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Production of the cysteine proteinase inhibitor cystatin C by rat Sertoli cells

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The Sertoli cells of the rat testis produce cystatin C, a cysteine proteinase inhibitor. Primary culture of Sertoli cells secreted both unglycosylated and glycosylated forms of rat cystatin C. Despite the low concentration of cystatin C in rete testis fluid, equilibrium dissociation constants (K_i) for the interaction between cystatin C and lysosomal cathepsins indicate that this molecule could be involved in the local regulation of testicular cysteine proteinase activity which may be necessary for spermatogenesis and spermiogenesis.

Cystatin C; Testis; Sertoli cell; Rat

1. INTRODUCTION

Sertoli cells, the major epithelial component of the seminiferous epithelium, are essential for spermatogenesis and spermiogenesis [1]. They are involved in supplying nutrients and controlling germinal cell multiplication and differentiation. Sertoli cells produce a great variety of proteins, including growth factors such as IGF or TGF β, and transport proteins such as transferrin, androgen binding protein and ceruloplasmin (for a review see [1]). However, only one proteinase inhibitor, alpha-2-macroglobulin, is presently known to be synthesized by these cells [2]. Although this molecule inhibits all four classes of proteases [3], it is likely that specific inhibitors must also play a role in controlling the proteolytic activities which participate in the migration of germ cells and maturation of spermatozoa, or are liberated during late spermatid degeneration [4].

The proteolytic activities described so far are due to the serine type proteinases including acrosin (EC 3.4.21.10) [4], plasmin (EC 3.4.21.7) [5], plasminogen activator (EC 3.4.21.31) [6] or to metalloproteinases [7]. Little information is available on the contribution of lysosomal cysteine proteinases to the cellular or extracellular proteolytic events accompanying the germinal cell transformations. Dramatic changes in the morphology and number of rat Sertoli cells lysosomes has been reported [8] and a latent cathepcin L has also been described in the spermatozoa of guinea pigs [9]. This report demonstrates that rat Sertoli cells produce cystatin C, a specific inhibitor of cysteine proteinases belonging to family 2 of the cystatin superfamily [10]. Rat cystatin C is a 120 amino acid molecule which is found mostly in secretions and extracellular fluids such as saliva, cerebrospinal fluid, blood plasma and seminal vesicles fluid [11-13]. It inhibits the lysosomal cysteine proteinases, cathepsins B, H and L [11]. This study shows that cystatin C is produced in the rat testis and indicates that it may participate in spermatogenesis and spermiogenesis.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Sertoli cells were isolated from the testes of 18-day-old rats (Wistar INRA 03) [14]. The preparation also contained 17.5±4.5% germ cells and 4±2% myoid cells, which were almost completely removed during the washing step after the first day of culture. These cells were cultured at a density of 10^6 cells/ml in either plastic dishes coated with Matrigel (Collaborative Research) or uncoated dishes. Cells were grown in Dulbecco's minimum essential medium supplemented with 2 mM glutamine, 2 μg/ml insulin, 5 μg/ml human transferrin, 50 ng/ml retinol, 100 U/ml penicillin and 100 μg/ml streptomycin. Leydig cells were isolated from the testes of 52-day-old rats (Wistar INRA 03) [15] and cultured at a density of $5 \cdot 10^6$ cells/ml on plastic dish in the same medium. The culture medium from both Sertoli and Leydig cells was changed after 1, 2, 3 and 6 days of culture, and the conditioned medium stored.

2.2. *Collection of biological fluids*

Blood was obtained from rats by cardiac puncture, rete testis fluid (a gift of J.L. Dacheux) was obtained by the method of Tuck et al. [16], and interstitial fluid was collected according to the method of Sharpe [17]. Rat and mouse seminal vesicle homogenates were prepared as previously described [11].

