Dimeric Transferrin Inhibits Phagocytosis of Residual Bodies by Testicular Rat Sertoli Cells

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ABSTRACT

Transferrin is well known as an iron transport glycoprotein. Dimeric or tetrameric transferrin forms have recently been reported to modulate phagocytosis by human leukocytes. It is mainly synthesized by the liver, and also by other sources, such as Sertoli cells of the testis. Sertoli cells show a strong phagocytic activity toward apoptotic germ cells and residual bodies. Here, we provide evidence that purified human dimeric transferrin from commercial sources decreased residual body phagocytosis, unlike monomeric transferrin. The presence of iron appeared essential for dimeric transferrin inhibitory activity. Importantly, dimeric transferrin could be visualized by immunoblotting in Sertoli cell lysates as well as in culture media, indicating that dimeric transferrin could be physiologically secreted by Sertoli cells. By siRNA-mediated knockdown, we show that endogenous transferrin significantly inhibited residual body ingestion by Sertoli cells. These results are the first to identify dimeric transferrin in Sertoli cells and to demonstrate its implication as a physiological modulator of residual body phagocytosis by Sertoli cells.

INTRODUCTION

Sertoli cells are the supportive and nutritive epithelial cells of mammalian testis seminiferous tubules, and they play vital roles in spermatogenesis. They form the blood-testis barrier, which creates a specialized protected environment, and provide all nutrients and growth factors required for germ cell development [1, 2].

Sertoli cells show strong phagocytic activities toward specific substrates: apoptotic germ cells and residual bodies (RBs). Phagocytic elimination of apoptotic spermatogenic cells appears to be necessary for efficient progression of spermatogenesis [3, 4]. RBs are made of the cytoplasmic portions of elongated spermatids that are shed during extrusion of differentiating sperm into the lumen of the seminiferous tubule [5]. Interestingly, RB external membrane contains apoptotic markers (e.g., ANXA5) [6].

Little is known about the molecular mechanisms underlying phagocytosis by Sertoli cells.

Two multiligand receptors from the class B receptor family seem to be implicated: SCARB1 for selective cholesteryl ester uptake [4, 7, 8], and CD36 for fatty acid transport [9]. Both receptors recognize apoptotic germ cells and RBs and are only partly responsible for phagocytosis by Sertoli cells [10]. This type of phagocytosis by nonprofessional phagocytes takes longer (hours) than by natural phagocytes, such as macrophages (minutes) [11]. As shown in different model systems, the rate of phagocytosis by Sertoli cells can be modulated in vivo and in vitro by numerous factors: extracellular calcium, insulin, epithelium growth factor, hydrocortisone, beta-endorphin [12], follicle-stimulating hormone (FSH) [13], and ANXA5 [4]. The physiological relevance of these factors in the control of Sertoli cell phagocytosis remains unclear, since no variation of their concentration at the stages VIII and IX of the seminiferous epithelium cycle, where phagocytosis occurs, has ever been evidenced.

A strong feature associated with RB phagocytosis is the loss of synthesis and secretion of the iron transport protein transferrin (TRF) by Sertoli cells at stages VIII and IX [14–17]. TRF is one of the most abundant proteins secreted by Sertoli cells and is involved in testicular iron homeostasis [18]. In blood, TRF has been shown to act as a phagocytosis modulator. Indeed, two macromolecular factors released from human platelets, s-MAPP and l-MAPP (shorter and longer multiligand activators of phagocytosis from platelets), have been reported as stimulators of phagocytosis by human leukocytes [19]. Partial amino acid sequence and immunochromatographic studies have identified dimeric and tetrameric iron-saturated TRF as constituents of s-MAPP and l-MAPP, respectively [19].

The purpose of the present study was to investigate whether TRF could be detected as oligomers in Sertoli cells and whether it could modulate RB phagocytosis.

