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Differential Age-Associated Regulation of Clusterin Expression in Prostate Lobes of Brown Norway Rats

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Abstract

Background—Serum androgen concentrations decline with age in male Brown Norway rats and castration induces apoptosis of luminal secretory epithelial cells in the ventral but not in the dorsal and lateral prostate lobes. Clusterin has been described as an androgen-repressed gene and a protein with either anti- or pro-apoptotic actions.

Methods—We measured clusterin mRNA and protein levels, the effects of aging and castration on clusterin protein levels and clusterin immunolocalization within the prostatic ductal network in the prostate lobes of young and aged rats.

Results—Whereas levels of clusterin mRNA and protein expression measured by RT-PCR and Western blot, respectively, were higher in the ventral and lateral lobes of aged (24 months) compared to young (4 months) rats, no age-dependent differences were observed in the dorsal lobe. Clusterin expression was localized by immunohistochemistry exclusively to the proximal duct segment of young rats, but extended to the distal segment of the ventral and lateral lobes of aged rats. Despite an age-related decrease in serum testosterone concentration, clusterin gene expression was not altered in the dorsal lobe. After castration, levels of clusterin expression increased significantly in the ventral and lateral lobes despite the absence of epithelial cell apoptosis in the latter. In castrated rats, clusterin expression extended throughout the proximal-distal duct regions of the prostate lobes of young and aged rats.

Conclusion—Regulation of clusterin expression in the prostate lobes of aging rats appears complex and is neither directly repressed by androgen nor dependent on apoptotic-induced stress.

Keywords

prostate; androgen; hyperplasia; apoptosis; age; clusterin

Introduction

The prostate gland is universally recognized as the prototypic androgen-responsive tissue that depends upon androgen for its normal development and growth as well as for maintenance of its function. In rats, the prostate is anatomically divided into distinct lobes, referred to as the ventral, lateral, and dorsal. Each lobe consists of a tree-like structure of

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glandular ducts lined by secretory epithelial cells that produce fluid and proteins that upon entry into the urethra contribute to the seminal fluid [1]. The ventral lobe is the largest of the lobes in the rat and it has been the primary focus for studies of androgen-responsiveness. Upon androgen withdrawal by castration, the ventral lobe of the rat prostate undergoes rapid regression exemplified by reduced protein synthesis and most notably by a wave of apoptosis that consumes 70-80% of the glandular luminal secretory epithelial cells [2,3]. This process is reversed upon androgen replacement where upon the regressed ventral lobe regenerates its full complement of luminal epithelial cells and normal adult size and function are restored. By contrast, the effects of androgen withdrawal on the lateral and dorsal lobes differ from the ventral lobe [3]. Specifically, few luminal epithelial cells undergo apoptosis in the lateral and dorsal lobes following androgen withdrawal despite functional regression as evidenced by loss of secretory protein synthesis, diminished stature of the luminal epithelial cells and a reduction in tissue mass. As in the ventral lobe, androgen replacement restores secretory function and size of the lateral and dorsal lobes. We previously reported that differences in apoptosis between the prostatic lobes were reflected in the differential expression of the pro- and antiapoptotic proteins, bax and bcl-2, respectively, before and following androgen withdrawal [4]. Moreover, differences in the patterns of androgen receptor expression following androgen withdrawal further suggested that lobe-specific differences in androgen-sensitivity might contribute to the incidence of epithelial cell apoptosis [5].