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2.3. *Immunochemical detection of rat cystatin C*

The concentration of rat cystatin C in Sertoli cell medium, rete testis fluid, Leydig cells' medium, interstitial fluid, seminal vesicles fluid and plasma was measured using a double antibody sandwich ELISA. Immulon plates M129B (Dynatech) were coated with rabbit anti-rat cystatin C immunserum, and goat immunserum against human cystatin C was used as the second antibody. Immunoreactive material was stained with peroxidase-conjugated rabbit anti-goat immunoglobulins (Dakopatts) and *o*-phenylenediamine as a substrate.

2.4. *Purification of rat cystatin C*

Rat cystatin C was concentrated from Sertoli cell culture medium (375 ml, cystatin C concentration 0.025 mg/l) by ultrafiltration on a Diaflo YM5 membrane (Amicon) and loaded onto a TSK 2000SW column (0.75 × 60 cm). Proteins were eluted with 0.1 M Tris-HCl, pH 7.4, 0.5 M NaCl at a flow rate of 0.5 ml/min and positive fractions detected by immunoblotting. These fractions were concentrated on a Diaflo YM5 membrane and separated by reverse phase HPLC on Aquapore BU-300 column (2.1 × 30 mm) (Applied Biosystems) using a 0–60% gradient of acetonitrile in 0.075% trifluoracetic acid.

2.5. *Sequence analysis*

N-terminal amino acid sequence analyses were performed on an Applied Biosystems 477A protein sequencer and PTH derivatives were identified using an on-line model 120A analyser.

2.6. *Deglycosylation of rat cystatin C*

The unfractionated Sertoli cell culture medium was deglycosylated by overnight incubation with 1 U/ml endo-beta-*N*-glucosaminidase F (EC 3.2.1.96) (Boehringer) in 20 mM potassium phosphate buffer, pH 6.5, 10 mM EDTA, 1% Triton X-100, 0.2% SDS and 1% mercaptoethanol.

2.7. *Electrophoretic techniques*

Electrophoresis was performed according to Laemmli [18] on a Hoefer SE200 slab unit. Electroblotting on nitrocellulose sheets (Sartorius) was performed as decribed [11]. Cystatin C was detected using rabbit anti-rat urinary cystatin C, peroxidase conjugated swine immunoglobulins to rabbit immunoglobulins (Dakopatts) and 4-chloro-naphthol as a substrate. Alternatively the enhanced chemiluminescent Western blotting detection system RPN 2106 (Amersham) was used.

2.8. *Analysis of inhibitory activity*

Inhibitory activity of concentrated culture medium towards papain (EC 3.4.22.2) was measured as previously described [11] using Z-Phe-Arg-NMec as a substrate. Checks showed that the concentrated culture medium contained no hydrolytic activity towards Z-Phe-Arg-NMec.

3. RESULTS AND DISCUSSION

Primary cultures of Sertoli and Leydig cells were found to secrete cystatin C in a time-dependent manner (Fig. 1). Production by Sertoli cells was maximal after two days of culture in uncoated dishes, and after three days in dishes coated with Matrigel (not shown) and reached 60 × 4 ng/10^6 cells/24 h. However, there was no hormonal regulation of the synthesis by FSH, retinol or isoproterenol, although these components have been shown to dramatically change the concentration of other proteins secreted by Sertoli cells [14,19]. Cystatin C production by Leydig cells was maximal on the first day in culture and decreased thereafter. It was also far lower than in Sertoli cells (4 ± 0.4 ng/10^6 cells/24 h) (Fig. 1). Therefore, Sertoli cells appears to be an important if not the major source of cystatin C in the rat testis.

