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Exosomes Secreted by Human Placenta Carry Functional Fas Ligand and TRAIL Molecules and Convey Apoptosis in Activated Immune Cells, Suggesting Exosome-Mediated Immune Privilege of the Fetus

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Apoptosis is crucially important in mediating immune privilege of the fetus during pregnancy. We investigated the expression and in vitro apoptotic activity of two physiologically relevant death messengers, the TNF family members Fas ligand (FasL) and TRAIL in human early and term placentas. Both molecules were intracellularly expressed, confined to the late endosomal compartment of the syncytiotrophoblast, and tightly associated to the generation and secretion of placental exosomes. Using immunoelectron microscopy, we show that FasL and TRAIL are expressed on the limiting membrane of multivesicular bodies where, by membrane invagination, intraluminal microvesicles carrying membranal bioactive FasL and TRAIL are formed and released in the extracellular space as exosomes. Analyzing exosomes secreted from placental explant cultures, to our knowledge, we demonstrate for the first time that FasL and TRAIL are clustered on the exosomal membrane as oligomerized aggregates ready to form death-inducing signaling complex. Consistently, placental FasL- and TRAIL-carrying exosomes triggered apoptosis in Jurkat T cells and activated PBMC in a dose-dependent manner. Limiting the expression of functional FasL and TRAIL to exosomes comprise a dual benefit: 1) storage of exosomal FasL and TRAIL in multivesicular bodies is protected from proteolytic cleavage and 2) upon secretion, delivery of preformed membranal death molecules by exosomes rapidly triggers apoptosis. Our results suggest that bioactive FasL- and TRAIL-carrying exosomes, able to convey apoptosis, are secreted by the placenta and tie up the immunomodulatory and protective role of human placenta to its exosome-secreting ability.

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Mammalian reproduction has to face and solve the immunological challenge of accepting a semiallogeneic fetus and supporting its development and growth. The human hemochorial placenta, known to preferentially express paternal Ags, is in direct contact with the maternal blood, and thus, the syncytiotrophoblast (STB) covering the chorionic villi is physically exposed to a risk of a potential attack from the maternal immune system. Despite this, the human placenta and the fetal allograft evade a harmful maternal immune attack and enjoy an immunologic privilege in the uterine cavity.

Multiple maternal and fetal mechanisms of tolerance work in concert to prevent fetal rejection (reviewed in Ref. 1). Several of these mechanisms are mediated by the placenta that not only supplies hormonal, nutritional, and oxygen support but also stands out as an important immunomodulatory organ. Apoptosis, or programmed cell death, is one of the proposed maternalfetal mechanisms of tolerance that plays an important role in implantation and pregnancy (2). Apoptosis is detected in the placenta throughout pregnancy and has been shown to participate in trophoblast invasion, differentiation, and turnover during placentation formation (3, 4) and in the creation of maternal immune tolerance toward the fetus (5, 6). Apoptotic cell death can be initiated through an intrinsic pathway by intracellular signals sensed by mitochondria or an extrinsic pathway via death receptors, members of the TNF superfamily (7, 8). Of all members, the death messengers Fas ligand (FasL) and the TRAIL are physiologically the most relevant.

FasL (apoptosis Ag [APO]-1L and CD95L), a homotrimeric 37-kDa type II membrane protein, signals cell death by engagement of its cognate receptor Fas (APO-1 and CD95). Initially, FasL was described as an apoptosis-inducing protein expressed on activated immune cells. Later studies revealed that it is involved in organ development and homeostasis and is expressed on various other cells and tissues including cells associated with immune privileged sites such as the eye, brain, testis, and placenta (9, 10). The intracellular and extracellular domains of FasL are located in the N- and C-terminal regions, respectively. The proapoptotic activity is associated with membranal expression of FasL where oligomerization of the molecule is needed in the formation of the death-inducing signaling complex (DISC) (11). A cleavage of the extracellular domain by the metalloprotease metralsyn produces a 26-kDa soluble form of FasL (12) that loses its apoptotic activity and can even inhibit the action of membranal-bound (m)FasL.

TRAIL (APO-2L and CD253) was first identified as a TNF superfamily member capable of inducing apoptotic cell death

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mainly in tumor cells. TRAIL, a type II membrane protein, spontaneously trimerizes around a centrally positioned Zn atom important for its stability, solubility, and biological activity as a death ligand (13) and binds to four membrane-bound receptors—TRAIL-R1/death receptor 4, TRAIL-R2/death receptor 5, TRAIL-R3/decoy receptor 1, and TRAIL-R4/decoy receptor 2 (3); and the soluble receptor osteoprotegerin (14). The ability of TRAIL to kill transformed cells by apoptosis is well established, but the overall physiological role of TRAIL is not fully defined; however, TRAIL-induced apoptosis has also been implied in other processes such as activation-induced cell death, homeostasis, intrathymic negative selection, suppression of autoimmunity, and immune surveillance (14). Studies of the cytotoxic immune response showed that both FasL and TRAIL could be recovered from the supernatant of cultured activated T cell blasts and leukemia cells in a bioactive microvesicular form (15). These findings were supported by our morphological studies of the cytotoxic machinery of activated decidual γ6T and NK cells, which revealed that FasL and perforin were preformed and stored in microvesicles in multivesicular body (MVB)—like membrane-bound cytotoxic granules (5).

