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Residual Bodies Stimulate Rat Sertoli Cell Plasminogen Activator Activity

Francesco Sigillo,* Gilles Pernod,† Lucien Kolodie,†
Mohamed Benahmed,* and Brigitte Le Magueresse-Battistoni*

**InsermU407, Batiment 3B, Centre Hospitalier Lyon-sud, 69495 Pierre-Bénite cédex, France; and †Laboratoire d'Hématologie, Centre Hospitalier Universitaire de Grenoble, B.P. 217, 38043 Grenoble cédex 9, France*

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Previous studies suggested indirectly that residual bodies (RB) may play a role in the regulation of the proteolytic system within the testis and in the coordination of the spermatogenetic process. In the present study, we examined the effects of RB recovered from adult rat testes by centrifugal elutriation on Sertoli cell plasminogen activator (PA) levels, by zymography and ELISA procedures. Addition of RB to Sertoli cell cultures prepared from 20-day-old rat testes resulted in a dramatic stimulation of PA. Effects were dose- and time-dependent. Phagocytosis of RB by Sertoli cells leads to a rapid stimulation of Sertoli cell interleukin-1α (IL-1α), a cytokine potentially involved in the regulation of spermatogenesis; the effects of IL-1α were investigated. We found that IL-1α augmented PA levels and that immunodepletion of Sertoli cell–RB cocultures with anti-IL-1α antibodies abrogated the stimulatory effects of RB on PA. Together, the present findings indicate that RB enhance Sertoli cell PA and that IL-1α may be involved in that control.

Spermatogenesis is a strictly coordinated and highly regulated process, whereby stem cells undergo massive division and differentiation to ultimately give rise to spermatozoa. In mammals, spermatogenesis proceeds apically towards the lumen of the seminiferous tubules. It is arranged in stages (14 in the rat testis) that correspond to specific and constant associations of Sertoli cells and germ cells at defined periods of development (1, 2). In a pioneering work, Regaud (3) suggested that phagocytosis of residual bodies (RB) by Sertoli cells could constitute a signal that would coordinate the process of spermatogenesis. RB are the cytoplasmic portion of the elongated spermatids that are shed at spermiation [i.e. when sperm is extruded in the lumen of the seminiferous tubule]. This hypothesis was based on the temporal correlation that exists between the emission of the RB, their phagocytosis and the initiation of a new wave of spermatogenesis, all occuring in the rat at stages VII-VIII of the cycle (1, 3).

Proteases and antiproteases are likely to be involved in this restructuring step (4). Plasminogen activators (PAs) are among those identified in the testis. PAs are highly specific serine proteases that convert latent plasminogen into the active protease plasmin. Two types of PA have been described, the urokinase-type (u-PA) and the tissue-type (t-PA) (5). The two are secreted by Sertoli cells, and are under the control of the trophic hormone FSH (4, 6). Interestingly, greatest rates of PA secretion occur at stages VII-VIII, and the *in vitro* PA secretion is strongly enhanced in rat Sertoli cells that had phagocytosed autoclaved E. Coli (Ref. in 4).

These observations prompted us to analyze *in vitro* the direct effects of a fraction of RB recovered by centrifugal elutriation on Sertoli cell PA activity. We observed that RB stimulated PA activity, and that the action of RB might be mediated through interleukin-1α (IL-1α), a cytokine rapidly induced in Sertoli cells phagocytosing RB (7, 8).

