Soluble TRAIL is present at high concentrations in seminal plasma and promotes spermatozoa survival

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Abstract

The expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL(TNFSF10)) and of its receptors (TRAILR1, TRAILR2, TRAILR3, and TRAILR4) have been documented in testis, but the presence of soluble TRAIL in seminal fluid, as well as the potential physiopathological role of the TRAIL/TRAILR system in spermatozoa, has not been previously investigated. Male donors ($n=123$) among couples presenting for infertility evaluation were consecutively enrolled in this study. The presence of soluble TRAIL was analyzed in seminal samples by ELISA, while the surface expression of TRAIL receptors was investigated by flow cytometry. High levels of soluble TRAIL were detected in seminal plasma (median, 11 621 pg/ml and mean $\pm$ s.d., $13\,371 \pm 8367$ pg/ml) and flow cytometric analysis revealed a variable expression of TRAIL receptors in the sperm cellular fraction among different subjects. In addition, the effect of physiologically relevant concentrations of recombinant TRAIL was investigated on survival and motility of spermatozoa. Of interest, the *in vitro* exposure of capacitated spermatozoa to recombinant TRAIL (10 ng/ml) significantly preserved their overall survival. Therefore, the present study demonstrates for the first time the presence of elevated levels of the anti-inflammatory cytokine TRAIL in seminal fluids. Moreover, the demonstration that recombinant TRAIL promotes spermatozoa survival after capacitation suggests potential therapeutic implications.

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Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL(TNFSF10)) is expressed as a type 2 membrane protein or as a soluble cytokine (Secchiero & Zauli 2008), and it is present in detectable amounts in the plasma/serum as well as in other body fluids of normal individuals (Secchiero *et al.* 2009, 2010, Bernardi *et al.* 2012). TRAIL acts as a homotrimer, interacting with any one of five cognate receptors, four transmembrane (TRAILR1, TRAILR2, TRAILR3, and TRAILR4), and one soluble (osteoprotegerin (OPG)) receptor, albeit with different affinities (Zauli *et al.* 2007, 2009). The best-characterized biological function of the two TRAIL-receptors containing a death-domain (TRAILR1 and TRAILR2) is to elicit an apoptotic response upon binding of TRAIL. However, in a series of studies, we have demonstrated that alternative TRAIL signaling is unmasked in cells resistant to TRAIL-induced apoptosis, in which the predominant result of TRAILR2 engagement is the activation of intracellular signal transduction pathways, such as

ERK/MAPK, AKT, and NF- κ B (Secchiero *et al.* 2001, 2008, Milani *et al.* 2003, Zauli *et al.* 2005, 2008). TRAILR3 and TRAILR4 are considered as membrane neutralizing or regulatory receptors. Although OPG has been initially identified as a member of the soluble tumor necrosis factor (TNF)-receptor family, inhibiting RANKL-mediated osteoclastogenesis, several *in vitro* studies have shown its ability to also neutralize TRAIL (Emery *et al.* 1998, Miyashita *et al.* 2004, Zauli *et al.* 2009).

Among different tissues, it is noteworthy that TRAIL and its receptors are abundantly expressed in human testis (Grataroli *et al.* 2004). Although TRAIL has been proposed to contribute to the control of the number of spermatogonia (Coureuil *et al.* 2010), the physiopathological role of TRAIL in testis remains to be fully elucidated. In addition, the presence of TRAIL in seminal plasma has never been evaluated. It should also be taken into consideration that a growing number of studies from different groups of investigators have shown that TRAIL displays anti-inflammatory activity (Li *et al.* 2012, Marcuzzi *et al.* 2012, Keuper *et al.* 2013,

Table 1 Demographics and descriptive analysis of semen parameters of male subjects ($n=90$).

Variables	Mean (s.d.) or median (IQR) ^a
Age (years)	39.7 (5.5)
BMI	24.9 (23.1–27.5)
Semen parameters ^b	
Sperm concentration (million/ml)	35 (15–85)
Rapid progressive sperm motility (A, %)	15 (5–20)
Slow progressive sperm motility (B, %)	15 (10–25)
Morphology (% normal form)	22 (18–26)
Leucocytes in semen (million/ml)	0.03 (0.00–0.36)
Male etiology of infertility (no. of cases)	
Negatives	54
Varicocele	21
Cryptorchidism	7
Testicular hypotrophy	3
Erectile dysfunction	2
Epididymal cyst	1
Hematospermia	1
Testicular hydrocele	1

IQR, interquartile range; WHO, World Health Organization.