Both FasL and TRAIL were found to be constitutively expressed by the placenta and proposed as cooperating players for inducing apoptosis in activated lymphocytes (16–18) that might pose a potential threat to the fetus. As stated above, membrane-bound Fas ligand (mFasL) but not soluble FasL (sFasL) promotes apoptosis of Fas-bearing cells (19, 20). Convincing reports by us and others have shown that the human placenta lacks cell surface membranal FasL expression (21, 22). Instead, the FasL expression is located inside the cytoplasm in the endosomal compartment of the STB of the chorionic villi and could be secreted in active microvesicular form (22).

Exosomes are 30- to 100-nm–sized membrane-bound microvesicles formed and released through the late endosomal compartment by a variety of cells. They contain both cytosolic and transmembrane proteins, and mRNA and microRNA and can be viewed as a way of intercellular communication without the need for cell–cell contact, thus conveying a potential biologic activity to more distant targets. Depending on their origin and composition, the exosomes can have either immunostimulatory or immunosuppressive properties (23–25). Lately, the role of microvesicles/exosomes in the multifactorial ways of promoting immunotolerance has gained elevated focus in many different areas, including tumor escape and fetal survival during pregnancy (23–25).

In previous immunoelectron microscopy (IEM) studies of the human placenta, we showed that FasL expression takes place in the STB and is solely confined to the endosomal compartment where FasL-expressing microvesicles were revealed in the MVBs as well as signs of MVB fusion with the plasma membrane, suggesting a release of FasL-carrying exosomes (22). In the current study, we have extended and deepened our investigation to include the biogenesis, expression, and precise intracellular location of the other major proapoptotic TNF superfamily member TRAIL. In this study, we provide evidence that similarly to FasL, TRAIL expression in the human placenta is restricted to STB and is present in MVBs on microvesicles released in the extracellular space as exosomes. Furthermore, to our knowledge, we show for the first time that FasL and TRAIL were expressed on the exosomal membrane in an oligomerized complex of two homomeric FasL and/or TRAIL molecules required for DISC formation and triggering apoptosis, and show evidence for their biologic activity in exosome-induced apoptosis in Jurkat T cells and activated PBMC from healthy donors.

## Materials and Methods

### Samples and Abs

Early and term placental specimens were donated by healthy women undergoing elective termination of pregnancy (8–14 wk of gestation) or normal delivery after ethical committee approval and informed consent. Clones and specificities of Abs were as follows: mouse anti-human FasL (G247–4; BD Pharmingen), goat anti-human TRAIL (K-18), mouse anti-human CD63 (MX-49-129.5; Santa Cruz Biotechnology), mouse anti-human pan MIC (clone 6D4; BD Biosciences), FITC-conjugated mouse anti-human CD63 (Immunotech), mouse anti-human CD95 (EO9; 1B9), and TRAIL (K-18). Antibodies against the following proteins were used: mAbs against TRAIL-R1 (clone number 69036) and TRAIL-R2 (clone number 71908) (R&D Systems), CD56 (My31; BD Biosciences), mAbs against CD4, CD8, CD19, and isotype-matched control mAbs IgG1, IgG2a, and IgG2b (DakoCytomation), normal goat serum (Vector Laboratories), and IRBP-conjugated secondary Abs (Jackson ImmunoResearch Laboratories).

### RNA extraction, RT-PCR, and quantitative PCR from isolated and laser-microdissected STB

Trophoblast from placental villi (three early and three term placental samples) was isolated using a mild optimized protocol as described in Ref. 26. STB was separated and enriched from the isolated trophoblast by positive selection with anti-human pan MIC–coated Dynabeads as previously described (26), put immediately in dissolving solution and frozen for total RNA isolation. In addition, STB was obtained by laser microdissection of chorion villi (two early and two late placental samples) and dissolved for RNA extraction (27). Total RNA extraction and real-time quantitative RT-PCR were performed as described previously (5, 26, 27). In brief, RNA was extracted according to the acid guanidinium thiocyanate-phenol-chloroform method, and reverse transcription was performed at 40°C for 15 min using random hexamer primers (Life Technologies/Applied Biosystems) and murine leukemia virus reverse transcriptase (Promega). Assays-on-Demand Gene Expression probes (Applied Biosystems) (FASLG, assay ID Hs00181226_g1; and TNFSF10, assay ID Hs00921974_m1) were used. Multiplexed real-time PCR assay with 18S rRNA as an endogenous control (Life Technologies/Applied Biosystems) was performed on a 7900HT instrument with the factory default thermocycle conditions for 40 cycles, and raw data were analyzed using a relative quantitation method. Gene expression levels are presented as a relative fold change between the studied samples. Each gene test included PBMC stimulated by PMA/ionomycin as a positive control and a negative control omitting addition of template.