MATERIALS AND METHODS

Sertoli cell culture and coculture with RB. Sertoli cells were prepared from 20-day-old Sprague–Dawley rats according to methods described elsewhere (9, 10), and cultured at 32°C in a humidified atmosphere of 5% CO_2 in Ham's F12/DMEM (v/v). RB were isolated from adult Sprague–Dawley rats by trypsinization followed by a centrifugal elutriation using a rotor Beckman JE-6 (11). The RB fraction contained residual bodies and cytoplasts from elongated spermatids steps 10–19, detached mechanically throughout the isolation procedure. This fraction was enriched at 75–80%, and was mainly contaminated with sperm heads and cell debris such as broken spermatids (11, 12). However for more clarity this fraction will be referred to as RB. Sertoli cell cultures were treated on Day 5 post-plating, either with freshly elutriated RB, and/or with human recombinant IL-1α (Boehringer Mannheim, Meylan, France), and/or with a polyclonal rabbit anti-mouse anti-IL-1α antibody which cross-reacted with rat IL-1α (Genzyme, Paris, France). At the end of the experiment, culture and coculture media were collected, and concentrated 10-fold using microconcentrator centricon 10 (Amicon Divi-

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sion, Beverly, MA). Cells were lysed in a Tris buffer solution (0.5 M, pH 8.2) containing 0.5% Triton X-100.

Zymographic analysis. SDS–PAGE zymography was carried out under non-denaturing conditions as previously described (13), using 40 μl per sample. Fibrinolytic activity was revealed by placing the gel on a plasminogen fibrin agarose underlay, and incubated at 37°C for 16 h. Human u- and t-PA, and mol wt standards were loaded in each SDS–PAGE gel. PA activator specificity was determined by preliminary experiments (13). In the absence of plasminogen in the gels, no protease activity was evident (not shown).

Measure of t-PA antigen. t-PA antigen was assayed in a ELISA kit (Imulyse t-PA, Biopool, Diamed, Paris, France), since the monospecific human antiserum used in this kit cross-reacted with rat t-PA. There is no cross-reaction with urokinase. The assay measures free t-PA as well as t-PA related activities.

Data analysis. Each figure is a representative experiment out of (at least) the three performed independently with triplicate dishes. Significance was determined by use of analysis of variance and student's t test. Differences are accepted as significant at $p < 0.05$.

RESULTS

Addition of 8×10^6 RB per Sertoli cell culture for 40h (RB had no detectable PA activity in our experimental conditions, data not shown) resulted in a dramatic increase in the activity of u- (a 45 kDa lytic band) and t- (a 70 kDa lytic band) PA either in lysates or media (Fig. 1a). The extra-lytic bands of 90 and 120 kDa present in media (but not in lysates) correspond probably to u- and t-PA complexed respectively with plasminogen activator inhibitor-1 (PAI-1), a fast-acting and specific inhibitor of the two PAs (4, 5). The basis for this assumption is two-fold: (i), a previous report (14) demonstrated that PAI-1 could complex to PAs in SDS-gels at migrating sizes compatible with our results; (ii), we developed experiments that consisted in incorporating to the plasminogen fibrin layers either amiloride or a polyclonal anti-t-PA antibody. We could that way eliminated all u- and t-PA-related activities, respectively (data not shown). Total PAI-1-related activity was also found to increase when Sertoli cells were cocultured with RB (Fig. 1a). Action of RB on t-PA antigen levels was dose-dependent (Fig. 1b). A maximal 4.5-fold-increase ($p < 0.01$) was observed with 8×10^6 RB added per dish after 16 h.

In addition, action of RB was time-dependent (Fig. 2). In Sertoli cell only cultures, we observed a time-dependent increase of the u-, t-, and PA-PAI-1 bands. Addition of RB (6×10^6/dish) resulted after 16h of coculture in a stimulation of the u- and t-PA activity, and in a decrease of the PAI-1-related activity. The RB-induced increase in PA activity was still evident at 24 and 40 h. It was accompanied by an increasing PAI-1-related activity. At 64 h of coculture, the u-PA and t-PA lytic bands did not further increase, contrasting with the enhancement of the intensity of the PAI-1 complexes (all compared to their respective time-matched control). RB action on t-PA antigen levels measured in the media (Fig. 2b) reached its maximum (5-fold increase; $p < 0.01$, vs respective time-matched controls) after 16–24 h of treatment. Thereafter, RB action declined slowly to a 3.5 and 2.7-fold after 40 and 64 h of treatment ($p < 0.01$ and $p < 0.05$, vs respective time-matched controls). The assay measuring t-PA and t-PA-PAI-1 complexes, the sustained stimulation resulted probably from an increase of the complexes observed on the corresponding zymographic profiles (Fig. 2a).