Our laboratory, as well as others, has used the Brown Norway rat as an animal model to study the effects of aging [6-8]. As occurs in aging men [9-11], serum testosterone levels decline with advancing age in Brown Norway rats [6,7]. Moreover, the effects of androgens on the prostate in this rat strain are both lobe-specific and age-dependent [5,6,12,13]. By two years of age, there is a significant age-dependent decline in serum testosterone concentration but this has no effect on the weight of the ventral lobe. Surprisingly, the weights of both the lateral and dorsal lobes in aged rats increase relative to young adult rats. The age-dependent increase in tissue weights of these lobes parallels increases in the numbers of epithelial cells based upon histological analyses and biochemical measures of total DNA content per lobe [6]. Morphologically, these changes in the lateral and dorsal lobes of the prostate in aging Brown Norway rats present the appearance of spontaneous, age-dependent epithelial cell hyperplasia, resembling the overgrowth associated with benign prostatic hyperplasia in aging men [14]. Further evidence for both lobe-specific and age-dependent differences in androgen sensitivity of the Brown Norway rat prostate comes from observations that androgen receptor levels decrease in the ventral lobe, but increase in the dorsal and lateral lobes, of aging rats [5]. Although apoptosis of epithelial cells occurs in the ventral lobe of young and old rats, the magnitude of the apoptotic response in older rats is muted by comparison to young rats [13]. Furthermore, we observed that overgrowth of the lateral and dorsal lobes but not the ventral lobe occurred in response to exogenous administration of testosterone but only in older rats, where the response was also dose-dependent [12].

Age-dependent changes in the levels of the androgen receptor and its ligands, testosterone and dihydrotestosterone, are likely to directly or indirectly affect the androgen-regulated gene networks within the different prostate lobes. Clusterin is a gene that was originally identified in the rat prostate as a testosterone repressed prostate message (TRPM-2), the expression of which increased in the ventral lobe during its regression following castration [15,16]. In general, clusterin was observed to be down-regulated during cell proliferation but up-regulated during conditions of cell stress, cell atrophy and organ involution. Thus, it gained recognition as a biomarker for cell death and mediator of apoptosis [17]. Subsequently, clusterin was implicated in a variety of physiological and pathological conditions that included tissue remodeling, lipid transport, phagocyte recruitment, cell adhesion, stress-induced chaperone-like cytoprotection, reproduction, complement cytotoxicity,

DNA repair, and cell cycle regulation [18-25]. In several human cancers including androgen-ablation resistant prostate cancer, clusterin expression was increased and postulated to provide protection against adjuvant therapies intended to induce apoptosis of tumor cells [26-30]. While clusterin is strongly associated with androgen withdrawal-induced apoptosis in the prostate [17,31,32], a specific functional role for clusterin in the death or survival of prostatic epithelial cells has yet to be clearly established.

Considering the anti-apoptotic properties of clusterin in the prostate and its responsiveness in the adult prostate gland to androgen withdrawal, we hypothesized that changes in clusterin expression might occur in response to the age-dependent decline in serum testosterone level as well as play a role in the age-dependent and lobe-specific involution and incidence of cell death that occurs following acute androgen withdrawal by castration. To evaluate the role of clusterin in the differential age-dependent and lobe-specific responses of the rat prostate to changes in serum androgen concentrations, we characterized the levels of clusterin mRNA and protein and its immunohistochemical localization and expression in the prostate lobes of young adult (4 months) and old (24 months) Brown Norway rats. Interestingly, we observed that levels of clusterin mRNA and protein increased in the ventral and lateral, but not the dorsal, lobes of aged rats. The predominant regional localization of clusterin expression observed in epithelial cells of the proximal duct segments in young rats extended to luminal epithelial cells lining the entire proximal-distal duct axis in aged rats. Despite the presence and absence of epithelial cell apoptosis in the ventral and lateral lobes, respectively, following castration, clusterin expression increased dramatically in both lobes of castrated young and aged rats.

Materials and Methods

Animals

Male Brown Norway rats of 4 (young adult) and 24 (old adult) months of age were purchased from Harlan (Indianapolis, IN) by special arrangement with the National Institute on Aging (Bethesda, MD). The animals were housed under standard conditions with access to food and water *ad libitum*. Animal protocols were approved by the Johns Hopkins University Animal Care and Use Committee.

Castration was performed under ether anesthesia via a midline incision in the scrotum. Epididymides were removed along with the testes. Rats were sacrificed at 1, 2, 3 and 7 days after castration. For harvest of prostate tissue, the entire urogenital complex was dissected from the abdominal cavity and placed in ice-cold phosphate buffered saline (PBS), pH 7.4. The ventral, dorsal and lateral prostate lobes were separated under a dissecting microscope, blotted on filter paper and weighed. The right and left portions of each lobe were subsequently fixed by immersion in 4% neutral-buffered paraformaldehyde for paraffin embedding or snap frozen in liquid nitrogen and stored at -80°C .

Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from prostate tissues using RNA STAT-60 reagent (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. The concentration of RNA was measured by reading the absorbance at 260 nm and the purity and integrity were verified by the 260/280 nm ratio ≥ 1.8 and by denaturing agarose gel electrophoresis. To generate first-strand cDNA, RNA (2 μg) from the ventral, dorsal or lateral lobe was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random primers according to the manufacturer's protocol. For PCR reactions, the QuantiTech SYBR green PCR kit (Qiagen, Valencia, CA) was used. Each PCR reaction volume of 50 μL contained 1 μL cDNA (1:5 dilution) and 10 pmoles each of sense and

antisense gene specific primers for clusterin: 5'-GAAATGAAGCTGAAGGCTTTCCCG-3' (sense) and 5'-GGAAGTGTAAAGCTGGGCTA TGGA-3' (antisense), GenBank accession number M16975 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-GAGAATGGGAAGCTGGTCAT-3' (sense) and 5'-GCATTGCTGACAATCTTGAGGGAG -3' (antisense), GenBank accession number M17701. PCR reactions were conducted under the following conditions: initial denaturation at 95°C for 15 min; followed by repetitive cycles of 94°C for 45 sec, 57°C for 45 sec, and 72°C for 1 min; and a final extension at 72°C for 10 min. All reactions used 24 cycles as determined to be within the linear range of the amplification reactions. Clusterin mRNA levels were normalized to GAPDH mRNA levels, the latter being used as a control. PCR products were separated using agarose gel electrophoresis and the intensities of SYBR green fluorescence were quantified on a Typhoon 9200 Imaging System using ImageQuant software (Amersham Biosciences, Piscataway, NJ).

Western blot analyses

Frozen prostate tissues were homogenized in RIPA buffer (10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) using a hand-held motorized homogenizer (Tissue-Tearor, Biospec Products, Bartlesville, OK). Tissue lysates were sonicated to shear genomic DNA and centrifuged. The protein concentration of each sample was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Aliquots of tissue lysate equivalent to 30 µg of total protein from the ventral, dorsal or lateral lobes were mixed with an equal volume of 2X loading buffer (100 mM Tris-HCl, pH 7.4, 4% SDS, 0.2% bromophenol blue and 20% glycerol), boiled for 5 min and separated by electrophoresis on 10% SDS-polyacrylamide gels. Proteins were transferred onto PVDF membrane (Millipore, Billerica, MA) with a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer's directions. The membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in PBS and probed for clusterin and β-actin. Blots were incubated with goat anti-clusterin antibody (1:250 dilution; SC-6420; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C in 5% non-fat dry milk in PBS. Membranes were washed three times in TBS-T (20 mM Tris-HCl, pH 7.5, 137 mM NaCl and 0.1% Tween-20) and incubated for 1 h at room temperature with anti-goat horseradish peroxidase-conjugated secondary antibody (1:10000 dilution; Amersham Biosciences). The chemiluminescent signal was detected on Blue Ultra autoradiographic film (ISC BioExpress, Kaysville, UT) using the SuperSignal WestPico Chemiluminescent kit (Pierce) according to the manufacturer's protocol. As a protein loading control, membranes were stripped using Restore Western Blot stripping solution and incubated with β-actin antibody (1:5000 dilution; A5441; Sigma, St. Louis, MO) in 5% non-fat dry milk in PBS for 30 min at room temperature. Membranes were washed three times in TBS-T, incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:10000 dilution; Amersham Biosciences) and the chemiluminescent signal was recorded as described above. Relative signal intensities for clusterin were normalized to β-actin by quantification on a Typhoon 9200 Imaging System using ImageQuant software (Amersham Biosciences).

