Clusterin Biogenesis Is Altered during Apoptosis in the Regressing Rat Ventral Prostate*

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Clusterin was first characterized as an apoptosis-associated transcript after it was identified as testosteronetreated prostate message (TRPM-2) that is expressed in the epithelial cells of the regressing rat ventral prostate. Increases in clusterin mRNA and protein have been consistently detected in apoptotic cell death paradigms, establishing clusterin gene expression as a prominent marker of apoptotic cell loss. However, enhanced protein expression has also been reported in surviving cells. This ambiguity makes it difficult to define the contribution of clusterin to apoptosis. To address this problem, a panel of polyclonal and monoclonal antibodies were raised against the clusterin α-chain, β-chain, and mixed αβ epitopes. These antibodies detect changes in the biogenesis of clusterin during apoptosis by Western analysis and immunohistochemistry. A 42-kDa glyco/isoform of clusterin appears to be up-regulated in dying epithelial cells. This glyco/isoform is apparently generated as a result of apoptosis-induced stimulation of a normal but under-utilized, synthetic pathway. These data demonstrate that clusterin synthesized by apoptotic cells can be immunologically distinguished from clusterin synthesized by surviving cells in damaged tissue.

Clusterin is the most widely accepted acronym for a highly conserved protein that has been identified independently by many different laboratories and named SGP2, S35-S45, apolipoprotein J, SP40,40, ADHC-9, gp80, GPHII, and testosterone-repressed prostate message (TRPM-2) (1–10). Clusterin mRNA was first identified as an apoptosis-associated transcript when it was cloned from regressing rat ventral prostate and localized to dying epithelial cells by in situ hybridization (11, 12). Increases in clusterin mRNA and protein levels have been consistently detected in apoptotic heart, brain, lung, liver, kidney, pancreas, and retinal tissue both in vivo and in vitro, establishing clusterin gene expression as a marker of apoptotic cell loss (8, 10, 11, 13–20). However, clusterin protein has also been implicated in physiological processes that do not involve apoptosis, including the control of complement-mediated cell lysis, transport of β-amyloid precursor protein, shunting of aberrant β-amyloid across the blood-brain barrier, lipid scavenging, membrane remodeling, cell aggregation, and protection from immune detection and tumor necrosis factor α-induced cell death (1, 5, 6, 9, 21–23).

A specific role for clusterin in apoptosis has yet to be established. The literature is complicated by controversy surrounding protein localization in apoptotic tissue. Enhanced expression has been alternatively reported in dying or surviving cells by different laboratories using the same cell death paradigms (9, 14, 19, 24–28). This ambiguity makes it difficult to define the contribution of clusterin to active cell death. To address this issue we have analyzed the biogenesis of the protein in the prostate before and after hormone ablation, which induces apoptosis of the secretory epithelial cells of the gland.

In mammals, clusterin is translated from a single 1,653-nucleotide-long mRNA, with a open reading frame of 1343 nucleotides. This mRNA codes for a primary translation product of 447 amino acids, the first 21 of which are a classical hydrophobic signal sequence (Fig. 1) (29). Proteolysis of the primary translation product between Arg205 and Ser206 generates the discrete 24-kDa α (amino acids 1–205) and 26-kDa β-chains (amino acids 206–426), which are linked by five interchain disulfide bonds. The unglycosylated holoprotein has a predicted molecular mass of 50 kDa but is glycosylated to produce a mature protein of 76–80 kDa, depending on the degree of glycosylation, which is species- and tissue-specific. Rat clusterin contains six N-linked glycosylation sites, two of which are on the ε-chain and four of which are on the β-chain; the separated α- and β-chains migrate on reducing SDS-polyacrylamide gels at 30–40 and 42–48 kDa, respectively. Mature glycosylated rat clusterin proprotein ranges in size from 75 to 80 kDa. A high mannose form of clusterin ranging in size from 64 to 68 kDa has been identified in endoplasmic reticulum (19). Both the mature protein and the fully glycosylated proprotein can be secreted and are believed to interact with a recently identified cell surface receptor, gp330/megalin (30).

The degree of variability in the post-translational modification of clusterin between species and between tissues of the same species (31, 32) suggests that different glyco/isoforms of clusterin may be distinguished from each other using immunological techniques. With respect to apoptosis, we reasoned that the controversy surrounding protein localization was evidence of different (and yet uncharacterized) affinities of existing antisera for distinct post-translational modifications of clusterin. We hypothesized that the biogenesis of clusterin is altered during apoptosis and that normal and apoptotic-associated
glyco/isoforms could be distinguished by conventional antibody-antigen interactions. To test this hypothesis, we developed a panel of polyclonal and monoclonal antibodies capable of recognizing discrete epitopes on either the α-chain, the β-chain, or both chains of the protein arising from tertiary structure. Using these antibodies, we have analyzed post-translational modifications of clusterin in the regressing rat ventral prostate.

