Presence, localization, and origin of clusterin in normal human spermatozoa

Zhijian Han · Zengjun Wang · Gong Cheng ·
Bianjiang Liu · Pengchao Li · Jie Li · Wei Wang ·
Changjun Yin · Wei Zhang

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Abstract

Purpose Clusterin in mammalian semen is a secretory form of clusterin (sCLU) with the heterodimeric structure. It is secreted by the epididymis and seminal vesicle. It is generally agreed that clusterin mainly exists on the surface of abnormal spermatozoa and is implicated in decreased sperm motility, sperm aggregation and infertility. However, few studies observe clusterin in normal spermatozoa, which is presumed to be a novel form. Up to now, the systematical information about the presence, localization, origin and function of clusterin in normal human spermatozoa has yet not been established. The aim of our current study is to systematically research clusterin in normal human spermatozoa.

Capsule A novel form of native clusterin is localized in the inner plasma membrane of human normal spermatozoa. It should be self-synthesized during the later stage of spermatogenesis.

Zhijian Han, Zengjun Wang and Gong Cheng contributed equally to this work.

Z. Han · B. Liu (✉) · P. Li · J. Li · C. Yin
Department of Urology, The First Affiliated Hospital of Nanjing Medical University,
Nanjing 210029, China
e-mail: bjliu@njmu.edu.cn

Z. Wang
Andrology Laboratory, The First Affiliated Hospital of Nanjing Medical University,
Nanjing 210029, China

G. Cheng · W. Wang
Human Sperm Bank, The First Affiliated Hospital of Nanjing Medical University,
Nanjing 210029, China

W. Zhang (✉)
Department of Surgery, The First Affiliated Hospital of Nanjing Medical University,
Nanjing 210029, China
e-mail: zhangwei@njmu.edu.cn

Methods We detected the presence of clusterin via western blot, explored the localization of clusterin using immunofluorescence, and investigated the origin and distribution of clusterin in human testis by western blot and immunohistochemistry.

Results We found native clusterin in the inner plasma membrane of normal human spermatozoa. It was derived from the testis and showed similar molecular weight and heterodimeric structure compared with sCLU in semen and on the surface of abnormal spermatozoa.

Conclusion Clusterin in normal spermatozoa should be self-synthesized during the later stage of spermatogenesis. The different localization and origin suggested that the clusterin observed by us may be a novel form compared with conventional sCLU on the surface of abnormal spermatozoa.

Keywords Clusterin · Human · Testis · Spermatozoa

Introduction

Clusterin was first identified in ram rete testis fluid in 1983. It was named for its ability to elicit clustering among Sertoli cells [5]. Clusterin is synthesized and secreted by a wide variety of tissues and cells in different species. There are a number of synonyms for clusterin, including T64, SGP-2, TRPM-2, gp80, and Gp-III. Clusterin has been found in all body fluids [36]. The human homolog of clusterin has also been given other names, including complement-lysis inhibitor (CLI), secreted protein 40 (SP-40), and apolipoprotein J (ApoJ). Clusterin participates in many biological processes such as cell adhesion, lipid transportation, complement inhibition, membrane recycling and apoptosis [15, 30, 31, 34, 36, 41]. High levels of clusterin expression have been found to be positively correlated with severe pathophysiological states

and conditions such as renal disease, neurodegenerative disease, atherosclerosis, myocardial infarction and cancer [6, 10, 27, 37, 40].

Human clusterin expression is complex, showing different patterns in different tissues and cells. Two forms of clusterin have been identified. The secretory form of clusterin (sCLU) is synthesized as a 50–60 kDa protein precursor and targeted to the endoplasmic reticulum (ER) by a leader peptide that is 22 amino acids long [18]. Then the precursor is glycosylated and proteolytically cleaved into α and β subunits, held together by disulfide bonds [41, 43]. Mature sCLU is secreted into body fluids as an ~80 kDa heterodimeric glycoprotein containing a ~40 kDa α and β protein subunit [43]. sCLU has been studied extensively. Current knowledge about clusterin mainly comes from research on sCLU. Relatively few studies have been performed on the nuclear form of clusterin (nCLU). nCLU is expressed as an ~49 kDa inactive protein precursor in the cytoplasm, which lacks the ER-targeting leader peptide and does not undergo α/β cleavage [28]. The nCLU precursor is transformed into an ~55 kDa protein and moves into the nucleus after certain cytotoxic events [42]. Functionally, sCLU protects cells against cytotoxic agents that induce apoptosis, and nCLU acts as a pro-death signal, inhibiting cell growth and survival [4, 18, 28, 42, 43].