Immunohistochemical Localization of Clusterin

Prostate tissue sections (6 µm) were cut from paraffin-embedded prostate tissue and placed on glass microscope slides. Tissue sections were deparaffinized through xylene and a graded series of ethanol, rehydrated and washed in PBS. For antigen retrieval, slides were immersed in 10 mM citrate buffer, pH 6.0, brought to boiling in a microwave oven and left to cool to room temperature. Endogenous peroxidase activity was blocked by treatment with 2% H₂O₂ in PBS for 30 min at room temperature. Non-specific protein binding was blocked by

incubating slides in a humidified chamber for 1 h at room temperature with PBS containing 1% crystalline grade bovine serum albumin (BSA) and 5% rabbit serum. Sections were then incubated overnight at 4°C with anti-clusterin antiserum (1:100 dilution; SC-6420; Santa Cruz) in PBS containing 1% BSA. Clusterin localization was visualized using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Sections were counterstained with 1% methyl green. Negative controls had primary or secondary antibody omitted. Clusterin immunostaining was observed on a Nikon Eclipse E800 microscope (Nikon, Melville, NY) and images were captured using a CCD camera (Princeton Instruments, Trenton, NJ) and IPLab Spectrum Analysis software (Scanalytics, Fairfax, VA) and converted in Adobe Photoshop (Adobe Systems, San Jose, CA). For clusterin immunostaining, tissue sections from each prostate lobe from three different rats per experimental group were examined and representative images are shown for publication.

Statistical Analyses

Data are expressed as the mean $\pm$ SEM. Statistical differences between young and old groups of rats were determined by student's t- test ($P < 0.05$). Within age group statistical differences between intact control and castrated rats were determined by one-way ANOVA followed by Scheffe's F-test ($P < 0.05$).

Results

Clusterin mRNA and protein levels in the ventral, lateral and dorsal lobes of young and old rats

Clusterin was originally identified as a testosterone-repressed prostate message (TRPM-2) that was up-regulated in the rat ventral prostate following androgen withdrawal [15,16]. In aging Brown Norway rats, serum testosterone levels decline with age [6]. We measured steady-state clusterin mRNA levels using a semi-quantitative RT-PCR assay to determine whether changes in clusterin gene transcription in the ventral, lateral and dorsal lobes of the prostate from young and old rats occurred relative to the age-dependent decline in serum testosterone level. As shown in figure 1A, clusterin mRNA levels were significantly higher in ventral (3-fold) and lateral (1.6-fold) lobes from old rats compared to young rats. By comparison, no age-dependent difference was seen in the level of clusterin mRNA in the dorsal lobes from young and old rats.

To determine whether age-dependent changes in clusterin mRNA abundance were conserved at the protein level, Western blot analyses were performed to compare levels of clusterin protein expression in tissue lysates of the ventral, lateral and dorsal lobes from young and old rats. Levels of clusterin protein were 4-fold higher in the ventral (Fig.1 B and C) and 2-fold higher in the lateral (Fig. 1 D and E) lobes of old than in young rats. Consistent with absence of an age-dependent difference in clusterin mRNA levels in the dorsal lobe, no difference was observed in clusterin protein levels between young and old rats (Fig. 1 F and G).

Immunolocalization of clusterin expression in the ventral, lateral, and dorsal lobes of young and old rats

In a previous study, clusterin protein expression was localized by immunohistochemistry to epithelial cells in the proximal duct segments of the ventral lobe from young adult rats [32]. In the current study, we investigated whether clusterin expression was similarly localized to the proximal duct segments in the lateral and dorsal lobes and if regional expression was altered in older rats in which total clusterin levels increased in the ventral and lateral lobes (Fig. 1). Significant age-dependent changes in clusterin immunostaining intensity and localization were observed in epithelial cells lining the proximal as well as distal duct

segments of the ventral (Fig. 2 A-D) and lateral (Fig. 2 E-H) lobes. In the ventral lobe, clusterin expression was apparent in cuboidal epithelial cells lining the proximal duct segment (Fig. 2A) but not the distal duct segment (Fig. 2C) of young rats. However, in the ventral lobe of old rats, clusterin expression was localized not only within epithelial cells of the proximal duct segment (Fig. 2B), but also became apparent within epithelial cells of the distal duct segment (Fig. 2D). Similar results were observed for the lateral lobe of young and old rats with immunostaining of epithelial cells lining the proximal duct segments apparent at both ages (Fig. 2, E and F) but only in epithelial cells lining the distal duct segment of old rats (Fig. 2G and H). Although clusterin immunostaining was also present in epithelial cells lining the proximal duct segments of the dorsal lobe from young (Fig. 2I) and old (Fig. 2J) rats, the relative staining intensity was less when compared to the ventral and lateral lobes. Moreover, clusterin expression in epithelial cells lining the distal duct segments was not observed in the dorsal lobes from young (Fig. 2K) or old (Fig. 2L) rats.