MATERIALS AND METHODS

Pharmacological Reagents

Human and rat TRF, lactoferrin, collagenase II-S, bovine serum albumin, biotin-amidocaproate N-hydroxysuccinimidemester, tetramethylrhodamine isothiocyanate (TRITC)-avidin conjugate, nitrotriacetic acid (NTA), FeCl3, latex beads (diameter 1 μm), and Dulbecco modified Eagle medium (DMEM) were purchased from Sigma Chemical Co. (Sigma-Aldrich Chimie, Lyon, France). Polyclonal anti-TRF antibodies (rabbit anti-human and rabbit anti-rat) were...
raised, purified, and characterized in our laboratory [20, 21]. The anti-human TRF antibody was highly specific of human TRF (0.001% of cross-reaction with rat TRF). The anti-rat TRF antibody was highly specific of rat TRF (0.001% of cross-reaction with human TRF). Secondary anti-rabbit antibodies conjugated to horseradish peroxidase were from GE Healthcare UK Limited. Monoclonal mouse antibodies raised against the N-terminal domain of human TRF were from AbD Serotec (Cergy Saint-Christophe, France).

Primary Cultures of Sertoli Cells

Sertoli cells were isolated from the testes of 19-day-old Wistar rats (Janvier, France). Animals were treated in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health guide). Sertoli cells were seeded in DMEM (Sigma) supplemented with penicillin G, streptomycin, glutamin, retinol, fungizone, and vitamin E (DMEMSC), as previously reported [22]. Sertoli cells were plated in 24- or 12-well dishes (Falcon, Grenoble, France) seeded with 300 000 or 600 000 cells per well, respectively, or in eight-well Labtek chamber slides (Nunc, Rochester, NY) seeded with 150 000 cells per well. On average, our Sertoli cell cultures were 90% pure, as previously quantified [23]. All assays were performed 48 h after initial cell seeding.

RB Purification

Residual bodies were isolated from the testes of adult male Wistar rats as described [6], with some modifications. Testis contents from 2- to 3-mo-old rats were gently recovered through an incision in the tunica albuginea and suspended in 25 ml of 0.01 M PBS (pH 7.2) containing 1% glucose, 3 mM lactate (PBSGL), and then centrifuged for 15 min at 17 000 g at 4°C. The resulting suspension containing RBs and germ cells was allowed to sediment for 3–4 min, and the supernatant was decanted. This process was repeated three times. The dispersed seminiferous tubules were allowed to sediment for 3–4 min, and the supernatant was decanted. This process was repeated three times. The seminiferous tubules were incubated for 15 min PBSGL containing 0.25 mg/ml trypsin for 20 min at 33°C, then cells were dispersed by gentle pipetting.

The suspension was filtered through sterile surgical gauze. Cells were pelleted by centrifugation at 800 × g for 10 min, washed twice, and resuspended in 40 ml PBSGL supplemented with streptomycin (100 μg/ml) and fungizone (2.5 μg/ml). Cell suspension was pelleted at 200 × g for 3 min. Supernatants were collected gently by pipetting and were pelleted twice at 200 × g for 3 min. The resulting suspension containing RBs and germ cells was allowed to sediment overnight at 4°C, then the supernatant was centrifuged at 800 × g for 10 min. The pellet containing mixed germ cells and RBs was discarded, and the supernatant was centrifuged again at 800 × g for 30 min. Pelleted RBs were used as phagocytosis substrates, either biotinylated or nonbiotinylated, and were stored at 4°C for less than 2 wk following purification. The quality of RB preparations was monitored by microscopic observation, following cresyl violet staining.

Biotin labeling was carried out essentially as previously described [24]. Briefly, RBs were transferred into labeling buffer (10 mM Na borate [pH 8.8], 150 mM NaCl), and then biotin from stock solution (50 μg/ml in DMSO) was added to a final concentration of 50 μg/ml. After 15 min of incubation at 20°C, 10 mM NH₄Cl was added to stop the reaction. Biotin-labeled RBs were washed twice, suspended in DMEMSC, and counted.