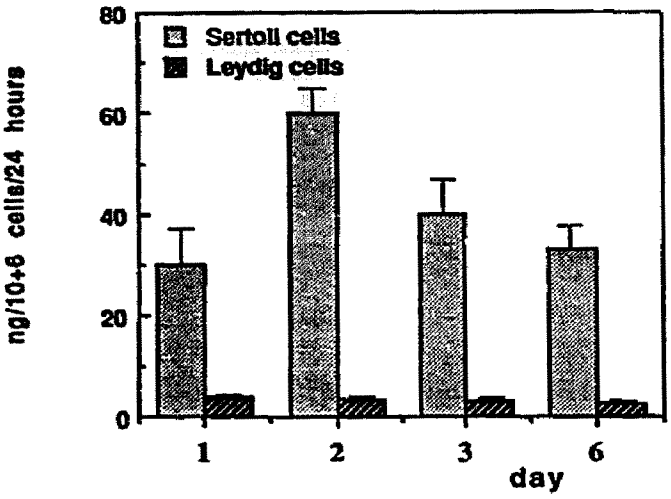

Fig. 1. Cystatin C production by rat Sertoli cells and Leydig cells in culture. The cystatin C concentration was determined by a double sandwich ELISA. Production is expressed in ng protein per million cells per day. (Mean values of four experiments).

Cystatin C from concentrated Sertoli cell culture medium appeared as two almost equivalent, closely migrating bands after SDS gel electrophoresis and electroblotting (Fig. 2a, lane 2). Using the more sensitive chemiluminescent substrate RPN 2106 these two bands were hardly distinguishable whereas an additional band of higher apparent M_r was revealed on the blot (Fig. 2b, lane 1) This latter component was susceptible to endo-beta-*N*-glucosaminidase F treatment (Fig. 2b, lane 2), and corresponded to the previously characterized *N*-glycosylated form of rat cystatin C [12,13]. Mouse cystatin C was also found to be partially *N*-glycosylated (Fig. 2b, lane 3,4). Both molecules bear the same potential glycosylation site at Asn-79 [13,20], which is located opposite the inhibitory region of the molecule [21], and therefore does not interfere with the inhibition.

The electrophoretic mobility of the two major forms of Sertoli cell medium cystatin C (Fig. 2a, lane 2) was intermediate between those of the glycosylated and the non-glycosylated form of seminal vesicle cystatin C (Fig. 2a, lane 1). This specific pattern could be the result of post-translational modification of the molecule in Sertoli cells. It could also be related to the specific pattern of cystatin C mRNAs in testis, since this organ contains two mRNAs, one of 700 and the other of 500 bases [22].

A specific procedure was developed for purifying cystatin C from Sertoli cells in culture, due to the low concentration of the molecule secreted. Concentrated medium was filtered through a TSK 2000SW column (Fig. 3a). Two peaks of immunoreactive material eluting at $M_{r,app}$ 30,000 and 16,000 were found after SDS gel electrophoresis and electroblotting (Fig. 3b). The