Apoptosis in the rat ventral prostate can be induced by castration, which results in the systemic loss of the hormone-dependent, tall columnar epithelial cells lining distal and intermediate zones of the prostatic ducts. Tissue regression begins as early as 12 h after surgery, and within 6–8 days, 80–90% of the secretory epithelial cells undergo apoptosis (33, 34). Stromal, basal epithelial, and cuboidal nonsecretory epithelial cells are relatively unaffected (12). Although the exact mechanisms controlling epithelial loss are not known, cell death is clearly an active process that has the major hallmarks of apoptosis, including the requirement for de novo gene expression, protein synthesis, and specific cleavage of DNA into multi-nucleosomal fragments (20). We now report that clusterin expressed by apoptotic cells can be immunologically differentiated from clusterin expressed by surviving cells on the basis of post-translational modification, and we have identified two apoptosis-associated glyco/isoforms of clusterin by Western analysis. These data indicate that clusterin synthesized by apoptotic cells is distinct from protein processed by surviving cells.

MATERIALS AND METHODS

Antibodies—Monoclonal antibodies were raised against Pichia pastoris recombinant rat clusterin purified from conditioned medium by precipitation with 25% ethanol, pH 5.5 at −10°C. The precipitated protein was dissolved in 10 mM PBS* (10 mM phosphate buffer, 154 mM NaCl, pH 7.4) containing 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 1 μg/ml pepstatin A, dialyzed overnight against 10 mM PBS, and sterilized by filtration through a 0.22-μm filter prior to immunization. 4–6-week-old female BALB/c mice (Taconic, Germantown, NY) were injected intraperitoneally with 60 μg of protein in 200 μl of MPL+TDM adjuvant (RIBA Immunochemical Research, Hamilton, MT). Spleenocytes were collected by sterile technique and fused to NS1 cells (ATCC, Rockville, MD) essentially as described previously (35). Culture supernatants were titrated by enzyme-linked immunosorbent assay using recombinant rat clusterin as the coating antigen, horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000; Caltag, Missassauga, ON, Canada) and O-phenylenediamine dihydrochloride (Sigma) as the substrate. Monoclonal antibodies were typed using a mouse monoAB ID/SP kit (Zymed Laboratories Inc., San Francisco, CA).

A clusterin polyclonal antibody, designated 301, was raised against four synthetic peptides derived from the α-chain of the mature clusterin protein. These peptides included residues 1–17 (EQEFSNDNLQEL-STOGSC), residues 32–48 (HKHTLIEKTNAERKS), residues 52–72 (LEEAKKKKEGALDDTRDSEC), and residues 133–148 (NGDRIDSTOGSC). In all but peptide 32–48, a nonencoded cysteine residue was included in the C-terminal amino acid to facilitate covalent coupling of the peptide to myoglobin. New Zealand White rabbits (Biobreeding, Ottawa, ON, Canada) were immunized subcutaneously at two sites with 0.5 mg of peptide mixture in Freund’s complete adjuvant (Sigma). Animals were boosted at 2, 4, 9, and 14 weeks with 0.5 mg of peptide mixture in Freund’s incomplete adjuvant. And test sera were 1 and 2 weeks after each booster injection and titred by enzyme-linked immunosorbent assay using the peptide immunogen as the coating antigen. 12 days after the fourth booster shot, animals were exsanguinated by cardiac puncture. The polyclonal antibody was affinity purified by standard chromatography on protein A-Sepharose (Amersham Pharmacia Biotech). A second polyclonal antibody, anti-SGP-2, raised against the sulfated form of testicular rat clusterin, was kindly provided by Dr. M. Griswold (Washington State University).

Western analysis of recombinant clusterin fusion proteins. cDNA coding for clusterin holoprotein (amino acids 22–447), α-chain (amino acids 1–205), and β-chain (amino acids 205–426) were amplified by polymerase chain reaction from the plasmid containing the full-length protein. These peptides included residues 1–17 (EQEFSNDNLQEL-STOGSC), residues 32–48 (HKHTLIEKTNAERKS), residues 52–72 (LEEAKKKKEGALDDTRDSEC), and residues 133–148 (NGDRIDSTOGSC). In all but peptide 32–48, a nonencoded cysteine residue was included in the C-terminal amino acid to facilitate covalent coupling of the peptide to myoglobin. New Zealand White rabbits (Biobreeding, Ottawa, ON, Canada) were immunized subcutaneously at two sites with 0.5 mg of peptide mixture in Freund’s complete adjuvant (Sigma). Animals were boosted at 2, 4, 9, and 14 weeks with 0.5 mg of peptide mixture in Freund’s incomplete adjuvant. And test sera were 1 and 2 weeks after each booster injection and titred by enzyme-linked immunosorbent assay using the peptide immunogen as the coating antigen. 12 days after the fourth booster shot, animals were exsanguinated by cardiac puncture. The polyclonal antibody was affinity purified by standard chromatography on protein A-Sepharose (Amersham Pharmacia Biotech). A second polyclonal antibody, anti-SGP-2, raised against the sulfated form of testicular rat clusterin, was kindly provided by Dr. M. Griswold (Washington State University).