^aMean ($\pm$ s.d.) are reported for variable distributing normally according to skewness and kurtosis joint test; medians and IQR are reported otherwise. ^bSemen quality parameters according to the WHO.

Secchiero *et al.* 2013, Walczak 2013). On these bases, the aim of our present study was i) to analyze the presence and the levels of soluble TRAIL in the seminal samples and the surface expression of TRAIL receptors in the sperm cellular fraction and ii) to evaluate the potential biological activity of soluble TRAIL, by assessing the *in vitro* response of spermatozoa in terms of vitality/survival and motility upon exposure to recombinant TRAIL.

Materials and methods

Subjects and semen collection

The study has been conducted according to the principles expressed in the Declaration of Helsinki. Institutional Review Board approval was obtained from the Internal Ethical Committee of the Institute for Maternal and Child Health, IRCCS 'Burlo Garofolo' (Trieste), that authorized the use of semen samples submitted to semen examination for experimental purposes. Each donor gave informed consent allowing the use of his semen for our study. Sperm samples were obtained by masturbation after 4–5 days of sexual abstinence from a total of 123 male donors among couples presenting for infertility evaluation. All samples were allowed to liquefy for at least 30 min at 37 °C, and then were evaluated for sperm concentration, vitality, motility, and morphology according to the guidelines of World Health Organization (WHO). Motile spermatozoa were then capacitated by the swim-up technique (37 °C for 75 min in a 5% CO₂ atmosphere) using sperm washing medium (SWM), as described previously (Miraglia *et al.* 2010). After swim-up, the motile sperm-rich fraction was centrifuged at 600 *g* for 10 min, the supernatant was discarded, and the pellets were re-suspended in SWM before evaluation for sperm concentration, vitality, motility, and morphology according to the guidelines of WHO.

Measurement of soluble TRAIL

Seminal plasma was collected from liquefied semen samples by centrifugation at 1000 *g* for 10 min, followed by a second centrifugation of the supernatants at 16 000 *g* for 15 min to remove debris and insoluble components. After centrifugations, the seminal plasma samples were collected and stored in aliquots at –80 °C. In selected cases ($n=10$), TRAIL was measured in both plasma and cellular fractions of the same semen samples. Analysis of TRAIL levels was carried out by using specific, commercially available ELISA Kit (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions and as previously described (Secchiero *et al.* 2009, 2010). Sensitivity of the assay was 2.86 pg/ml with the intra- and inter-assay coefficients of variation being 3.9 and 6%

Figure 1 Soluble TRAIL levels in seminal samples. Levels of soluble TRAIL were measured in seminal samples by ELISA. (A) TRAIL levels were assessed in 90 seminal samples. In ten samples, TRAIL levels were comparatively determined in the unfractionated (total) sample, plasma, and cell lysate. Horizontal bars are median, upper and lower edges of box are 75th and 25th percentiles, lines extending from box are 10th and 90th percentiles. * $P<0.01$ compared with the levels measured in the seminal plasma and total seminal samples. (B) Lack of significant correlation between the seminal levels of TRAIL and the number of spermatozoa. Correlation coefficient (R), calculated by Spearman's analysis, and P value are reported in the text.

Figure 2 Surface expression of TRAIL receptors in spermatozoa. Surface expression of TRAILR1, TRAILR2, TRAILR3, and TRAILR4 was determined by flow cytometric analysis of seminal samples. Shaded histograms represent cells stained with mAbs specific for the indicated surface antigens, while unshaded histograms represent the background fluorescence obtained from the staining of the same samples with isotype-matched control mAbs. (A) Representative phenotypes of seminal samples analyzed before capacitation are shown. (B) Comparison of the TRAILR phenotype of the seminal samples before and after capacitation, showing the enrichment of a subpopulation (arrow) with higher TRAILR2 expression upon capacitation. Data are representative of three different cases.

respectively and the upper limit of detection being 1000 pg/ml. Serial dilutions (1:5) of each sample were run in duplicate. Some samples were run in each ELISA plates, as internal controls, confirming the reproducibility of the determinations over times.