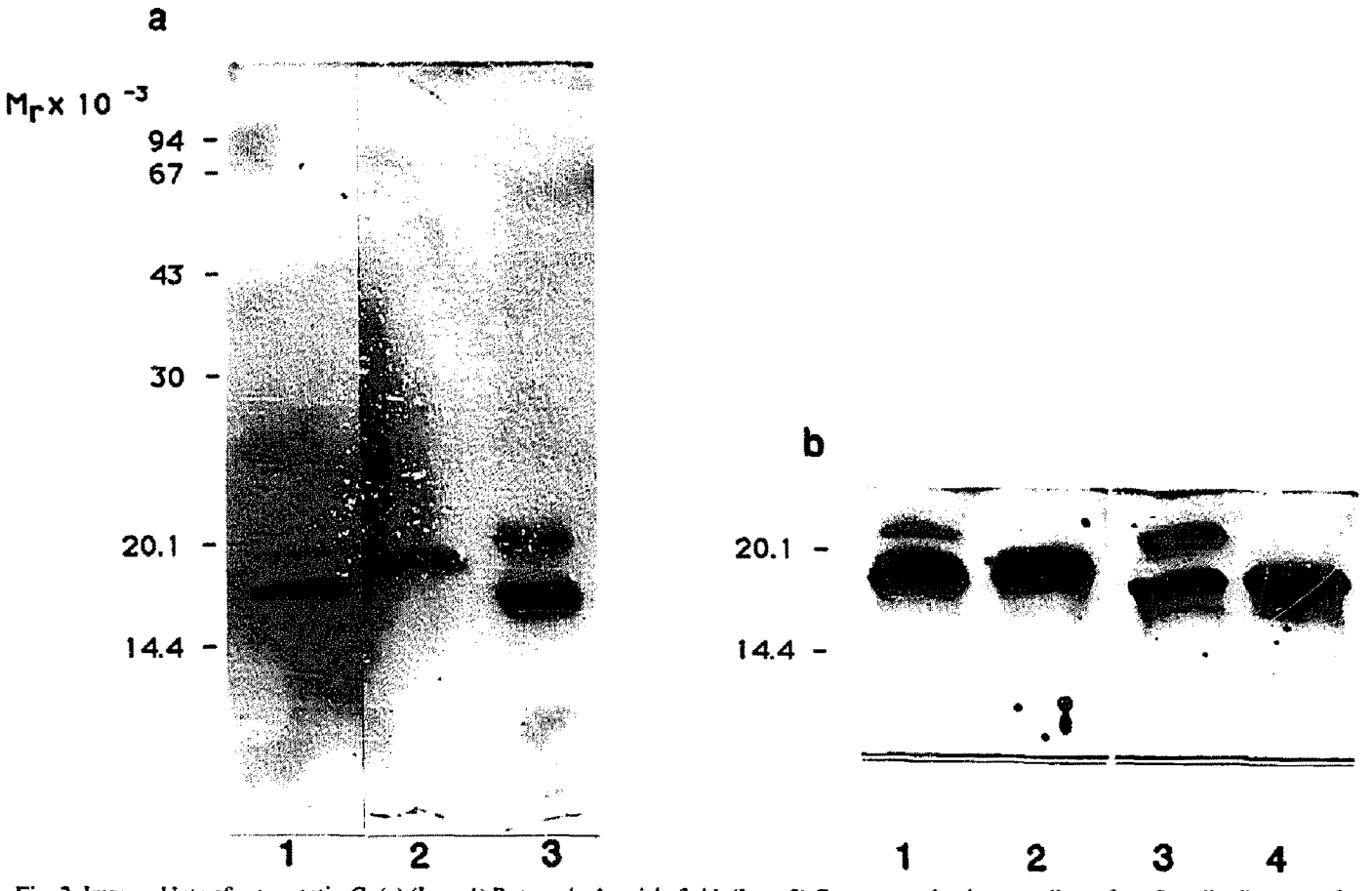

Fig. 2. Immunoblots of rat cystatin C. (a) (Lane 1) Rat seminal vesicle fluid. (Lane 2) Concentrated culture medium of rat Sertoli cells. (Lane 3) Urinary rat cystatin C. Cystatin C was detected using 4-chloro-naphthol as a substrate. (b) Concentrated culture medium of rat Sertoli cells: (Lane 1) untreated medium; (Lane 2) medium with endo-β-*N*-glucosaminidase F. Mouse seminal vesicle fluid: (Lane 3) untreated fluid; (Lane 4) fluid treated with endo-β-*N*-glucosaminidase F. Rat and mouse cystatins C were detected using the chemiluminescent detection system RPN 2106.

latter peak strongly inhibited papain and was retained for further fractionation by reverse phase chromatography on a C4 column (Fig. 4a). Eluted fractions were analysed by SDS gel electrophoresis and electroblotting which demonstrated the presence of two closely migrating immunoreactive forms in the quantitatively major peak (1') of elution. Only one amino acid sequence was obtained from N-terminal sequencing of this heterogeneous peak. Wether this is due to the complete identity between the two forms at their N-terminal end or to the presence of a N-terminally blocked sequence is not known so far. This sequence was identical to that of native cystatin C which indicates that cystatin C was