RB Binding and Ingestion Assay

We have developed an original assay in order to measure RB phagocytosis by Sertoli cells. Primary Sertoli cells seeded in eight-well Labtek chambers were incubated with 1.5 × 10⁴ biotinylated RBs for 2, 4, 6, or 24 h at 34°C in a humidified atmosphere of 5% CO₂. Then, unbound RBs were washed away with DMEM, and cells were fixed for 5 min with 4% paraformaldehyde/PBS (pH 7.4). To distinguish ingested and plasma membrane-bound RBs, samples were divided into two groups, each in duplicate wells. Group 1 was permeabilized with 0.5% Triton X-100 for 5 min, and group 2 remained unpermeabilized. Residual bodies were labeled with avidin-TRITC for 1 h at 25°C. Then, nuclei were stained with 4’,6’-diamidino-2-phenylindole for 5 min at 25°C. The total number of RBs (plasma membrane bound + ingested) was obtained from group 1, and the number of plasma membrane-bound RBs was obtained from group 2. The number of ingested RBs was obtained by subtracting plasma membrane-bound RBs from total RBs. Residual bodies and nuclei were numbered using Visilog 6.3, Macro VBA software (Noesis SA, Les Ulis, France). The phagocytic index was calculated as the ratio of RB/nuclei number in each well.

Small Interfering RNA Transfection

Three distinct small interfering RNA (siRNA) sequences targeting nonoverlapping regions within the rat Trf coding sequence were designed using the www.dharmacon.com web site. Sequences were:

1. 5’-AAAAACCGTCTCTCCAGCTGAT-3’ (position 155–171)
2. 5’-AACAGACTTCTTGCAGCTAAC-3’ (position 388–409)
3. 5’-AAGATTGGAATGTGAGAACA-3’ (position 1202–1221).

Indicated position numbers are relative to the start codon. These three sequences were blasted against the entire rat genome in the NCBI database.

One siRNA duplex that has no silencing effect was used as a control: 5’- AAUUCUCCGACGGUGACCU-3’. All siRNAs have been chemically synthesized (Qiagen, Courtaboeuf, France). Primary Sertoli cells were transfected with 50 nM siRNA using Transit-TKO transfection reagent (Mirus BioCorporation, Montluçon, France) according to the manufacturer’s recommendations.

A total of 1 μl transfection reagent was added to 50 μl DMEMSC and incubated for 10 min at room temperature. At the end of the incubation, 50 nM siRNA control or Trf-specific siRNA was added and incubated at room temperature for 15 min. The mixture was dropped onto Sertoli cells. Forty-eight hours after transfection, TRF content in the culture media was measured by radioimmunoassay, and the phagocytosis assays were carried out. Similar results were obtained with the three siRNA. Results presented here have been obtained with the siRNA in position 388–409.

Purification of Dimeric TRF

Dimeric TRF was purified by gel filtration of human holo-TRF and apo-TRF solutions on an Ultragel AcA44 (IBF Biotechnics) column. The TRF solution (20 mg/ml in PBS) was loaded onto a PBS-equilibrated, 17-ml column. Elution was carried out with PBS at a flow rate of 0.5 ml/min. Fractions of 0.3 ml following Vₑ were collected, subjected to discontinuous native PAGE [25] in 7.5% acrylamide gels, and then Coomassie blue stained.

Characterization of Dimeric TRF by Surface Plasmon Resonance

TRF dimerization was confirmed by surface plasmon resonance (SPR) on a Biacore 1000 (Biacore, Uppsala, Sweden). The anti-hTRF monoclonal antibody reactive with the N-terminal domain of hTRF was immobilized on a CM5 sensor chip (Biacore). Immobilization was carried out as recommended by the manufacturer. The concentration of monoclonal antibody injected was adjusted in order to obtain 4000 units of resonance immobilized on the activated surface. A flow cell control was carried out by activation-deactivation without coupling. The interactions between the purified fractions of hTRF and the monoclonal antibody were studied in 10 mM PBS (pH 7.4), 150 mM NaCl, 0.005% Tween 20 with a flow of 10 μl/min. Flow cells were regenerated in 100 mM H₃PO₄ with a flow of 30 μl/min. Results have been subtracted from those obtained with the flow cell control.

Rat apoTRF Saturation With Iron

Rat apoTRF was saturated with iron as previously described [26]. The A465/ A280 ratio estimated to control TRF saturation.

Radioimmunoassay for TRF Content

Radioimmunoassay measurement of TRF was performed as previously described [20]. The range of the assay was 0.2–200 ng/tube, with an intrassay variation coefficient of 8% for samples within the 20%–70% range of specific binding. All standards and samples were assayed in triplicate.