Phenotypic analysis and assessment of sperm vitality, apoptosis, and motility

Surface TRAIL receptor expression in both pre- and post-capacitated samples was analyzed by using PE-conjugated mAbs anti-human TRAILR1 (FAB347P), TRAILR2 (FAB6311P), TRAILR3 (FAB6302P), and TRAILR4 (FAB633P; all from R&D Systems), as described previously (Rimondi *et al.* 2006). Assessment of sperm vitality was carried out by using the one-step eosin–nigrosin staining technique as previously reported (Björndahl *et al.* 2003) and according to the WHO guidelines of 2010. In parallel, in capacitated samples, assessment of apoptosis was evaluated by double staining with PI and FITC-conjugated Annexin V (Alexis Biochemical, Lausen, Switzerland) and flow cytometry analysis as previously detailed (Re *et al.* 1993, Zauli *et al.* 1995, Vitale *et al.* 1997).

The sperm motility parameters were assessed by CASA (CGA-WLJY-9000; CGA Distribution, Florence, Italy; Krause & Viethen 1999). The following kinetic parameters were determined: percentage of spermatozoa exhibiting a forward progressive motility (A and B WHO classes), *in situ* motility (C WHO class), or no motility (D WHO class). For the analysis of the potential effects of TRAIL on spermatozoa vitality and motility, aliquots of sperm suspension (200 μ l) were incubated in the absence or presence of recombinant TRAIL (used in the range of 1–10 ng/ml), prepared as described previously (Milani *et al.* 2003, Zauli *et al.* 2008). For specificity assays, sperm samples were treated with either recombinant human OPG (R&D Systems; 10 ng/ml) or recombinant TRAIL pre-incubated with OPG used at the same (1:1) concentration.

Statistical analysis

Box plots were used to show the median, interquartile ranges, and minimum and maximum values for each group of data. After verifying that TRAIL values of this study did not distribute normally (skewness and kurtosis joint normality test),

Table 2 Semen variables in samples employed for the *in vitro* assays in response to recombinant TRAIL.

Subject no.	Pre-capacitated						Capacitated				
	Volume (ml)	Sperm concentration ($\times 10^6$ /ml)	Motility WHO classes (%)				Sperm concentration ($\times 10^6$ /ml)	Motility WHO classes (%)			
			A	B	C	D		A	B	C	D
101	3	120	30	30	20	20	65	80	15	5	0
102	5.6	12	10	10	30	40	0.6	70	20	10	0
103	3	80	19	14	7	60	2.5	64	26	10	0
104	3.5	26	10	34	14	42	3	80	10	20	0
105	5	80	15	35	20	30	1.4	90	10	0	0
106	2.7	65	10	15	25	50	3	100	0	0	0
107	3.6	135	17	44	17	22	75	100	0	0	0
108	2.5	150	20	30	10	40	30	90	10	0	0
109	2.1	55	27	30	22	21	10	100	0	0	0
110	3	60	25	25	15	35	45	100	0	0	0
111	5	45	22	22	11	45	5	80	20	0	0
112	3.7	35	26	20	24	30	7	90	10	0	0

we applied the nonparametric Mann–Whitney *U* or the Kruskal–Wallis tests for the equality of populations to compare the TRAIL values among different populations. Correlation coefficients were calculated with the Spearman's rank coefficient ρ . *P* value <0.05 was considered statistically significant.

Results

Presence of high levels of soluble TRAIL in seminal samples

Although previous studies have documented the presence of TRAIL in the testis (Grataroli *et al.* 2004, Coureuil *et al.* 2010), no studies have addressed the presence of TRAIL in semen. Therefore, in the first group of experiments, we have analyzed the presence and levels of TRAIL in a large group of seminal samples ($n=90$; Table 1). As shown in Fig. 1A, concentration of soluble TRAIL in semen samples of donors, among couples presenting for infertility evaluation, was very high in all individuals, showing a median of 10 924 pg/ml (mean $\pm$ s.d., 14 109 $\pm$ 9334 pg/ml). The levels of TRAIL did not correlate (Spearman's $\rho = -0.0334$, $P=0.75$) with the number of spermatozoa (Fig. 1B). In additional experiments, to evaluate whether TRAIL was associated to the liquid and/or to the cellular components of the seminal samples, we have comparatively analyzed the levels of TRAIL in the seminal plasma and in the corresponding cellular fraction of a subset of samples ($n=10$). While the seminal plasma showed levels of TRAIL comparable (median, 11 621 and mean $\pm$ s.d., 13 371 $\pm$ 8367) to those of unfractionated samples (median, 11 551 and mean $\pm$ s.d., 11 995 $\pm$ 6955), the amount of TRAIL associated to the cells was markedly lower (median, 221 and mean $\pm$ s.d., 302 $\pm$ 201) (Fig. 1A). Taken together, these data clearly indicate that most of the soluble TRAIL was present in the liquid fraction rather than being associated to the spermatozoa.