In the male mammalian reproductive tract, clusterin is synthesized and secreted by the testis, epididymis and seminal vesicle [1, 25, 32]. Clusterin is a major glycoprotein in mammalian semen. Abnormal increases and decreases in clusterin are often indicative of poor-quality semen [12, 14, 20, 23, 24]. Regardless of whether or not the semen is normal, it is generally agreed that clusterin mainly exists on the surfaces of immature, low motile, or morphologically abnormal spermatozoa [7, 13, 26]. Clusterin in the semen and on abnormal spermatozoa belongs to sCLU. Animal experiments have indicated that clusterin in the rete testis fluid is synthesized by Sertoli cells and adheres to the surface of testicular spermatozoa. When spermatozoa move through the rete testis and efferent duct, testis-derived clusterin is replaced by clusterin from the epididymis or seminal vesicle [3, 8, 9, 32]. Clusterin on abnormal spermatozoa is implicated in decreased sperm motility, sperm aggregation and infertility [7, 12, 13, 26]. It is thus regarded as a marker of pathological spermatozoa. However, this is in direct contrast with the results of other reports, in which clusterin was observed in all rat and ram spermatozoa [33, 38]. In human, clusterin has also been reported in normal spermatozoa. This is presumed to be a novel form of clusterin distinct from conventional heterodimeric sCLU [2, 26, 35]. Until now, there has been little systematic information regarding the presence, localization, origin and function of clusterin in normal mammalian spermatozoa. The aim of this study is to extend the knowledge of clusterin in human motile and morphologically normal spermatozoa.

Materials and methods

Approval for this study was granted by the ethics committee of Nanjing Medical University (China) prior to sample collection and informed written consent was received from all participants. All chemicals and reagents used in this study were molecular biology grade and purchased from Sigma-Aldrich (St. Louis, MO, U.S.) unless otherwise stated.

Semen preparation

Freshly ejaculated human semen samples were obtained at Human Sperm Bank of The First Affiliated Hospital of Nanjing Medical University from donors who had practiced 3–7 days of sexual abstinence. The mean $\pm$ SD age of the 8 donors was 23.9 ± 3.9 years. Routine semen analysis monitored by a computer-assisted semen analyzer (IVOS; Hamilton-Thorne, Beverly, MA, U.S.) revealed that spermatozoa were within the normal range stipulated by WHO1999 guidelines. To separate high motile and morphologically normal spermatozoa from semen, the liquefied ejaculate was washed on a two-layer (90 % and 45 %) discontinuous Percoll (GE Healthcare; Piscataway, NJ, U.S.) gradient, a modified protocol described previously [16, 17, 19]. After centrifugation at 400 g for 18 min, the cell pellets from the 90 % layer were washed twice in Earle's balanced salts (Sigma; St. Louis, MO, U.S.). As the controls, ejaculated semen samples were directly centrifuged and washed to collect unseparated spermatozoa.

Sperm protein extraction

The sperm pellets prepared as above were resuspended in lysis buffer (50 mM Tris, 150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, 2 mM Na_3VO_4 , 50 mM NaF, 10 $\mu\text{g}/\text{ml}$ leupeptin) containing a protease inhibitor cocktail (Thermo Scientific; Rockford, IL, U.S.) for 2 h at 4 °C, sonicated for 1 min on ice, and centrifuged at 12,000 g for 15 min at 4 °C. Hydrophobic and hydrophilic proteins were acquired from the supernatant as reported previously [39]. Briefly, the supernatant was extracted with chloroform and methanol ($v/v=3:1$) and centrifuged at 12,000 g for 10 min at 4 °C. The organic-aqueous interface and aqueous phase were collected as hydrophobic and hydrophilic protein extractions, respectively, and stored at -20 °C for later use.