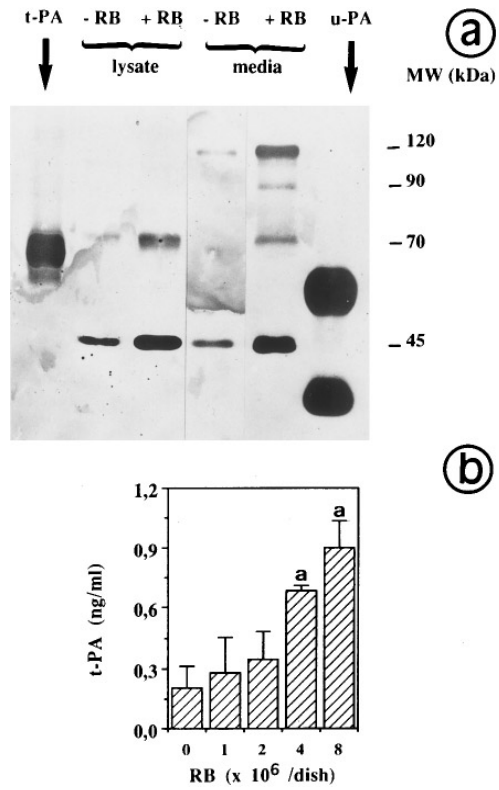

FIG. 1. RB stimulated PA activity and t-PA antigen levels. (a) Upper panel, Sertoli cells were cocultured with 8×10^6 RB/dish for 40 h, after which cell lysates and media were analyzed by zymography. t-PA and u-PA are from human origin. (b) Lower panel, Sertoli cells were cocultured with RB (from 1 to 8×10^6/culture dish) for 16 h, after which media were analyzed by ELISA for measuring t-PA related activities. Results are expressed in ng/ml concentrated media, and are the mean ± SEM of 3 dishes. a, $p < 0.01$ versus control (no RB added).

RB inducing Sertoli cell IL-1α production rapidly [first significant effects observed 3h after RB addition with a plateau reached by 24h (8), we designed two experiments: 1), we tested the action of this cytokine on Sertoli cell PA activity with a dose of 100U/ml i.e. compatible with the amount of IL-1α secreted by Sertoli cells having phagocytosed 8×10^6 RB/dish in 24 h (7, 8). Results presented in Fig. 3 indicate that IL-1α was a potent stimulator of Sertoli cell PA activity, and of t-PA antigen levels; 2), we immunodepleted RB-Sertoli cell cocultures with anti-IL-1α antibodies (dilution 1:500). Results presented in Fig. 4 indicate that anti-IL-1α-antibodies prevented the RB-induced enhancement of Sertoli cell u- and t-PA, normally observed after 16h of coculture with 8×10^6 RB/dish (Fig. 4).