Clusterin protein levels in the ventral, lateral and dorsal lobes of young and old rats following castration

Upon androgen ablation by castration, the various lobes of the rat prostate undergo regression [13]. In the ventral lobe, this regression is marked by apoptosis of a majority of the luminal secretory epithelial cells whereas in the lateral and dorsal lobes relatively few luminal epithelial cells die. Because clusterin has been postulated to possess anti-apoptotic biological activity, we investigated by Western blot analyses whether clusterin expression was differentially regulated in the ventral, lateral and dorsal lobes of young and old rats following castration (Fig. 3). In the ventral lobe of both young and old rats (Figs. 3A and 3B), clusterin expression was up-regulated on the first day following castration and progressively increased on days 2 and 3 after castration. Interestingly, the high level of clusterin expression on day 3 following castration coincides with the time point of maximal apoptotic cell death in the ventral lobe (13). Similar to the ventral lobe, clusterin levels were up-regulated in the lateral lobe of young rats on the first day after castration and increased by 90% on day 3 following castration (Figs. 3C and 3D). In the lateral lobe of old rats, clusterin levels on days 1 and 2 following castration were comparable to levels in old intact control rats, but increased dramatically by day 3 post-castration (Figs. 3C and 3D). In contrast to the ventral and lateral lobes, no pronounced changes in the levels of clusterin expression were observed in the dorsal lobes of young rats whereas in old rats a small increase was seen during the immediate 3 days following castration (Figs. 3E and 3F).

When the observational time course was extended beyond the peak of maximal epithelial cell apoptosis that occurs in the ventral lobe on day 3, clusterin levels at 7 days post-castration remained elevated or declined slightly in the ventral (Fig. 3A) and lateral (Fig. 3C) lobes of young and old castrated rats. By comparison, clusterin protein levels increased between days 3 and 7 following castration in the dorsal lobe of young and old castrated rats (Fig. 3E).

Immunolocalization of clusterin expression in the ventral, lateral and dorsal lobes of young and old rats following castration

In Fig. 2 we showed immunohistochemical evidence for the expression of clusterin by epithelial cells lining the distal duct segments of the ventral and lateral lobes of old rats but not young rats, a finding in old rats coincident with the approximate 40-50% age-dependent decline in serum testosterone levels. Similarly, castration was previously shown to affect the immunolocalization of clusterin expression in the ventral lobe of young Sprague-Dawley rats [32]. Increased expression of clusterin in the ventral lobe of castrated Brown Norway rats (Fig. 3A) is mirrored by time-dependent progressive increases in immunostaining intensity and the numbers of epithelial cells within the distal duct segment that express

clusterin in both young (Fig. 4 A-C) and old (Fig. 4 D-F) castrated rats. Similarly in the lateral lobe, the intensity of immunostaining and numbers of epithelial cells that express clusterin increase in young castrated rats (Fig. 4 G-H) and even more dramatically increase in old castrated rats (Fig. 4 J-L). In the dorsal lobe of young and old castrated rats, relatively few epithelial cells within the distal duct segments expressed clusterin on the first day following castration (Fig. 4 M and P) and small increases in the numbers of epithelial cells expressing clusterin occurred on days 2 and 3 after castration (Fig. 4 N, Q, O and R). No age-dependent differences in clusterin immunostaining were observed for the dorsal lobe after castration. Comparing the three prostate lobes, the immunostaining intensities and numbers of cells within the distal duct segments that expressed clusterin on days 1-3 following castration increased in a time-dependent pattern and showed much higher relative levels of expression in the ventral and lateral lobes than in the dorsal lobe.