Western blot analysis

The protein concentration was determined by bicinchoninic acid assay. Each protein sample was divided into two aliquots. Under reduced conditions, one aliquot was boiled in sodium dodecyl sulphate (SDS) sample buffer containing

2 % (v/v) β -mercaptoethanol and resolved by SDS-PAGE on 5–12 % polyacrylamide gels followed by transfer onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad; Hercules, CA, U.S.). Under non-reduced conditions, the other aliquot was mixed with sample buffer without β -mercaptoethanol prior to electrophoresis. The membrane was blocked with 5 % non-fat milk in Tris-buffered saline (TBS) for 2 h before being incubated with rabbit anti-human clusterin antibody (1:1000; Abcam; Cambridge, U.K.) and diluted in blocking solution at 4 °C overnight. After washing, the membrane was probed with horseradish peroxidase (HRP) conjugated second antibody (1:5000; Zhongshan Goldenbridge Biotechnology, Beijing, China) at 37 °C for 1 h. An ECL reaction kit (Cell Signaling Technology, Danvers, MA, USA) was used to detect the peroxidase activity and images were captured by FluorChem 5500 (Cell Biosciences, Santa Clara, CA, U.S.).

Immunofluorescence

Sperm pellets prepared as above were resuspended in phosphate buffered saline (PBS), air-dried onto slides, and fixed with 4 % paraformaldehyde in PBS for 1 h at 4 °C. Sperm samples that were not separated on Percoll and had been washed only in PBS were also fixed onto slides. These served as controls. After three 5-min washes in PBS, slides were blocked in goat serum (Zhongshan Goldenbridge Biotechnology, Beijing, China) for 2 h. For observing the intracellular localization of the protein, several slides were permeabilized with 0.2 % Triton X-100 before blocking. Then slides were incubated with a 1:50 dilution of anti-clusterin antibody or, as a control, with normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.) overnight at 4 °C. After incubation with goat anti-rabbit IgG labeled with FITC (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.) in dark room for 45 min at room temperature, slides were washed in PBS and coverslipped. Slides were viewed with an Axioskop 2 plus fluorescent microscope (Carl Zeiss, Thornwood, NY, U.S.) and images were captured with a digital camera.

Testis protein extraction and western blot analysis

Human testis tissues were obtained from four patients on The First Affiliated Hospital of Nanjing Medical University. These patients received orchiectomy for the treatment of prostate cancer. Testicular tissues samples were homogenized in lysis buffer, sonicated on ice, and centrifuged at 12,000 g for 30 min at 4 °C. The supernatant was collected and analyzed via western blot as described above.

Immunohistochemistry

Formalin-fixed human testis tissues were embedded in paraffin, sectioned at 5 μ m and mounted on silane-coated slides. Sections were de-waxed and re-hydrated through descending grades of alcohol to distilled water. Slides were subjected to microwave antigen retrieval in 0.02 M EDTA and incubated in 3 % H_2O_2 to block endogenous peroxidase. Thereafter, they were stained using a ZYMED® Histostain-Plus Kit (Invitrogen; Carlsbad, CA, U.S.). Briefly, sections were washed in PBS, blocked with goat serum for 15 min at room temperature, and incubated overnight with anti-clusterin antibody (1:200 dilution) or, as a control, with normal rabbit IgG in a humidified chamber at 4 °C. After washing in PBS, slides were incubated with biotin-conjugated goat anti-rabbit IgG for 15 min at 37 °C, followed by incubation with a streptavidin/peroxidase complex for 15 min at 37 °C. Immunoreactivity was revealed with 3,3'-diaminobenzidine (DAB). Sections were counterstained with hematoxylin and mounted onto coverslips.

Results

In order to study the presence of clusterin, normal human spermatozoa were separated from semen samples. Then hydrophobic and hydrophilic protein were extracted and analyzed via western blot. As shown in Fig. 1, lane 1, an ~40 kDa protein band was detected from hydrophobic extractions using anti-clusterin antibody under reduced conditions. Under non-reduced conditions, the antibody recognized an ~80 kDa protein (Fig. 1, lane 2). In contrast, no bands were observed when anti-clusterin antibody was omitted (Fig. 1, lane 3–4). In addition, no protein bands were generated from hydrophilic extractions using this specific anti-clusterin antibody (Fig. 1, lane 5–6). As the controls, similar protein bands (~40 kDa and ~80 kDa) were observed in unseparated spermatozoa (Fig. 1, lane 7–8).