### Immunohistochemistry

Chorionic villi were fixed in 4% paraformaldehyde for 2 h, washed in PBS with 3.5% sucrose and 1% fish gelatin, and snap-frozen in liquid nitrogen. Eight-micrometer cryosections were stained as described previously (22, 26). FcRs were blocked by a mixture of horse serum (ImmPRESS reagent kit) and 10% normal human serum (AB+), aldehyde groups were blocked by PBS with 0.1 M glycine, and 0.03% H2O2 in PBS was used for extinguishing the endogenous peroxidase activity. ImmPRESS anti-mouse or anti-goat Reagent Kit (Vector Laboratories) were used for enhanced detection with 3,3′-diaminobenzidine tetrahydrochloride (DAB) as substrate. Serial sections were included as negative controls stained by exchanging the first Ab with isotype-matched control mAbs or normal goat serum for the polyclonal primary Abs.

### IEM of chorionic villi

Freshly isolated placental samples were immediately fixed in 4% paraformaldehyde for 4 h, washed in 0.1 M phosphate buffer containing 7% sucrose and 0.05% saponin at 4°C overnight, and snap-frozen. Cryosections were processed by indirect immunoperoxidase method, as previously described (22, 27) using mAb clone G247–4 against FasL, and goat anti-human Ab (K18) against TRAIL. ImmPRESS anti-mouse or anti-goat Reagent kit (Vector Laboratories) was used for enhanced detection with DAB as substrate. One-percent normal human serum (AB+) and 0.5% BSA in PBS was used to block FcRs on STB in all blocking steps. Anti-goat and anti-rabbit ImmPRESS reagents and DAB were used to reveal specific staining. Ultrathin sections were examined in a Zeiss EM 900 electron microscope (Carl Zeiss) without additional staining. Sections in-
−10 mg wet weight pieces of chorion villi were cultured in ultracentrifuged medium of RPMI 1640 supplemented with 0.5% BSA (Sigma Aldrich) and antibiotics at 37°C in humid atmosphere with 5% CO₂. Supernatant was harvested after 24 h in culture and used for exosome isolation.

Isolation of exosomes

Supernatants from short-term placental explant cultures from individual donors were cleared from cell debris and larger particles by sequential centrifugations at 4,000 × g for 30 min and 17,000 × g for 25 min. After filtration through a 0.2-µm filter, the supernatant was ultracentrifuged at 110,000 × g for 2 h. The pellet was resuspended in sterile, particle-free PBS and ultracentrifuged through a 20 and 40% discontinuous sucrose gradient; the exosomes were collected and washed a couple of times with sterile PBS. The yield of exosomes was estimated with bicinchoninic acid protein assay kit (Pierce). Isolated exosomes were stored at −80°C in PBS or radioimmunoprecipitation assay buffer (Pierce) supplemented with protease inhibitor mixture (Roche Diagnostics) until use.

Western blot analysis

Isolated placental exosomes, snap-frozen pieces of chorion villi, and cultured Jurkat cells were solubilized with radioimmunoprecipitation assay buffer (Pierce). After separation on 12% SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare). Membranes were blocked for 1 h with 5% fat-free powdered milk in PBS with 0.05% Tween 20 (PBST) before incubation overnight with the appropriate Abs. HRP-conjugated secondary Abs were applied for 1 h. PBST was used in all washing steps. Signals were detected with Amersham ECL plus Western blot detection system with Amersham ECL developing films (GE Healthcare).

Immunoflow cytometry of protein expression on the surface of isolated placental exosomes

For analyzing the expression of proapoptotic molecules on the surface of isolated placental exosomes, surfactant-free ultracentrifuged 4-µm latex microbeads (Interfacial Dynamics) were used as described previously (27, 29). The beads were coated with anti-Fas-, anti–TRAIL-, or isotype-matched control Abs. After blocking of uncoupled sites, isolated exosomes were added and incubated 4°C overnight with end-to-end rotation. After washing, FITC-conjugated CD63 mAb was incubated for 30 min before final washing. The beads were analyzed by flow cytometry on FACScan (BD Biosciences) using CellQuest software.

IEM of protein expression on the surface of isolated placental exosomes

Isolated exosomes were absorbed on formvar/carbon-coated nickel grids, washed with PBS, and fixed with 2% paraformaldehyde in PBS for 10 min. Negative staining was performed on the grids using 1.9% methylcellulose containing 0.3% uranyl acetate. Excess fluid was removed and allowed to dry before examination with electron microscope. For IEM, after blocking in 0.1 M glycine and 0.3% BSA, grids with exosomes were incubated with appropriate monoclonal or polyclonal Abs, isotype-matched control Abs, or ultracentrifuged goat serum for 1 h in wet chamber. After washing, the grids were incubated with secondary Abs conjugated with 5- or 10-nm gold particles for 1 h. After washing and additional fixation with 2.5% glutaraldehyde, the grids were negatively stained as described previously (27). In double IEM staining after completed first staining with anti-Fas and after a blocking step with goat serum, the secondary IEM staining for TRAIL was performed. Conjugation of the secondary Abs with 5- or 10-nm gold particles was used to distinguish between exosomal FasL and TRAIL staining.