Variable expression of surface TRAIL receptors in human spermatozoa

In order to start the investigation about the potential significance of high levels of TRAIL in seminal plasma, we have assessed the surface expression of TRAIL receptors (TRAILR1, TRAILR2, TRAILR3, and TRAILR4) in spermatozoa. As exemplified in Fig. 2A, a variable expression pattern was observed among the different samples analyzed. It should be noticed that between the two TRAIL receptors able to transduce both apoptotic and nonapoptotic intracellular signals (TRAILR1 and TRAILR2), TRAILR2 appeared usually at higher expression levels than TRAILR1. Moreover, while TRAILR1, TRAILR3, and TRAILR4 expression did not show appreciable differences before and after capacitation, in some samples analyzed, TRAILR2 expression increased after capacitation, suggesting the enrichment of cell subpopulations exhibiting higher expression (Fig. 2B).

Exposure to recombinant TRAIL increased spermatozoa survival after capacitation

As TRAIL is known to affect cell survival/apoptosis in a cell type-dependent manner (Bernardi *et al.* 2012), we have then investigated the effect of physiologically relevant (1–10 ng/ml) concentrations of recombinant TRAIL on spermatozoa survival/apoptosis. For this purpose, fresh selected samples, with baseline characteristics reported in Table 2, were exposed *in vitro* to recombinant TRAIL. After 24–48 h of culture, pre-capacitated spermatozoa were virtually all immotile or dead/apoptotic irrespectively of the presence of recombinant TRAIL (data not shown). On the other hand, treatment with recombinant TRAIL significantly preserved the vitality of post-capacitated spermatozoa both at 24 and 48 h (Fig. 3A). The specificity of the effect was documented in three additional fresh sperm

Figure 3 Effect of recombinant soluble TRAIL on spermatozoa vitality. Capacitated spermatozoa were exposed to recombinant TRAIL (10 ng/ml) and cell vitality/mortality was assessed by two different operators using the one-step eosin–nigrosin staining technique. (A) Cell vitality/mortality was assessed at the indicated time points; data from 12 independent experiments are reported as mean $\pm$ s.d., * $P < 0.05$ compared with untreated sample. (B) Cell vitality/mortality was assessed at 48 h after the indicated treatments with recombinant TRAIL (10 ng/ml) and OPG (10 ng/ml), used either alone or in combination. Data from three independent experiments are reported as mean $\pm$ s.d., * $P < 0.05$ compared with untreated sample.

samples, in which we observed that pre-incubation of TRAIL with an equal (1:1) concentration of OPG completely abrogated the pro-survival effect of TRAIL (Fig. 3B).

In parallel, we have investigated whether the *in vitro* treatment with recombinant TRAIL might impact the spermatozoa motility, by analyzing the distribution among the different WHO classes: (class A) rapid progressive motility, indicating sperm swimming with a progression velocity $> 25 \mu\text{m/s}$; (class B) slow progressive motility, indicating sperm swimming with a progression velocity equal to $5\text{--}25 \mu\text{m/s}$; (class C) *in situ*

motility; and (class D) no motility. As shown in Fig. 4, after 24 h of culture, the situation spontaneously changed drastically with respect to the motility pattern of the fresh capacitated samples (Table 2), in that percentage of classes A+B spermatozoa significantly dropped in all samples, while classes C+D significantly increased. Of note, in several samples the exposure to recombinant TRAIL resulted in an increment of the progressive motility (WHO classes A+B), counterbalanced by a parallel decrease in both C and D classes spermatozoa (Fig. 4).