The localization of clusterin in normal human spermatozoa was further investigated using immunofluorescence. As shown in Fig. 2a–b, there were no intense fluorescent staining on the sperm surfaces. After permeabilization treatment, fluorescent staining appeared in the heads of spermatozoa, especially in the post-acrosomal regions (Fig. 2c–d). In the unseparated sperm sample, staining signals were detected in the heads, necks or tails of some abnormal spermatozoa (Fig. 2g–h). Control experiments with normal rabbit IgG did not show staining in any part of the spermatozoa (Fig. 2e–f and i–j). Similar images were observed when the primary antibody was omitted (not shown).

The presence and distribution of clusterin in human testis were investigated by western blot and immunohistochemistry. Western blot analysis of reduced samples showed that

Fig. 1 Protein extractions from normal human spermatozoa analyzed via western blot. Lane 1–2: hydrophobic membrane protein extractions were probed with anti-clusterin antibody respectively under reduced and non-reduced conditions; lane 3–4: hydrophobic membrane protein extractions were probed without anti-clusterin antibody under reduced and non-reduced conditions; lane 5–6: hydrophilic protein extractions were probed with anti-clusterin antibody respectively under reduced and non-reduced conditions; lane 7–8: protein extractions from unseparated spermatozoa were probed with anti-clusterin antibody respectively under reduced and non-reduced conditions. Each experiment was repeated three times. A representative experiment was shown ($n=8$)

anti-clusterin antibody recognized three protein bands of ~40, ~50 and ~60 kDa (Fig. 3, lane 1). Under non-reduced conditions, only two bands (~80 kDa and >130 kDa) appeared (Fig. 3, lane 2). Immunohistochemical analysis exhibited the distribution of clusterin at the testis level. As shown in Fig. 4a–c, intense signals were observed

in the myoid cells of seminiferous tubules, Sertoli cells, the immature spermatozoa in the lumen of seminiferous tubules, and Leydig cells. In contrast, almost no staining appeared in control experiments (Fig. 4d).

Discussion

Most previous studies have shown that clusterin is secreted into semen by the epididymis and seminal vesicle, and mainly adheres to the surfaces of abnormal spermatozoa [3, 8, 9, 25, 32]. Only a few researchers observed clusterin in normal human spermatozoa [2, 26, 35]. However, the presence, localization and origin of clusterin in normal spermatozoa have caused great deal of controversy. In our current study, we systematically studied clusterin in normal human spermatozoa. Discontinuous Percoll gradient centrifugation was performed to separate normal spermatozoa from the semen. This sperm separation technique could effectively acquire sperm populations with high motility and normal morphology free of seminal plasma, debris, microbial contamination, and residual cells (immature germ cells or polynuclear cells). This was confirmed by our microscopy inspection and has been shown in previous reports [16, 17, 19, 21, 22]. Sperm motility was monitored

Fig. 2 Immunofluorescent staining of clusterin in human spermatozoa. **a–b** Separated spermatozoa were probed with anti-clusterin antibody. **c–d** Separated spermatozoa were permeabilized and probed with anti-clusterin antibody. **e–f** Separated spermatozoa were probed with normal rabbit IgG. **g–h** Unseparated spermatozoa were probed with

anti-clusterin antibody. **i–j** Unseparated spermatozoa were probed with normal rabbit IgG. **a, c, e, g** and **i** Phase-contrast images; **b, d, f, h** and **j** Immunofluorescent images. Magnification was $\times 1000$. Each experiment was repeated three times. A representative experiment was shown ($n=8$)

Fig. 3 Protein extractions from human testis tissues analyzed via western blot. Lane 1: protein extractions were probed with anti-clusterin antibody under reduced conditions. Lane 2: protein extractions were probed with anti-clusterin antibody under non-reduced conditions. Each experiment was repeated three times. A representative experiment was shown ($n=4$)

using the computer-assisted semen analyzer, and a significant increase in motility was observed (from 74.85 ± 5.35 to 90.09 ± 3.27 ; $P < 0.05$).

Using anti-clusterin antibody, we identified the ~80 kDa native clusterin from hydrophobic membrane protein extractions of normal spermatozoa under non-reducing conditions. The ~80 kDa protein could be reduced to an ~40 kDa band. Clusterin was not found in hydrophilic extractions. It has been known that heterodimeric sCLU possesses binding activity to hydrophobic domains of various proteins [11]. Our results demonstrated the presence of clusterin in normal spermatozoa, within the membrane structures. A previous study also found clusterin in normal human spermatozoa