Isolation and activation of PBMC and culture of Jurkat cells

PBMC from healthy donors were isolated by Lymphoprep (Nycomed) gradient centrifugation and used for detection of exosome-induced apoptosis. One million isolated PBMC were activated for 24 h with PMA/ionomycin as previously described (27, 28), and the PBMC activation was measured by flow cytometry of Fas and TRAIL-R1 and TRAIL-R2 expression. Jurkat cells, purchased from the American Type Culture Collection, were cultured in RPMI 1640 medium, supplemented with 0.5% BSA (Sigma Aldrich) and antibiotics at 37°C in atmosphere of 5% CO₂ and humidity and used in apoptosis detection tests.

Assessment of the apoptotic effect of detection placental exosomes

Annexin V:PE Apoptosis Detection Kit I (BD Biosciences) and immunoflow cytometry were used for detection of exosome-induced apoptosis on Jurkat cells, according to the manufacturer’s instructions. In brief, Jurkat cells (1 × 10⁶/m) were cultured in a 96-well plate, treated with different concentrations of isolated placental exosomes for 24 h, harvested, washed with PBS, stained with Annexin V/7-aminoactinomycin D according to the manufacturer’s description, and analyzed by flow cytometry on FACScan (BD Biosciences). Activated Fas-expressing PBMC were incubated with exosomes for 24 h in RPMI 1640 medium, supplemented with 0.5% BSA (Sigma Aldrich) and antibiotics and thereafter analyzed by electron microscopy (EM) to assess apoptotic signs in their morphology.

Statistical analysis

The results, appropriate for statistical analysis, were subjected to two statistical tests—Student’s t test and two-way ANOVA performed with GraphPad Prism 5 program.

Results

FasL and TRAIL are transcribed and expressed in early and term human normal placentas

Fig. 1A shows the relative mRNA expression of FasL and TRAIL in STB of early and term pregnancy placentas assessed by real-time quantitative RT-PCR. STB from five individual samples of early and term normal pregnancy placentas were analyzed. As can be seen, mRNA for both FasL and TRAIL was found in all samples confirming these molecules’ expression in early and term placentas. FasL showed higher mRNA expression level in early pregnancy placentas, whereas TRAIL mRNA dominated in expression in term placentas; however, these differences were not statistically significant (Fig. 1A). Fig. 1B illustrates FasL and TRAIL expression at the protein level, revealed by immunohistochemical staining and light microscopy of serial sections of chorionic villi. Positive staining for both molecules was seen in the villous trophoblast, preferentially in the STB of the chorionic villi and on some Hofbauer cells (22) in the villous stroma. The staining was strong and seemed to be intracellularly localized. In light microscopy, the DAB-reaction product gave the impression that both STB and cytotoxophoblast (CTB) were stained; however, the following ultrastructural analysis by IEM excluded staining of CTB. No staining was observed in negative controls performed with isotype-matched control Abs. These results were consistent in all stained samples (n = 4). Similar results were obtained in term placentas (n = 3; data not shown). From these experiments, we conclude that FasL and TRAIL are constitutively transcribed and intracellularly expressed as proteins in the STB of human early and term placentas.

IEM revealed FasL and TRAIL expression on intraluminal microvesicles in the MVB of STB, consistent with the endosomal pathway of exosome biogenesis

We assessed the precise localization of FasL and TRAIL in placentas on the ultrastructural level by indirect immunoperoxidase staining and EM. Four individual early placental samples were examined, and no significant differences in the staining patterns were observed. Representative photomicrographs, illustrating IEM staining for FasL and TRAIL, are shown in Fig. 2. Both molecules exhibited a similar intracytoplasmic staining pattern. The staining of FasL (Fig. 2A, 2B) and TRAIL (Fig. 2C, 2D) in the placenta was mainly restricted to the STB of the chorionic villi and in scarce individual Hofbauer cells (22) in the villous stroma (data not shown). No staining was observed in the CTB cells. Furthermore, the apical microvillous (Fig. 2A, arrowheads) and the basal membranal surfaces of STB were generally devoid of staining indicating that there was no expression of these molecules on the apical and basal syncytiotrophoblastic membrane. Instead, the limiting membrane of cytoplasmic vacuoles and numerous microvesicles of ∼40–60–80 nm in diameter tightly packed inside the vacuoles were positively stained for FasL (Fig. 2A, 2B) and...
TRAIL2 (Fig. 2C, 2D). The stained cytoplasmic vacuoles displayed the classical morphology of MVBs (Fig. 2A, 2B, 2C, 2D). In all identified MVBs, the number of intraluminal FasL- or TRAIL-stained microvesicles was by far higher than 9, a number defined as the lower limit of vesicle content distinguishing between early and late endosomes (30). The MVBs were located at different levels in the syncytioplasm and were between 600–800 nm and up to 1.5–2 μm in size. Several of them were fused with the apical microvillous membrane of the STB, as illustrated in Fig. 2D, where a MVB, positively stained for TRAIL on its limiting membrane, is opening to the apical intervillous space and releasing its microvesicle content. The stained limiting membrane of the MVBs together with the stained intraluminal vesicles suggest vesicle generation by inward budding of the MVB’s limiting membrane consistent with biogenesis of exosomes. In summary, our IEM analyses demonstrated that both FasL and TRAIL are typically localized on intraluminal vesicles with the size and morphology of exosomes enclosed in the MVBs of the late endosomal compartment. Moreover, there were frequent morphological signs of microvesicle secretion/release in the intervillous apical space of the STB.