Discussion

Since its original identification as an androgen-repressed mRNA transcript in the rat ventral prostate lobe [15,16], clusterin has been variously described as an androgen-regulated gene and a protein with either anti- or pro-apoptotic activity in the prostate [30]. In the present study, we examined clusterin expression in the different prostate lobes of Brown Norway rats relative to changes in serum androgen levels and epithelial cell death in the contexts of aging and castration. We previously reported that serum testosterone concentrations decline by ~ 40% in aging Brown Norway rats [6] and that androgen ablation by castration provokes both lobe-specific and age-dependent differences in epithelial cell apoptosis in the ventral, lateral and dorsal lobes of the prostate [13]. Following castration, apoptosis of luminal epithelial cells is observed in the ventral lobe, but not in the dorsal or lateral lobes. Moreover, the magnitude of cell death in the ventral lobe of aged rats is less than that in young rats.

Based upon studies in intact and castrated rats, clusterin was originally postulated to be an androgen-repressed gene in the rat ventral prostate termed testosterone-repressed prostate message-2 (TRPM-2) by Montpetit et al. [15]. Its cDNA was cloned and identified by sequence homology as that of sulfated glycoprotein 2 [16]. The name of clusterin subsequently was adopted under a unifying nomenclature. Our observations that clusterin mRNA and protein levels are lower in the ventral and lateral lobes of intact rats than castrated rats and in young rats with higher levels of serum testosterone than in aged rats nominally support the premise that clusterin gene transcription is repressed by androgen. However, findings in our study and those of others suggest that this view is overly simplistic. The age-dependent increase in clusterin mRNA and protein levels in the ventral lobe of aged rats is consistent with testosterone-mediated repression of clusterin expression based upon our previous findings of reduced numbers of androgen receptors in this lobe and decreased serum levels of testosterone with increasing age [5,13]. The higher levels of clusterin mRNA and protein in the lateral lobe of aged rats suggest that clusterin escapes androgen repression in this lobe as well. However, this interpretation is tempered by our previous observation of increased numbers of androgen receptors in the lateral lobe of aged rats which could compensate for the age-related decline in serum testosterone concentration [5]. Moreover, levels of clusterin mRNA and protein are not different in the dorsal lobes of young and aged rats despite differences in serum testosterone concentrations and higher levels of androgen receptor expression in the dorsal lobe of aged than young rats [5]. Taken together, these results do not support the androgen-mediated repression of clusterin gene transcription in the rat prostate but rather infer a more complex regulation that is age-dependent and lobe-specific.

The general concept of androgen repression of clusterin expression is further refuted by a recent report that clusterin expression is in fact up-regulated by androgen when studies are performed outside the context of castration-induced apoptosis [33]. In this study, clusterin mRNA and protein expression in androgen-dependent LNCaP human prostate cancer cells was increased by treatment with DHT or R1881 and this effect was reversed in the presence of bicalutamide, an anti-androgen. More relevant to our studies was the observation that androgen was able to induce clusterin mRNA levels in organ cultures of ventral prostate tissue from castrated rats [33]. Androgen stimulation of human clusterin gene transcription was traced to an enhancer region within the first intron as demonstrated in luciferase reporter gene assays and by direct interaction of the androgen receptor with this enhancer region in chromatin immunoprecipitation assays. Interestingly, interaction of androgen receptor with this enhancer occurred with a latency of 48 h following initiation of androgen treatment, suggesting either that displacement of repressor proteins or remodeling of the chromatin structure was a temporal prerequisite to the binding of the receptor at this site [33]. Although these findings are provocative, they fail to reconcile the in vivo mechanisms by which levels of clusterin mRNA and protein expression are increased in the ventral and lateral lobes of aged or castrated rats with lower or absent serum testosterone concentrations, respectively. The increased levels of clusterin seen in our Western blot analyses in the ventral and lateral lobes of aged rats were associated with a dramatic induction of clusterin expression in epithelial cells lining the intermediate (data not shown) and distal duct segments of these prostate lobes. In young animals, clusterin expression was localized exclusively to cuboidal epithelial cells lining the proximal duct segment whereas in aged rats, expression of clusterin extended to columnar epithelial cells throughout the ductal tree similar to that seen by Sensibar et al. (32) in the ventral lobe of young rats following castration. These age-related changes in the levels and regional localization of clusterin expression occur within the prostatic ducts of intact aging rats and are independent of apoptosis. Moreover, the levels of clusterin expression in the prostate lobes of aged rats are not strictly dependent upon the lobe-specific levels of androgen receptor expression or the availability of testosterone as its ligand. These findings suggest the possibility that other forms of cellular stress associated with increasing age may induce clusterin expression in the secretory epithelial cells lining the intermediate and distal duct segments of the prostate lobes of aging rats. Heat shock factor (HSF)-1 binds to the clusterin gene promoter and induces its transcription [34]. Increases in clusterin expression may reflect a greater need for the chaperone-like activity of clusterin, similar to that commonly associated with small heat-shock proteins, in aging cells of the prostate [35,36].