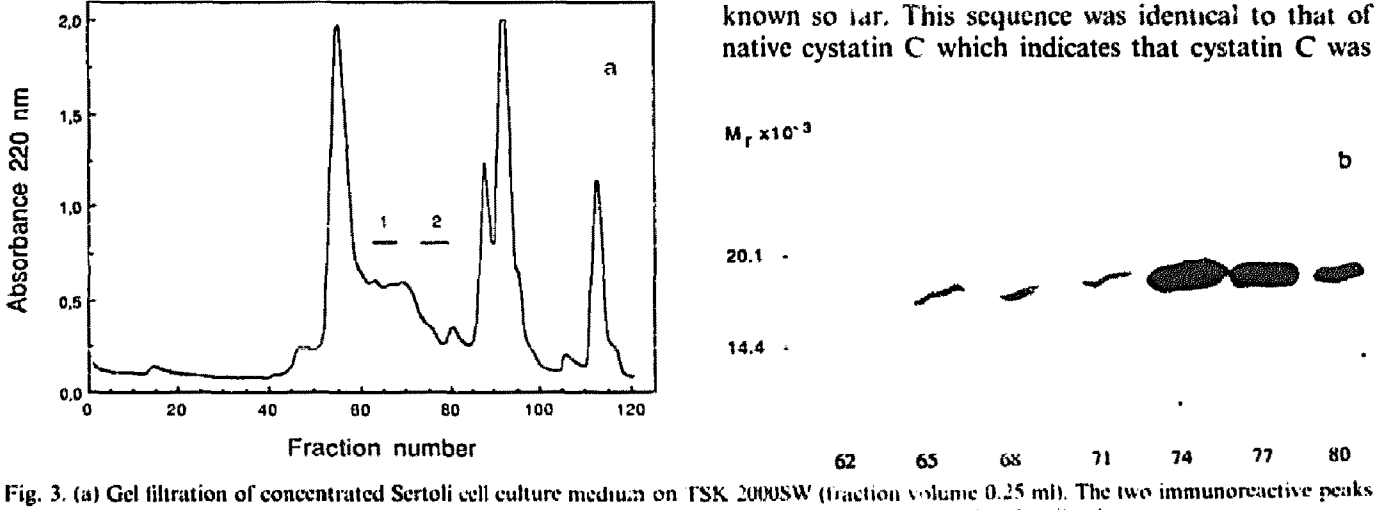

Fig. 3. (a) Gel filtration of concentrated Sertoli cell culture medium on TSK 2000SW (fraction volume 0.25 ml). The two immunoreactive peaks are indicated by 1 and 2 respectively. (b) Immunoblot of eluted fractions released by chemiluminescence.