Secreted placental exosomes express exclusively bioactive aggregated form of FasL and TRAIL

To further confirm the IEM results of generation and secretion of FasL- and TRAIL-bearing exosomes, 24-h cultures of placental explants were established in the presence of metalloprotease inhibitors. The short time of culture was chosen to ensure the explants’ good condition throughout the culture avoiding microvesicle release because of necrosis. Thus, the vast majority of nanovesicles in the culture supernatant emanate from exosome secretion. The exosomes, isolated and enriched by sucrose gradient ultracentrifugation from the culture supernatant, were subjected to analysis for FasL and TRAIL with three different methods—Western blot analysis, immunoflow cytometry, and IEM. The results from representative experiments are summarized in Fig. 3.
FIGURE 2. Immunoelectron microscopic localization of FasL and TRAIL in STB of human early placentas. (A) Low-power micrograph of STB stained for FasL. The electron-dense reaction product labels MVB in the syncytioplasm (arrow). Note that the apical surface membrane is not stained (arrowheads). (B) High-power micrograph of FasL-positive MVB containing tightly packed and intensively stained intraluminal microvesicles (arrowheads). (C) Micrograph of TRAIL-positive MVB (arrow) in perinuclear location showing stained limiting membrane and internal microvesicles. (D) High-power micrograph of an apically located MVB, which exhibits staining of the limiting membrane and internal microvesicles (arrowheads) and forms an opening to the intervillous space (star). Note that apical plasma membrane is TRAIL negative (arrow). Scale bars, 1 μm (A); 400 nm (B); and 200 nm (C, D). N, Nucleus.

Isolated placental exosomes, placental tissue, and a positive control of lysed Jurkat T cells were used in the Western blot analyses of FasL and TRAIL molecules (Fig. 3A). The tetraspanin CD63 was analyzed as an exosomal control. As can be seen, FasL was expressed in all exosomal fractions exclusively as a single protein band of ∼75 kDa corresponding to a membranal hexameric form of FasL, composed of two trimeric molecules (11), whereas in placental tissue, both a 37-kDa and a hexameric 75-kDa FasL were revealed (Fig. 3A). In contrast, lysed Jurkat cells showed a dominating band of ∼37 kDa. Similarly, TRAIL was enriched in the exosomal fractions and placentas as a 70-kDa band, whereas the major amount of TRAIL in Jurkat was expressed as a 34-kDa band and only a smaller amount as a 70-kDa protein band. To our knowledge, this is the first demonstration of FasL and TRAIL in human placental exosomes, showing that both FasL and TRAIL, the major apoptosis-inducing molecules of the TNF superfamily, are not only secreted by exosomes but exclusively enriched as an oligomerized membranal bioactive form.

To further prove that the localization of FasL and TRAIL is indeed on the exosomal membrane, experiments were performed by immunofluorescence staining and immunoflow cytometry with exosomes loaded on latex beads (Fig. 3B) and by immunogold staining of exosomes and IEM (Fig. 3C). Representative histograms of the flow cytometric analysis, shown in Fig. 3B, revealed membranal FasL and TRAIL expression. In Fig. 3C, using negative contrast staining, we illustrate the typical exosomal size and cup-shaped morphology of the isolated microvesicles, thus proving that they are exosomes. Besides a morphological characteristic, the positive immunogold staining of CD63 and placental alkaline phosphatase further depict them as exosomes of human placental origin. IEM staining of FasL and TRAIL was revealed on the surface of the exosomes, confirming that these molecules were expressed as membranal proteins. We made a double IEM staining with FasL and TRAIL, using 5- and 10-nm gold particles, conjugated to secondary Abs to distinguish between FasL and TRAIL localization. Interestingly, we found FasL (stained by 5-nm gold particles) and TRAIL (stained by 10-nm gold particles) on different exosomes, indicating that at least to a certain extend they might be generated in separate MVBS.