Western Blot Analysis

Sertoli cell culture media and cell extracts were analyzed by Western blot analysis. The extracts were obtained from 600 000 Sertoli cells. Cells were washed with PBS, then submitted to a hypotonic shock using 0.4 ml of 10 mM Tris-HCl, pH 7.4, scraped, homogenized in a Potter homogenizer, and centrifuged for 20 min at 17 000 × g. The pellets were discarded. Protein contents in the supernatants were quantified by Bradford assay. After discontinuous native PAGE as indicated above, proteins were transferred onto a polyvinylidene fluoride transfer membrane (Perkin-Elmer) by electroblotting as previously described [27], except that methanol was omitted in the transfer buffer. To detect TRF, the primary and secondary antibodies were diluted 1:10 000 and 1:6000, respectively.

Statistics

Results are presented as means ± SD. Data analyses were performed using Mann-Whitney test for evaluation of differences between control and treated
samples using Statview (SAS, Cary, NC). All tests were performed two sided, and $P < 0.05$ was considered statistically significant.

RESULTS

Identification and Purification of Dimeric TRF From Commercial Human TRF Preparation

In commercially prepared apo- and holo-human TRF, two bands corresponding to high- and low-electrophoretic mobility forms could be observed. The high- and low-mobility forms of holo-hTRF were purified by gel filtration (Fig. 1, A and B), and then fractions 6 and 12 were characterized further by mass spectrometry and SPR. Mass spectrometry confirmed that both fractions contained only hTRF. Importantly, fractions corresponding to the low-mobility band exhibited a molecular mass of 150 kDa, whereas the estimated mass of the fractions corresponding to the high-mobility band was 78 kDa (data not shown). These results suggest that the low-mobility band was a dimer form of TRF.

By SPR, only the fraction of high molecular mass gave an additional specific signal when the anti-hTRF monoclonal antibody was reinjected. This observation reveals the existence of two binding sites, which is indicative of a dimeric form of TRF. In contrast, this additional signal was not observed with the fraction of low molecular mass, which has only one binding site, immobilized on the sensor chip (Fig. 1C). These results
confirm the presence of monomeric and dimeric TRF in commercially available human TRF preparations.

**Inhibitory Role of Dimeric TRF in RB Phagocytosis by Sertoli Cells**

As a preliminary step, we sought to develop a reliable assay in order to quantify Sertoli cell phagocytic activity using the cells’ physiological substrates (i.e., purified and labeled RBs). Figure 2A shows a typical RB preparation, which was further biotinylated and used in phagocytosis studies. Biotinylation of RB membranes did not block their recognition by Sertoli cells (Fig. 2, B1 and B5). Total and plasma membrane-bound RBs were measured by comparing labeling of permeabilized versus unpermeabilized Sertoli cells, respectively. In permeabilized cells, both membrane-bound and ingested RBs were detected (Fig. 2, B6 and B8). The number of ingested RBs was obtained by subtracting plasma membrane-bound RBs to total RBs. Noteworthy, no specific interaction of RBs with the chamber support was observed. For each group of samples, the phagocytic index was calculated as the ratio of RB/nuclei number in each well. Using this method, we analyzed the time-dependent rate of RB binding and ingestion by Sertoli cells. The total number of RBs (bound + ingested, squares) and plasma membrane-bound RBs (circles) were evaluated by image analysis after 2, 4, and 6 h of incubation. The number of ingested RBs (triangles) was calculated by subtracting plasma membrane-bound RBs from total. The phagocytosis index was calculated as the ratio of RB/nuclei number in each well. Values represent the mean ± SD of three independent experiments.
Sertoli cell cytoplasm (data not shown). Thus, 2- to 4-h incubations were within the linear part of the phagocytosis kinetic curve and were applied throughout the present study.

The addition of TRF dimers did not modify the number of total RBs phagocytosed by Sertoli cells. However, the number of plasma membrane-bound RBs significantly increased in the presence of dimeric TRF, whereas ingested RBs were substantially reduced under the same conditions (Fig. 3A). Indeed, 100 nM of TRF dimers totally abolished RB ingestion by Sertoli cells. The same results were obtained when using rat TRF (data not shown). In contrast, neither albumin, which also exists in dimeric form, nor lactoferrin, another iron transporter, altered the ability of Sertoli cells to ingest RBs (data not shown). These results indicate that the inhibitory effect of TRF is specific. Furthermore, only the dimer is effective, since monomeric TRF was unable to modulate Sertoli cell phagocytosis (Fig. 3B).