Other studies have suggested that regulation of clusterin expression in the prostate is more closely associated with cellular stress than with the direct effects of androgen ablation on gene transcription mediated by the androgen receptor [30]. For example, up-regulation of clusterin expression in the androgen-dependent Shionogi tumor model after castration is inhibited when apoptosis is blocked with apoptosis-inhibiting calcium channel blockers [27]. Increased levels of clusterin expression have been observed in various malignant and normal tissues experiencing apoptotic stress [30]. In the rat prostate, apoptosis of luminal epithelial cells following castration is prominent only in the ventral lobe whereas relatively few cells die in the dorsal and lateral lobes [3,13]. However, castration caused significant stress within all three lobes as evidenced by the involution and regression of these lobes. Similar to the regional shift in clusterin expression along the luminal ducts of aging rats, castration shifted the localization of clusterin expression from predominantly epithelial cells in the proximal duct segment to epithelial cells throughout the proximal-distal duct axis in all three prostate lobes of young rats. These increases in clusterin expression did not parallel the lobe-specific changes in androgen receptor expression that we observed previously following castration [5]. Androgen receptor epithelial cell nuclear localization and protein levels decreased over the first 3 days following castration in the ventral and dorsal lobes, whereas androgen

receptor levels did not change in the lateral lobe of castrated rats. The increased expression of clusterin that was observed to extend throughout the ductal tree of aged rats, increased even further in the three lobes when aged animals were castrated. Despite apoptosis of many of the luminal epithelial cells that line the ducts in the ventral lobe, the magnitude of clusterin expression and its regional shift in localization in the apoptotic-resistant cells along the proximal-distal axis of the ductal tree increased progressively during the 3 days following castration, similar to that observed by Sensibar et al. [32] for the ventral lobe of young rats. Like the ventral lobe, large increases in clusterin expression and its regional shift in localization also occurred along the luminal ducts of the lateral lobe following castration. However, unlike the ventral lobe, these changes in the lateral lobe are not accompanied by extensive apoptosis of luminal epithelial cells, as previously shown [13]. In the dorsal lobe, levels of clusterin expression increased but with a marked latency following castration such that marked increases were not observed until day 7.

Both pro-apoptotic and anti-apoptotic actions of clusterin have been described in the literature [30]. The existence of multiple protein isoforms of clusterin has provided some insight into these divergent effects [21,24,30,33,37]. Clusterin is synthesized and secreted as a 70-80 kDa disulfide-linked heterodimer that appears as a 35-40 kDa α - and β -subunit smear on SDS-PAGE under reducing conditions [38]. This, in part, explains the appearance of multiple immunoreactive protein bands, including the diffuse band in the 30-40 kDa range, on Western blots shown in figure 3. The secreted glycosylated isoform of clusterin is cytoprotective. A cytoplasmic isoform of clusterin prevents apoptosis, in part, by binding to and inhibiting activated Bax [39], whereas a nuclear isoform of clusterin promotes apoptosis [21,24,38]. These findings help to explain how clusterin promotes cell survival in a number of different types of cancer and why its over-expression as the cytoplasmic isoform is commonly associated with resistance of cancer cells to hormone-, chemo- and radio-therapy [28,29]. In humans, the secreted and nuclear isoforms of clusterin are generated by alternative splicing whereas in rodents, the alternate splice sites are not conserved and the multiple isoforms of clusterin are thought to be primarily derived by post-translational glycosylation and differential proteolytic processing [30,33,38]. Moreover, evidence in rodents is lacking regarding the nuclear localization of clusterin and it is primarily localized within the cytoplasm of cells or secreted. Interestingly, as demonstrated in clusterin deficient (*Clu*^{-/-}) mice, the absence of clusterin did not affect involution and cell death in the prostate following castration nor regeneration of the prostate following androgen replacement [40].