In summary, with three independent methods—Western blot, immunoflow cytometry with latex beads, and IEM—we demonstrate that the placenta secretes exosomes that carry FasL and TRAIL, enriched on the exosomal membrane as single protein bands with sizes consistent with their bioactive membranal form.
FasL- and TRAIL-expressing placental exosomes induce apoptosis in vitro in a dose-dependent manner

We studied the proapoptotic ability of exosomes from separate placental samples in vitro in two model systems—Jurkat leukemia T cells and activated PBMC from healthy donors. The apoptosis was revealed by Annexin V-PE Apoptosis Detection Kit I (BD Bioscience) and by EM looking for early signs of apoptosis in activated PBMCs incubated with placental exosomes. Fig. 4A and 4B summarizes the results of Jurkat cell experiments with exosomes isolated from culture supernatants of individual placental explants and Fig. 4C–G the results from experiments with activated PBMC. As can be seen, the placental exosomes induced statistically significant apoptosis in Jurkat cells in a dose-dependent manner (n = 7; Fig. 4A). In contrast, the explant culture supernatants depleted of microvesicle after gradient ultracentrifugation were unable to trigger apoptosis of the target cells indicating that the apoptotic effect was exosome specific (data not shown). The in vitro proapoptotic effect on the Jurkat cells could differ between individual exosome samples (Fig. 4B). Although most exosomal samples had a good proapoptotic capacity, some had lower intensity, and two samples had very low effect. The reason for the variation in the apoptotic capacity is at present not known but may be explained by several factors—for example, less concentration of apoptotic molecules on the surface of some isolated exosomes because of insufficient protective effect of the protease inhibitor mixture during isolation and storage; variations because of differences in the STB or in the gestational stage of the placental samples; variations because of the condition of the samples and of the explant cultures’ performance; and/or biological differences between the donors. Exosomal samples, unprotected by matrix metalloproteinase (MMP) inhibitors lost their FasL and TRAIL expression and their apoptotic ability as shown in Supplemental Fig. 1. The presence of metalloproteinase inhibitors in the culture medium during storage and experimental procedures with exosomes is essential for the preservation of their proapoptotic effect. Despite a biological variation in the apoptotic potency in individual samples, the mean value ± SD of induced apoptosis was statistically significant and dose dependent (Fig. 4A).

In addition, we studied the in vitro apoptotic effect of exosomes on PBMC from healthy donors (n = 5) activated by PMA/ionomycin. The activation was assessed by measurement of Fas and TRAIL receptors by immunoflow cytometry as illustrated in a typical experiment in Fig. 4C–E where Fas and TRAIL recep- tor expression is shown on different subpopulations of activated PBMC from one donor. Apoptosis was assessed by Annexin V-PE Apoptosis Detection Kit (Fig. 4F) and EM (Fig. 4G). As can be seen in a representative PBMC experiment (Fig. 4F), the number of Annexin V–expressing apoptotic cells is doubled in the presence of 40 μg exosomes. In the EM analyses, we saw cells with intact cellular membrane but with clear early apoptotic signs like fragmentation of the nucleus, vacuolization of the cytoplasm, and membrane blebbing as illustrated in Fig. 4G. Taken together, our results indicate that human placenta secretes exosomes with membranal expression of preformed functional FasL and TRAIL molecules that are able to rapidly trigger apoptosis in vitro in Jurkat T cells and in activated PBMC from healthy donors.

Discussion

Three main points summarize the results presented in this paper: 1) In the STB of human placenta, the apoptosis-inducing molecules FasL and TRAIL are constitutively transcribed and expressed as proteins on the MVB’s membrane and on the membrane of intraluminal vesicles that are released in the extracellular space as exosomes. The protein expression of FasL and TRAIL is, thus, strictly confined to the endosomal compartment of STB, whereas the apical and basal syncytiotrophoblastic membranes are completely devoid of FasL and TRAIL expression. 2) The STB-derived exosomes enrich FasL and TRAIL on their membrane as bioactive oligomerized molecules able to form DISCs. 3) FasL- and TRAIL-carrying placental exosomes trigger apoptosis in Jurkat T cells and activated PBMC from healthy donors in a dose-dependent manner. Taken together, these results indicate that human early and term placenta constitutively secrete functional FasL and TRAIL on exosomes that are able to convey apoptosis and thus might contribute to protection of the fetoplacental unit from activated maternal immune cells.