and suggested that this form of clusterin was not a conventional heterodimeric sCLU, but rather represented a α subunit of clusterin or a combination of α - and β -subunits. This was not the conventional heterodimeric structure [26]. However, our study showed native clusterin in normal human spermatozoa to have similar molecular weight and heterodimeric structure similar to those of conventional sCLU in semen and on the surfaces of abnormal spermatozoa. Clusterin observed by immunofluorescence was mainly localized on the surfaces of abnormal spermatozoa [12, 13]. Similar results were observed in our study. We found clusterin to be localized on abnormal human spermatozoa. However, our immunofluorescence test did not find clusterin on the membrane surfaces of normal spermatozoa, although native clusterin was identified during sperm membrane extraction. After permeabilization treatment, fluorescent staining was observed in the head of the sperm, especially in the post-acrosomal region. This demonstrated that this form of clusterin existed in the inner plasma membrane. It suggested that the clusterin found in normal human spermatozoa is not secreted by the epididymis or seminal vesicle and adheres to the surfaces of spermatozoa. This form of clusterin may be a kind of sperm own protein synthesized during the spermatogenic process. Our results were inconsistent with those of another research team, who found clusterin in the acrosomal region of human normal spermatozoa [2, 26]. Different antibodies and methods of observation might have caused these contrasts. Another possibility was that clusterin observed by us was neither the clusterin from the acrosomal region nor the conventional sCLU on the surfaces of abnormal spermatozoa. Our results indicated differences in clusterin localization between human abnormal spermatozoa and abnormal spermatozoa. It is noteworthy that the spermatozoa

Fig. 4 Immunohistochemical localization of clusterin in human testis. **a–c** Testis tissues were probed with anti-clusterin antibody. The stainings in typical myoid cells, Sertoli cells, immature spermatozoa, and Leydig cells were respectively indicated using *red arrows*. No staining was observed in spermatogonia (*green arrow*). **d** Testis tissues were probed with normal rabbit IgG as the control. Magnification was $\times 100$ in **a**, $\times 400$ in **b** and **d**, and $\times 1000$ in **c**. Each experiment was repeated three times. A representative experiment was shown ($n=4$)

came from normal semen samples. The result might be different if the spermatozoa came from abnormal semen samples. In conclusion, the accurate form of clusterin in human normal spermatozoa and its localization at the ultrastructural level still remain to be determined through further research.

In human testis protein extractions, protein bands (~40 and ~80 kDa) identical to those that appeared in the sperm extractions were detected. However, several extra bands showed the complexity of clusterin expression at the testis level. The >130 kDa band might indicate the presence of multimers or protein complexes containing clusterin. Two faint bands (~50 and ~60 kDa) might represent nCLU or other novel forms of clusterin. Previous investigations have shown that nCLU was present in undamaged cells at low levels and would increase after some cytotoxic events [18, 43]. It might be for this reason that nCLU was not detected in normal sperm protein extractions. Immunohistochemical analysis showed that clusterin was present in spermatogenic cells besides Sertoli cells in the lumen of seminiferous tubules. As mentioned in the introduction, clusterin derived from Sertoli cells bound to the surface of testicular spermatozoa in the rete testis fluid and would be replaced by clusterin from the epididymis or seminal vesicle during sperm migration [3, 8, 9, 32]. Therefore the clusterin observed by us in the inner membrane of normal spermatozoa may have been synthesized by the spermatogenic cells. It was interesting that clusterin was found in immature spermatozoa, but not in spermatogonia. This suggests that this form of clusterin might be synthesized during the later stage of spermatogenesis.

sCLU in mammalian semen usually adheres to the surfaces of abnormal spermatozoa. No detailed data about nCLU in spermatozoa were reported. In our present study, we identified a form of clusterin in normal human spermatozoa, which showed molecular weight, heterodimeric structure, and hydrophobic binding activity similar to that of sCLU in semen and on the surfaces of abnormal spermatozoa, but it was not found on the sperm surfaces. The different localizations and origins suggest that it might be a novel form. Clusterin in the mammalian reproductive tract is not always involved in decreased sperm motility, sperm aggregation, or infertility. It has been reported that clusterin can protect bull spermatozoa against oxidative damage and enhance sperm motility and survival [29]. In a recent study, clusterin has also been found to bind to and react with some of the sperm surface proteins responsible for semen liquefaction and antibacterial activity [39]. Further research on clusterin in normal spermatozoa may help researchers uncover new functions of clusterin during spermatogenesis and the maturation of spermatozoa.

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