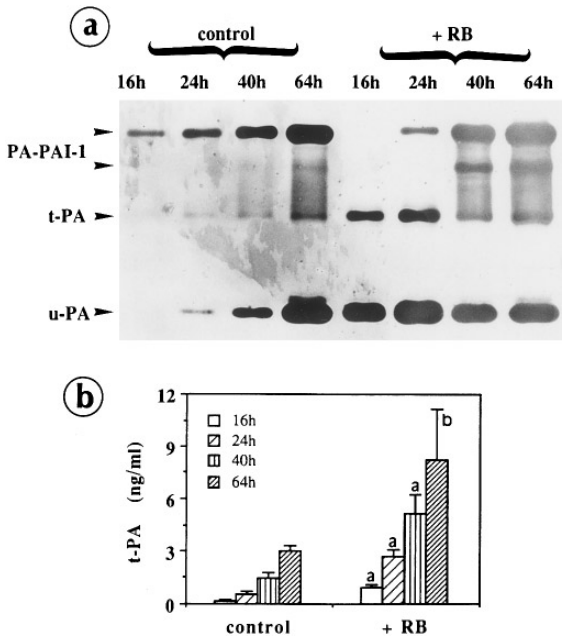

FIG. 2. RB stimulated Sertoli cell PA in a time-dependent fashion. Sertoli cells were cocultured with 6×10^6 RB/dish for 16, 24, 40, or 64 h, after which culture and coculture media were collected, concentrated and analyzed by zymography (a) and ELISA (b). Results in b are expressed in ng of t-PA-related activity /ml concentrated culture media, and are the mean ± SEM of 3 dishes. a, $p < 0.01$; b, $p < 0.05$ versus its time-matched control.

DISCUSSION

We here investigated whether RB modulated Sertoli cell PA production and whether IL-1α could be involved in that control. The *in vitro* system consisted in elutriating a fraction of RB from adult rat testes, and in coculturing RB with highly purified Sertoli cells recovered from 20-day-old rat testes. Procedures developed included zymography and t-PA antigen ELISA procedures.

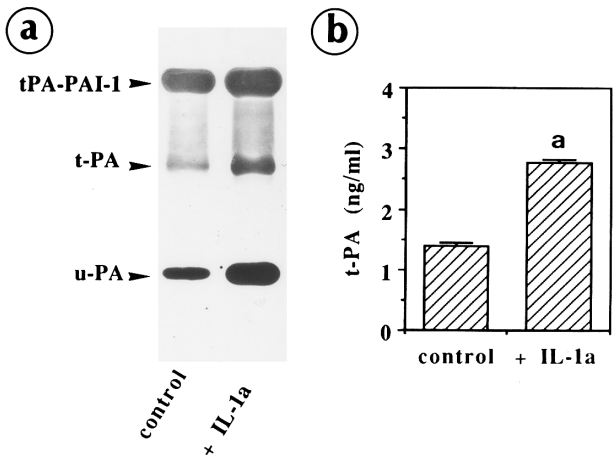

FIG. 3. IL-1α stimulated Sertoli cell PA. Sertoli cells were treated for 24 h with IL-1α (100 U/ml), after which culture media were collected, concentrated and analyzed by zymography (a) and ELISA (b). Results in (b) are expressed in ng of t-PA-related activity/ml concentrated culture media, and are the mean ± SEM of 3 dishes. a, $p < 0.001$ versus control (no IL-1a added).

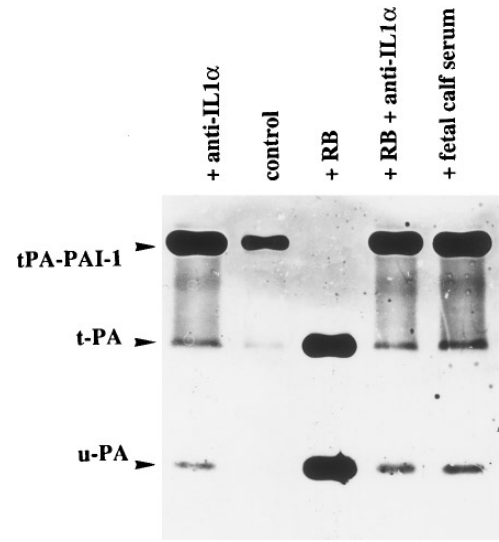

FIG. 4. Addition of anti-IL-1α prevented the RB-induced enhancement of PA. Sertoli cells were cocultured with 8×10^6 RB/dish for 16 h in the presence or absence of anti-IL-1α. Concentrated media were analyzed by zymography. Note the presence of complexes in Sertoli cells treated with anti-IL-1α (supplied as neat hyperimmune antiserum; dilution 1:500) and/or with fetal calf serum (dilution 1:500) versus Sertoli cells only.