The intracellular localization of FasL and TRAIL in human placenta is intimately bound to the biogenesis of exosomes. To our knowledge, this is the first demonstration of TRAIL protein expression on the ultrastructural level and the first report that TRAIL is solely exosomally expressed in STB of human placenta. Furthermore, a constitutive release of FasL- and TRAIL-expressing exosomes from the apical microvillous surface of STB was demonstrated. Our results are in tune with previous investigations by others and us (22, 31, 32) showing that FasL is targeted to the MVB of secretory lysosomes and is expressed on exosome-like microvesicles (5, 15, 33, 34). Two independent pathways that control the internalization of FasL on microvesicles in MVBs have been identified—phosphorylation of FasL by binding of its proline-rich domain to Fgr, Fyn, and Lyn tyrosine kinases and a direct ubiquitinylation at the lysine residues flanking the proline-rich domain (35–37). The extracellular domain of FasL, TRAIL, and other members of the TNF family contain cleavage sites for MMPs that generate soluble proteins by proteolytic shedding and substantially reduce the proapoptotic activity of the shed proteins. The mFasL is the proapo- totic molecule, whereas sFasL is 1000 times less active in inducing apoptosis (38, 39). Translocation of FasL to the plasma membrane as a way to provide mFasL will expose the molecule to proteolytic cleavage reducing apoptotic activity. Moreover, FasL expressed on the cellular membrane, instead of dampening the immune responses, induces inflammation and promotes allograft rejection (19, 39–42) as compared with sFasL that was proposed to be anti-inflammatory (40, 41, 43). Thus, different molecular forms of FasL do mediate different functions. The exact mechanisms are not completely understood, but compartmental expression, levels of membranal and/or shed form, and the surrounding microenvironment where it is expressed seem to act together to influence its biological functions (10). Similarly to FasL, there are differences in TRAIL activity depending on whether it is in a membrane-bound form or cleaved to generate a soluble form. Initially, it was found that both forms were equally effective (44), whereas later reports singled out membrane-bound TRAIL as the molecular form preferably inducing cell death (45–47). Recently, it was reported that TRAIL protein in STB cultures could be at least partly localized to the cytoplasm (18). Previously proposed function as a protein repairing syncytiotrophic damage and maintaining the placental barrier integrity could not be confirmed. Instead, a similar function for TRAIL as for FasL as a contributor to immune privilege was suggested, and an intracellular compartmental restriction of TRAIL expression in the placenta similar to that of FasL was assumed but not documented (18). In line with this assumption, we show that TRAIL is restricted to STB’s endosomal compartment and expressed and released on exosome-like microvesicles. The previously described cell membrane expression on CTB and STB (18) could not be confirmed despite detailed EM analyses. Similar results of exosomal TRAIL and FasL expression have been shown in other cells and sites such as dendritic and T cells (5, 48, 49),
melanoma, and ovarian cancer cells (50, 51) and in joint fluid of rheumatoid arthritis patients (52). Interestingly, our double immunogold staining of FasL and TRAIL revealed these molecules on separate exosomes, suggesting that in human placenta these molecules might be enriched and processed through separate MVBs. This is consistent with a previous finding of only partial colocalization of endosomal FasL and TRAIL expression in human melanoma (46).

**FIGURE 4.** Human placenta-derived exosomes carry functional FasL and TRAIL on their membrane. (A and B) FasL- and TRAIL-bearing placental exosomes trigger apoptosis of Jurkat T cells in a dose-dependent manner. (A) Staple diagram showing average percentage ± 1 SD of dose-dependent apoptosis in Jurkat cells induced by different concentrations of exosomes isolated from supernatant of placental explant cultures (n = 7). (B) Induction of apoptosis by individual exosomal preparations derived from explant cultures from seven consecutive samples of human early placenta from normal pregnancies showing biological variation in apoptotic activity. (C–G) FasL- and TRAIL-expressing exosomes trigger apoptosis in activated PBMC from healthy donors. (C–E) Immunoflow cytometry showing Fas- and TRAIL-R1– and -R2–expressing cells in subpopulations of PBMC from healthy donors, activated by PMA/ionomycin. (F) Immunoflow cytometry analysis of apoptotic cells in activated PBMC before and after addition of 20 μg placental exosomes. Note that the percentage of the apoptotic PBMC is doubled in the presence of FasL- and TRAIL-bearing placental exosomes. (G) Electron micrograph of a representative experiment out of three, showing classical apoptotic signs of nuclear fragmentation, vacuolization of the cytoplasm and plasma membrane blebbing of activated PBMC incubated with FasL- and TRAIL-bearing placental exosomes. Original magnification ×5000. ***p = 0.0001; **p = 0.0016; *p = 0.0498.
Soluble and membranal FasL and TRAIL molecules have separate biologic activities; thus, exosome-ally expressed FasL and TRAIL may be viewed as a “soluble” form that preserves membranal expression and a proapoptotic biologic activity. Using the highly specific anti-FasL mAb G247-4 (53), we revealed a single FasL protein band of 75 kDa in the exosomal fractions and a single TRAIL protein band of 70 kDa. FasL and TRAIL, as well as other members of the TNF superfamily, are membranal-ly expressed as homotrimeric molecules. However, aggregation by self-induced oligomerization is necessary for formation of DISC and rapid triggering of apoptosis. Holler et al. (11) proved that a hexameric FasL, consisting of two homotrimers at close proximity, represents the minimal ligand structure required to form DISC and signal apoptosis. Both trimeric and hexameric FasL bound equally well to Fas but trimeric FasL failed to induce a DISC and is thus inefficient in triggering of apoptosis (11). A hexameric FasL would have a m.w. between 75 and 80 kDa depending on glycosylation level. We suggest that the single 75 kDa FasL and the 70 kDa TRAIL protein bands in the placental exosomal fractions represent two homotrimeric membranal molecules aggregated in a bioactive hexameric form of FasL (11) and TRAIL, respectively. Our suggestion is based on the fact that in our experiments exosomes carrying the 75-kDa FasL and 70-kDa TRAIL were able to trigger exosomal dose-dependent apoptosis in Jurkat cells and activated PBMC, implicating that a functional DISC was formed. When the expression of these oligomerized ligands was lost from the exosomal membrane, the proapoptotic ability was lost as well as shown in Supplemental Fig. 1. The functional experiments are in-tune with apoptosis triggered by exosomes from other sources such as activated T cells and DC cells (5, 15, 34, 48, 49) and various cancer cells (31, 33, 50, 51, 54). To our knowledge, this is the first report of expression of preformed oligomerized FasL and TRAIL on exosomal membrane. The sphingolipid-, cholesterol-, and tetranspan-rich exosomal membrane emanates from the endosomal compartment and resembles in its composition the cellular membrane’s lipid rafts, known to promote attachment and aggregation of signaling proteins as well as recycling (55). Therefore, the exosomal membrane is exceptionally suitable for the oligomerization and selective enrichment of biologically active TNF superfamily ligands, able to form DISC (11). Limiting the expression of oligomerized FasL and TRAIL to exosomes in MVB protects these molecules from MMP cleavage and keeps them in a molecular form ready to trigger cell death in stringently defined events such as delivering of “cytotoxic hit” to a target cell (5, 15, 48) or protection of specific tissues/organs or sites from an immunologic attack (9), rendering these sites an immunologic privilege.

