cell lines.

Discussion

In this study, high levels of EDA were detected in sera of fetuses and newborns, in good agreement with the known timing of EDA function in the development of ectodermal appendages (Mikkola 2008). Lower levels of EDA were also found in adult sera and only background levels in XLHED sera. The size of endogenous EDA under native and denaturing conditions, its glycosylation and receptor binding patterns correspond to what we know about the protein (Kowalczyk-Quintas and Schneider 2014).

Expression of *EDA* mRNA has been reported in various adult human tissues (Montonen et al. 1998) and, in adult mice, EDA and EDAR have been implicated in regulation of the hair cycle (Fessing et al. 2006). In addition, hair-associated sebaceous glands respond to EDAR stimulation, suggesting that sebaceous glands are likely targets of EDA in the adult (Kowalczyk-Quintas et al. 2014a). Other tissue candidates for the action of EDA in adults are salivary glands (Hill et al. 2014) and wounded skin (Garcin et al. 2016), both of which respond to EDAR agonists. It therefore makes sense to find EDA expressed in adults.

The measurement of EDA combined with pre-depletion on recombinant receptors showed that the main circulating isoform is EDA1 but that lower levels of EDA2 are also present in fetal calf serum. In principle, depletion of sera with recombinant XEDAR should give direct information on the proportion of circulating EDA2; however, in practice, the reduction observed was too low for reliable interpretation. The co-existence of EDA1 and EDA2 in serum is interesting in terms of possible heteromer formation. In the TNF family, heteromers of lymphotoxin-α/lymphotoxin-β as well as heteromers of BAFF/APRIL, whose receptor binding specificities can be distinct from those of homotrimers, have been structurally characterized (Sudhamsu et al. 2013; Schuepbach-Mallepell et al. 2015). It will be interesting in the future to investigate whether EDA1/EDA2 heteromers can be produced, and, if so, determine the receptor(s) to which they bind.

In the adult, EDA expression has been described in skin epithelium, hair follicles and teeth but also in organs where EDA has no known functions, such as the central nervous system, kidney or prostate (Montonen et al. 1998). The origin of circulating EDA reported here in 3 different species remains to be determined. It may not originate exclusively from the skin because mice overexpressing EDA1 in the epidermis, where it induces pronounced morphological alterations in various appendages (Mustonen et al. 2003), had normal levels of circulating EDA. The same was true for complete deletion of *Edar* in OVE1B mice (Headon and Overbeek 1999), indicating that endogenous EDAR does not deplete or regulate EDA levels in the circulation. EDA contains a basic domain that promotes its binding to heparin and proteoglycans (Swee et al. 2009), and it is conceivable that EDA produced in the skin, even in EDA1 transgenic mice, exerts its effects locally without systemic implication.

Regarding diagnosis of EDA-deficiency, the detection of EDA in serum or in dried blood spots offers an alternative to phenotypic or genetic screening of newborns. In routine dental practice, blood samples are not taken but saliva is readily accessible. The measurement of EDA in saliva could provide useful information with regard to the cause of missing teeth. Selective tooth agenesis can be caused, among other reasons, by mutations in genes encoding master regulators of tooth formation such as paired box gene 9 and Msh homeobox 1, elements of the WNT signaling pathway, such as WNT10A or axis inhibition protein 2, the WNT target bone morphogenetic protein-4, or EDA, EDAR or EDAR associated death domain (Stockton et al. 2000; Bergendal et al. 2011; van den Boogaard et al. 2012, Huang et al. 2013). In cases of non-syndromic tooth agenesis, the EDA protein must be produced in vivo at least to some extent; however, its capacity to bind to EDAR is often decreased (Mues et al. 2010). The measurement of EDA with or without pre-depletion on recombinant EDAR provides information about both expression levels and functionality of EDA.

In conclusion, this study provides the first description of soluble EDA in blood and saliva, characterizes several of its properties, including receptor binding, and documents its expression levels throughout life and disease. Although the origin and function of circulating EDA remains to be determined, its concentration and receptor-binding capacity correlate well with its function in vivo and could therefore be used as a specific biomarker to facilitate the diagnosis of XLHED or unexplained tooth agenesis.

Author Contributions

J. Podzus, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; C. Kowalczyk-Quintas, contributed to conception, design, data acquisition, critically revised the manuscript; S. Schuepbach-Mallepell, L. Willen, G. Staehlin, M. Vigolo, and A. Tardivel, contributed to data acquisition, critically revised the manuscript; D. Headon, contributed to data acquisition and interpretation, critically revised the manuscript; N. Kirby, contributed to conception, design, and data acquisition, critically revised the manuscript; M.L. Mikkola, contributed to data acquisition, critically revised the manuscript; H. Schneider, contributed to conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript; P. Schneider, contributed to conception, design, data acquisition, analysis, and interpretation, wrote the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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