Iron Requirement in Dimeric TRF Inhibitory Activity

Next, we have examined whether or not iron was involved in dimeric TRF-mediated inhibition of RB phagocytosis by Sertoli cells. Clearly, iron-saturated TRF dimer was a potent inhibitor of RB ingestion by Sertoli cells, whereas iron-free TRF dimer was totally inactive (Fig. 4).

Identification of Two Forms of TRF in Sertoli Cells

Since TRF modulates platelet phagocytosis as oligomers, we then sought to visualize by native gel electrophoresis and immunoblotting the TRF present in the culture media and in Sertoli cell lysates. In order to visualize extracellular TRF secretion, cells were incubated with FSH and with insulin, two well-known stimulators of TRF expression [28]. With a highly specific antibody to rat TRF, two bands with different electrophoretic mobility, identified as the monomeric and dimeric forms of TRF, were detected in the media from cultured Sertoli cells (Fig. 5, lane 2) and in Sertoli cell lysates (lane 4). In the cell lysates, the dimer was found to be substantially more abundant than the monomer.

Inhibition of Phagocytosis by Sertoli Cell Endogenous Transferrin

To investigate whether endogenously synthesized TRF was implicated in Sertoli cell-mediated phagocytosis, endogenous
TRF expression was knocked down by siRNA transfection. Forty-eight hours after Trf siRNA but not control siRNA transfection, a 2-fold reduction in TRF expression was observed (Fig. 6A). Similar results were observed with three siRNA targeting nonoverlapping regions within the rat Trf sequence (data not shown).

Interestingly, we found that the total number of phagocytosed RBs (bound + ingested) was 2-fold higher in Trf siRNA-transfected Sertoli cells when compared to control siRNA-transfected cells (Fig. 6B). Both plasma membrane-bound and ingested RBs were significantly increased in TRF-depleted Sertoli cells. Thus, TRF expression seems to inhibit RB phagocytosis.

Importantly, the dimeric but not the monomeric form of TRF was able to counteract the Trf siRNA-mediated activation of RB phagocytosis by Sertoli cells (Fig. 7A), which supports that Sertoli cell endogenous TRF could act as a dimer to physiologically inhibit RB phagocytosis. Furthermore, we found that the dimer inhibited the ingestion step of phagocytosis but not the adhesion step (Fig. 7, B and C).

**DISCUSSION**

Two macromolecular factors, s-MAPP and 1-MAPP, corresponding to dimeric and tetrameric TRF, respectively, have been reported to act as potent phagocytosis activators for human leukocytes [19]. In the present study, we have been able to accurately quantify RB phagocytosis by Sertoli cells. We have shown that TRF dimer prevents RB ingestion by Sertoli cells. The involvement of dimeric TRF as a regulator of RB phagocytosis by Sertoli cells is strongly supported by our siRNA-mediated knockdown of Trf. Even a partial inhibition of TRF expression is sufficient to increase RB phagocytosis by Sertoli cells (i.e., both plasma membrane binding and ingestion are increased). The addition of TRF dimer but not TRF monomer to Sertoli cells transfected by Trf-specific siRNA abolished the siRNA-mediated stimulation and even decreased the rate of RB ingestion. This observation is also supported by previous data showing that FSH accelerates RB binding to Sertoli cells but markedly reduces the number of ingested RBs [13]. On the other hand, FSH is well known to be the main activator of TRF expression [28]. In that context, it can be speculated that the FSH modulatory effects on Sertoli cell phagocytosis might be due to an increase in TRF dimers that arrest RB ingestion. If this hypothesis holds true, other factors controlling TRF expression, such as vitamin A, insulin, or growth factors [2], should also be able to control the rate of phagocytosis by Sertoli cells. Further studies will be necessary to test this hypothesis.