In contrast to our results, confirmed in other settings (5, 15, 31–34, 49–51, 54), Abrahams et al. (21) could not detect surface expression of FasL on trophoblast cell line–derived microvesicles and could only induce apoptotic activity when the vesicles were disrupted by Triton X treatment, concluding that FasL was localized inside the microvesicles. The reasons for this discrepancy are not clear but might have several explanations, for example, loss of apoptotic activity because of shedding by proteolytic cleavage. Protection against metalloprotease activity is of crucial importance, because the different forms of these molecules—membranal homotrimeric, oligomerized, and shed—all have separate biological functions and fates. The retrieval of a 37-kDa protein band after Triton X membrane disruption treatment (21) cannot be the membranal exosomal FasL described by us in this study. Maybe it emanates from cross-linking or trimerization of the Triton treatment–produced sFasL. It has been reported that, if the cleavage of mFasL occurs by MMP7 at a particular protease cleavage site, the generated sFasL may form functional trimers (11, 56, 57). Another explanation could be that we have studied different microvesicles because they were isolated from different sources and by different isolation procedures.

The physiological importance of apoptosis in normal pregnancy is associated with the fetal immune privilege in the pregnant uterus a phenomenon similarly regulated as other immune privileged sites, the eye being the one more thoroughly studied (9, 58, 59). We can speculate that the release of proapoptotic placental exosomes is in highest concentration near the placenta and builds an exosomal gradient in the maternal blood nearest the placenta, which functions as a protective shield of the fetoplacental unit. The results presented in this study suggest that the placenta secretes exosomes expressing preformed bioactive FasL and TRAIL molecules able to convey apoptosis and might partly contribute to the immune privilege of the fetus. Thus, the immunomodulatory and protective role of the placenta seems to be associated to its exosome-secreting ability.

Several published reports (reviewed in Ref. 25), including this one, concern normal placenta and depict exosomal secretion as beneficial to pregnancy. A logical question is how the exosomal secretion is affected in pathological pregnancies. So far, there are few conclusive exosome studies regarding such conditions. In preterm pregnancy, exosomal concentration in the maternal peripheral blood was shown to be lower compared with that in women with normal term delivery and nonpregnant women (60). Previous studies (61) of preeclampsia highlighted another type of extracellular microvesicles, the much larger shedding microvesicles generated from the apical microvillous syncytial membrane, and showed that they are highly elevated in the circulation of preeclamptic women and seemed to be involved in the enhanced inflammation, cytokine production, and endothelial destruction and dysfunction that are cardinal characteristic for this enigmatic disease. One can speculate that there may be a balance in the STB between generating shedding vesicles and generating exosomes, and a disturbance of this balance by overproducing or underproducing either type of extracellular STB vesicles might affect the fine-tuning of the placental function. Many more well-designed future studies of syncytiotrophoblast microparticles and exosomes in pathological pregnancy conditions are needed to elucidate this question.

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Disclosures
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5. Mincheva-Nilsson, L., O. Nagaeva, K. G. Sundqvist, M. L. Hannström, S. Hannström, and V. Baranov. 2000. sFasL in the eye being the one more thoroughly studied (9, 58, 59). We can speculate that the release of proapoptotic placental exosomes is in highest concentration near the placenta and builds an exosomal isolation procedures.

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