Our interest stems from a previous hypothesis based on the temporal correlation that exists between the emission of RB, their phagocytosis and the initiation of a new wave of spermatogenesis, all occuring in the rat at stages VII-VIII of the cycle (1–3). Moreover, greatest rates of PA secretion occur at stages VII-VIII, and the *in vitro* PA secretion is strongly enhanced in rat Sertoli cells that had phagocytosed autoclaved E. Coli (Ref. in 4). Furthermore RB have been found *in vitro* to induce Sertoli cell IL-1α release (7, 8). IL-1α has initially been described as a macrophage-derived protein and is now known to be synthesized by a large variety of cells (15). Apart from its multiple effects on cells of the immune system, recent studies reported that IL-1α may regulate the functions of several endocrine cells such as testicular cells (Ref. in 16, 17). Interestingly, IL-1α is a testicular product with Sertoli cells being one source (7, 8, 18) and (i) the onset of Sertoli cell IL-1α production coincides with the establishment of the meiotic process (18); (ii) maximum levels of IL-1α are reached at stages VIII and IX-X (19); (iii) rodent germ cells contain IL-1 receptor 1 transcripts (20); (iv) IL-1α promotes DNA synthesis and differentiation of spermatogonia and *preleptotene* spermatocytes (19, 21).

RB isolated by centrifugal elutriation and in the *in vitro* conditions described previously and used here,

adhere rapidly and in a great proportion to rat Sertoli cells. They are thereafter phagocytosed following a scheme close to the *in vivo* situation (12, 22). Sperm heads contaminating the RB fraction are also phagocytosed by Sertoli cells (22). Therefore our coculture model allowed us and others (7, 8, 17) to investigate the consequences of inducing Sertoli cell phagocytic activity by a fraction highly enriched in RB/cytoplasts. We here demonstrated that the addition of such an elutriated fraction containing RB induced a dose- and time-dependent stimulation of Sertoli cell PA levels. The highest PA activity was detected at 16h (high PA levels, no PAI-1-related activity). A high PA activity was still detected at 24h. However past that time, the PA lytic bands did not further increase. They rather decreased when compared to the PA lytic bands observed in cocultures at 24 h. In addition there was an increase in PAI-1 activity (u-PA–PAI-1 and t-PA–PAI-1 complexes), which is probably of Sertoli cell origin (23). These data suggest the existence of a two-step mechanism, probably in order to counteract the proteolytic action resulting from the phagocytic activity of Sertoli cells induced by the addition of the RB fraction. We are now investigating whether the RB-inducing effects (increased PA activity followed by an increase in PAI-1 activity) which are observed in this study at the protein activity level, are exerted at the mRNA level.

We next analyzed whether IL-1α might be involved in the RB-induced increase of PA. We showed in a first step that IL1-α at a dose compatible with the amount of IL-1α secreted by Sertoli cells that had been cocultured with RB (7, 8), stimulated PA activity and t-PA antigen levels. In addition and the most striking evidence that action of RB on Sertoli cell PA could be mediated by at least an increase of IL-1α was given by the experiment that consisted in immunodepleting Sertoli cell-RB cocultures with anti-IL-1α antibodies. We that way, prevented the RB-induced action on Sertoli cell PA activity.

In conclusion, an elutriated fraction highly enriched in RB/cytoplasts can regulate Sertoli cell PA activity, through probably an enhancement of IL-1α production. Although this work was made *in vitro*, it might be tempting to speculate that RB, once phagocytosed by Sertoli cells induce PA through at least enhancing IL-1α production. This might lead in the stimulation of DNA synthesis in spermatogonia and spermatocytes, and in the opening of the tight junctions required for germ cells to migrate in the adluminal compartment, and to enter the meiotic program. In a next step, RB would induce PAI-1 to avoid detachment of the elongating and not yet mature spermatids. IL-1α inducing IL-6 (8), we are now determining the effects of IL-6 on the plasminogen activation system within the testis.

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