It is likely that in Sertoli cells, dimeric TRF results from stable binding of two monomers of TRF via at least one S-H bond, since the dimers are disrupted under reducing conditions. Dimeric TRF was detected in Sertoli cell lysates and in Sertoli cell culture media, but the monomeric versus dimeric ratio was quite different intracellularly and extracellularly: dimeric TRF appeared to be less abundant in culture media than in cell lysates, where it was the prominent form. However, we cannot exclude that part of the dimeric TRF visualized in Sertoli cell lysates could be trapped with fragments of plasma membrane embedding the TRF receptors. In support of this possibility, we found that dimeric TRF isolated from commercial human TRF preparation binds to TRF receptors present at the Sertoli cell plasma membrane with a better affinity than the TRF monomer, with an ED50 of 0.3 nM versus 4.9 nM (our unpublished observations). We have also detected the previously identified Trf1 and the as-yet undescribed Trf2 mRNA in Sertoli cells (our unpublished observations). In addition, it is generally accepted that once iron dissociates from TRF in the endosomes, the TRF/Trfr complexes are addressed back to the plasma membrane [29].

TRF dimers inhibit the ingestion phase of RB phagocytosis by Sertoli cells. It could play this physiological role by modulating intracellular signaling pathways, leading to phagocytosis. Iron is strictly required for dimeric TRF to exert its effects, and thus might represent a major element in the control of phagocytosis by Sertoli cells. This possibility raises another speculative but exciting question: is the role of TRF in phagocytosis control related to iron transport? We have previously shown that in the adult brain, the effects of TRF in oligodendrocyte maturation do not solely relate to iron transport, since the levels of iron and ferritin remain constant during the process [30].

The variations in TRF expression that occur during the course of the seminiferous epithelium cycle support the physiological relevance of a regulatory role for TRF in RB phagocytosis by Sertoli cells. More specifically, in the stages devoid of Sertoli cell-mediated phagocytic activity, TRF expression by Sertoli cells is high. In contrast, during stages VIII–IX of the epithelium cycle, where RB phagocytosis is known to occur, TRF expression is low [17]. Together with our results, these data suggest that the decrease of TRF expression during spermatogenesis facilitates RB phagocytosis by Sertoli cells. Whether dimeric TRF also modulates apoptotic germ cell phagocytosis by Sertoli cell remains to be investigated.
FIG. 6. Knockdown of Sertoli cell-secreted TRF by specific siRNA transfection. A) Sertoli cells in culture were transfected with control or Trf siRNA. After 48 h, supernatants were collected, and TRF was measured. B) After 48 h, Sertoli cells were incubated with RsB for 2 h. The number of plasma membrane-bound, total, and ingested RBs was evaluated. The phagocytosis index was calculated as in Figure 1C. Values represent means ± SD of three independent experiments. ***P < 0.001.

FIG. 7. Rescue of dimeric TRF inhibitory effect in Trf knocked down Sertoli cells on RB phagocytosis. Sertoli cells in culture were transfected with control siRNA (black bars) or Trf siRNA (white bars). After 48 h, holomonomeric or holo-dimeric TRF (holo-TRF monomer and holo-TRF dimer, respectively) was added. After 2 h of incubation, the total number of RBs (A) (bound + ingested) and plasma membrane-bound RB (B) were quantified. C) The number of ingested RBs was calculated by subtracting plasma membrane-bound RBs from the total. The phagocytosis index was calculated as in Figure 1C. Values represent means ± SD of three independent experiments. *P < 0.05; ***P < 0.001.
In conclusion, TRF is synthesized by the liver and also by tissues, such as the choroid plexus, retina, testis, and mammary gland, which all display strong phagocytic activities. Our study raises the exciting possibility that TRF could be a universal phagocytosis modulator in professional [19] as well as nonprofessional phagocytes in mammals. Further studies, including conditional Trf knockouts, are now required in order to further understand the role of TRF in the phagocytosis process.

ACKNOWLEDGMENTS

We thank Dr. Rustem Uzbekov for his help with microscopic studies; Dr. Pascal Rainard for neutrophil studies; Daniel Tanguy for design of phagocytosis analysis software; Maya Belghazy for mass spectrometry studies; and Dr. Michel Blanc for critical reading of the manuscript. We also thank Claude Cahier, Jean-Claude Braguier, and Christelle Ramé for rat breedings.

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