Discussion

The role of seminal fluid in the regulation of human reproductive processes has been demonstrated in various studies (Eggert-Kruse *et al.* 2007, Robertson *et al.* 2009). Several cytokines secreted by the seminal vesicle and prostate gland modulate the response of various cell populations, including spermatozoa and white blood cells. It has also been demonstrated that seminal fluid affects the levels of cytokines and chemokines in human cervix, showing profound immunological effects (Sharkey *et al.* 2012). In this respect, the first major finding of the

Figure 4 Effect of recombinant soluble TRAIL on spermatozoa motility. Capacitated spermatozoa were exposed to recombinant TRAIL (10 ng/ml) before the assessment of sperm motility by two different operators. Results of six selected capacitated samples (out of 12 samples) are shown. Results are reported as mean $\pm$ s.d. of experiments carried out in triplicate. * $P < 0.05$ compared with untreated sample.

present study was that TRAIL, a TNF family member, is present at high levels (median, 11 621 pg/ml) in seminal plasma. To appreciate how seminal plasma is enriched in TRAIL cytokine, it should be considered that the levels of TRAIL measured in human plasma, with the same commercial assay, are usually between 60 and 90 pg/ml (Secchiero *et al.* 2009, 2010, Volpato *et al.* 2011). Thus, seminal plasma contains >2 log higher concentrations of soluble TRAIL with respect to human plasma. Moreover, in this study, we have documented a variable expression of the surface TRAIL receptors in the spermatogenic cellular fraction. Although TRAIL/TRAILR system is generally considered to be a component of the cell anti-tumorigenic activity, its physiopathological role is broader than originally thought (Di Pietro & Zauli 2004). In particular, the ability of TRAIL to suppress inflammatory reactions has been demonstrated in a variety of studies (Li *et al.* 2012, Marcuzzi *et al.* 2012, Keuper *et al.* 2013, Secchiero *et al.* 2013, Walczak 2013).

The second major finding of our study was that recombinant TRAIL, used at concentrations comparable to those found in seminal plasma (10 ng/ml), significantly increased the overall survival of capacitated spermatozoa. On the other hand, the survival/apoptotic rate of uncapacitated spermatozoa did not vary upon culture for 24–48 h in the absence or presence of recombinant TRAIL, probably due to the high spontaneous mortality/apoptosis. However, it is possible to speculate that the effect of TRAIL on capacitated spermatozoa could be due to the increase in surface TRAILR2 expression in capacitated spermatozoa as compared with pre-capacitated spermatozoa. The pro-survival/anti-apoptotic activity of TRAIL on spermatozoa was not totally unexpected, because we and other authors have previously demonstrated that the effect of TRAIL on survival/apoptosis is cell-type specific (Di Pietro & Zauli 2004). Indeed, while recombinant TRAIL induces apoptosis of most cancer cells *in vitro*, when added to primary normal cells can either exhibit no effects or it can activate intracellular pathways, such as ERK/MAPK, AKT, and NF- κ B, which have been involved in the mediation of cell survival in different cell types (Secchiero *et al.* 2001, 2008, Milani *et al.* 2003, Zauli *et al.* 2005, 2008). In this respect, it is noteworthy that these pathways have been involved also in promoting the survival of spermatozoa in response to progesterone and other molecules (Rogers *et al.* 2008, Awda & Buhr 2010, Espino *et al.* 2011, De Amicis *et al.* 2012, Gómez *et al.* 2012, Kim *et al.* 2012, Maheshwari *et al.* 2012, Sagare-Patil *et al.* 2013).

The third major finding of our study is that TRAIL, by promoting the cell survival, predominantly increased the capacitated spermatozoa of A+B motility classes, although this effect was not documentable in all the cases analyzed. Thus, although we have not a direct proof that the presence of TRAIL on seminal fluid has a direct impact on male semen fertility, the ability of TRAIL to

prolong the survival of cells with forward progressive motility strongly suggest that TRAIL might play an important role as a trophic cytokines for capacitated spermatozoa. Taking into account that recombinant TRAIL is now in phase I and II clinical trials, the therapeutic potential of this cytokine in promoting spermatozoa survival/mobility should be further investigated.

In conclusion, our data indicated that soluble TRAIL is present in extremely high levels in seminal plasma and suggest that spermatozoa are the direct target of TRAIL, which acts by affecting the vitality of capacitated spermatozoa. It is particularly noteworthy that while our manuscript was under consideration for publication, a study performed in the TRAIL^{-/-} C57BL/6J model has demonstrated a fundamental role of TRAIL in preserving spermatogenesis, with adult TRAIL^{-/-} mice suffering a marked decline in the production of mature spermatozoa (Lin & Richburg 2014). Therefore, our current data obtained in humans are in agreement with the study reported by Lin & Richburg, extending the protective activity of TRAIL to mature spermatozoa and further underlining the importance of TRAIL in the whole process of spermatogenesis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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