In summary, we observed that aging and castration-induced stress had similar effects to increase the expression of clusterin in the different lobes of the rat prostate. Clusterin expression appeared to be independent of epithelial cell apoptosis and only indirectly related to diminished levels of androgen that accompany aging or castration. The increased expression of clusterin in prostate epithelial cells may have a role in their cytoprotection and survival in response to stress-related stimuli such as occur during aging and following androgen ablation.

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Figure 1.

Age-dependent and lobe-specific expression of Clusterin in the Brown Norway rat prostate.

A. Steady-state clusterin mRNA levels were determined by semi-quantitative RT-PCR analyses in the ventral, lateral and dorsal prostate lobes of young (Y; 4 month-old) and old (O; 24 month-old) Brown Norway rats. The histogram shows the quantification of clusterin mRNA normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in the same samples. Western blot analyses of clusterin and β -actin protein levels from three different young and old Brown Norway rats are shown for the ventral (**B**), lateral (**D**) and dorsal (**F**) prostate lobes. Equal amounts (30 μ g) of total protein were loaded in each lane. β -actin protein levels were used to show equal loading of protein in each lane and as a control for quantification of clusterin levels as shown in the histograms representing the ventral (**C**), lateral (**E**) and dorsal (**G**) prostate lobes from young (Y) and old (O) rats. Values are the mean $\pm$ SEM, $n = 3$ rats/age group. **a**, significantly different from young rats ($P < 0.05$)

Figure 2.

Immunohistochemical localization of clusterin protein expression and immunostaining in tissue sections of ventral, lateral and dorsal lobes from young and old Brown Norway rats. Clusterin immunostaining is shown within the proximal duct segments of the ventral lobe (VP) from young (**A**) and old (**B**) rats, the lateral lobe (LP) from young (**E**) and old (**F**) rats, and the dorsal lobe (DP) from young (**I**) and old (**J**) rats and the distal duct segments of the ventral lobe (VP) from young (**C**) and old (**D**) rats, the lateral lobe (LP) from young (**G**) and old (**H**) rats, and the dorsal lobe (DP) of young (**K**) and old (**L**) rats.

Figure 3.

Western blot analyses of clusterin protein levels in prostate lobes of young and old Brown Norway rats following castration. Clusterin expression was detected in the ventral (A), lateral (C) and dorsal (E) lobes of intact control (C) rats and 1-, 2-, 3- and 7- days (d) following castration in young (Y) and old (O) rats. Equal amounts (30 ug) of protein were loaded in each lane. β -actin was used to show equal loading of protein in each lane. Clusterin levels were normalized to β -actin levels and quantified for the ventral (B), lateral (D) and dorsal (F) lobes of young (Y) and old (O) rats; mean $\pm$ SEM, $n = 3$ rats/group. **a**, significantly different from young rats, $P < 0.05$. **b**, significantly different from intact control, $P < 0.05$.

Figure 4.

Immunohistochemical localization of clusterin protein expression in distal duct segments of the prostate lobes of young and old Brown Norway rats following castration. Clusterin immunostaining is shown on days 1 (1d), 2 (2d) and 3 (3d) following castration within the distal duct segments of the ventral lobe (VP) from young (**A-C**) and old (**D-F**) rats, the lateral lobe (LP) from young (**G-I**) and old (**J-L**) rats, and the dorsal lobe (DP) from young (**M-O**) and old (**P-R**) rats.