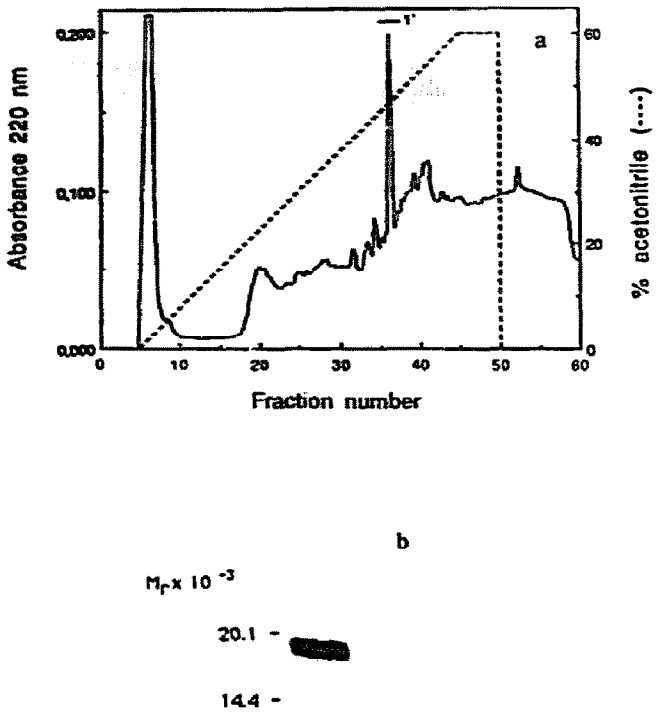

Fig. 4. (a) Reverse phase chromatography on Aquapore BU300 of the immunoreactive peak 2 from TSK 2000SW (fraction volume 0.20 ml). Immunoreactivity is indicated by 1'. (b) Immunoblot of peak 1' revealed by chemiluminescence.

secreted into the culture medium as a non-proteolysed form (Table I), and confirmed the functionally active state of the secreted molecule [23].

The rat cystatin C concentrations in the rete testis and testis interstitial fluid were determined using a double sandwich ELISA assay. They were 0.22 ± 0.02 mg/l ($1.65 \cdot 10^{-8}$ M) in the rete testis fluid based on the M_r of the full length 120 amino acid molecule [13] and 0.24 ± 0.05 mg/l ($1.80 \cdot 10^{-8}$ M) in the interstitial fluid. These concentrations are in the same range as that of blood plasma cystatin C (0.14 ± 0.02 mg/l ($1.05 \cdot 10^{-8}$ M)). However, compared to total proteins, the relative concentration of cystatin C in the rete testis fluid is 32 times greater than in blood plasma and 48 times greater than that of interstitial fluid [24].

Cystatin C's are potent inhibitors of lysosomal cysteine proteinases cathepsins B, H and L [11,25,26]. Cathepsin L, which is the most efficient lysosomal cysteine proteinase [27,28] is also preferentially inhibited by all the cystatin C's studied so far [11,25,26]. The equilibrium dissociation constant (K_i) of rat cystatin C for homologous cathepsin L is below 0.01 nM and its association rate constant (K_{on}) is about 10^7 $M^{-1}\cdot s^{-1}$ [11]. This means that cystatin C, in spite of its low concentration on the rete testis fluid (0.01 μM), remains a putative biological inhibitor of lysosomal cathepsin L in the testis [29].

Table I

Amino terminal sequences of rat cystatin C

a)	G-X-X-R-P-P-P-R-L-L-G-A-P-
b)	G-T-S-R-P-P-P-R-L-L-G-A-P-
c)	G-T-S-R-P-P-P-R-L-L-G-A-P-L-L-G-A-P-

a) cystatin C from Sertoli cell culture medium (peak 1' from Fig. 4a); b) cystatin C from seminal vesicle fluid [12]; c) cystatin C from urine of chromate-treated rats [12]

Intracellular proteinases, including the lysosomal cysteine proteinases, can probably be released into the seminiferous tubules in the course of the dramatic tissue changes that occur during germ cell transformation and degeneration. This is probably the case for lysosomal cathepsin L, which is present in a latent form in spermatozoa [9]. This study shows that cystatin C, a specific inhibitor of cysteine proteinases, is synthesized by rat Sertoli cells in addition to the non-specific proteinase inhibitor alpha2-macroglobulin [2]. Rat cystatin C concentration and equilibrium dissociation constants of the inhibitor towards its homologous cathepsins indicate that rat cystatin C could play a significant role in the control of the proteolytic activity of cathepsin L. The in vivo conditions in which inhibition occurs remains to be demonstrated. Rat cystatin C messenger RNAs are found in the testis and also in epididymis, prostate and seminal vesicles [22]. The seminal vesicle fluid contains the highest concentration of this molecule (40 mg/l, i.e. $3.0 \cdot 10^{-6}$ M) detected to date. This suggests that this molecule could be involved not only in spermatogenesis and spermiogenesis in the testis, but also in the entire transit of the spermatozoa along the male genital tract.

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