The specificity of each antibody was determined by Western analysis of recombinant clusterin fusion proteins. cDNA coding for clusterin holoprotein (amino acids 22–447), α-chain (amino acids 1–205), and β-chain (amino acids 205–426) were amplified by polymerase chain reaction from the plasmid containing the full-length cDNA for rat clusterin, pG17H (29), and subcloned in frame as C-terminal fusions with the maltose-binding protein in the expression vector pMalP1 (New England Biochem, Beverly, MA) according to the protocol provided by the manufacturer. Forward primers GGCGATTCCAGCCAGGATTTTCTCTGACCTAGG for the α-chain and holoprotein and GGCGATTCCAGCTATCGCGCTCTCTCCTCCACT for the β-chain had 5′ extensions with an EcoRI site in frame with both the clusterin and MalE coding sequences. Reverse primers GGCGATTCCATTCTGCGATGTTTTTTCCTGCGGT for the β-chain and holoprotein and GGCGATTCCATCGCCG GCCCTCCTGCGGT for the α-chain had 5′ extensions with a BamHI site following a stop codon. The MalE fusion proteins containing clusterin α-chain, β-chain, or holoprotein were purified from Esherichia coli lysates by amylase-affinity chromatography (New England Biolabs). Purified fusion protein was separated on 10% SDS-polyacrylamide gels under reducing conditions and electroblotted to nitrocellulose membrane. Western analysis was performed using monoclonal anti-SGP2 (1:1000), polyclonal 301 (1:1000), or a panel of monoclonal (1:10) primary antibodies and detected by horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:2000; Caltag) secondary antibodies. Antibodies were diluted in 10 mM PBS containing 3% heat-denatured casein. Immunoreactive bands were visualized by chemiluminescence using ECL reagents according to the protocol provided by the manufacturer (Amersham Pharmacia Biotech).

Hormonal Ablation: Induction of Apoptosis in Rat Ventral Prostate—

**FIG. 1.** Amino acid sequence and post-translational processing features of rat clusterin. Inverted triangles, N-linked glycosylation sites; squares, cysteine residues required for disulfide linkage of α- and β-chains; arrows, potential Arg/Ser cleavage sites.
Male Sprague-Dawley rats (Taconic) weighing 250–300 g were maintained on a 14:10-h light-dark cycle with food and water available ad libitum. Rats were castrated via the scrotal route under light halothane anesthesia. Untreated control animals (day 0; n = 10) and castrated animals on day 4 after surgery (n = 40) were sacrificed by cervical dislocation. Prostate glands were excised, flash-frozen in liquid nitrogen-chilled isopentane, and stored in liquid nitrogen until Western analysis or excised, fixed for 24 h in 4% paraformaldehyde in 100 mM phosphate buffer, pH 7.5, followed by routine paraffin embedding. Sections (10 μm) were cut on a rotary microtome and mounted on sterile, gelatin-coated, positively charged microscope slides (Fisher).

Western Analysis of Normal Prostate, Regressing Prostate, and Serum Proteins—Protein was extracted from normal prostate tissue and from regressing prostate on day 4 after androgen ablation. Extracts were prepared in RIPA buffer (10 mM phosphate buffer, 154 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 30 μl/mg aprotenin, 10 mM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride). Normal rat serum was isolated by Ficoll-Hypaque density chromatography under reducing conditions on 12.5% SDS-polyacrylamide gels and electroblotted to nitrocellulose membrane. Western analysis was performed as described above with the exception that biotinylated goat anti-rabbit (1:10 000; Amersham Pharmacia Biotech) or goat anti-mouse (1:10 000; Sigma) IgG were used as the secondary antibody, and extravidin-peroxidase (1:1000; Sigma) was used as the tertiary reagent. In control reactions, primary antibodies were incubated with a 10-fold excess of recombinant rat clusterin for 1 h at room temperature and at 4 °C overnight prior to immunoblotting to establish the specificity of the observed banding patterns for clusterin.

Immunohistochemistry—Sections were deparaffinized, rehydrated, and equilibrated in 10 mM PBS (10 mM sodium phosphate buffer, pH 7.2, 154 mM NaCl). Immunoperoxidase staining was used to localize immunoreactivity as described previously (36). After overnight incubation with the primary antibody at 4 °C, sections were labeled for 1 h at room temperature with biotinylated goat anti-mouse IgG (1:300; Amersham Pharmacia Biotech) or biotinylated donkey anti-rabbit IgG (1:200; Amersham Pharmacia Biotech), incubated for 1 h in extravidin-peroxidase (1:20, Sigma), and reacted with 1 mg/ml diaminobenzidine in 50 mM Tris-HCl, pH 8.0, containing 0.003% H2O2. Antibodies and tertiary reagents were diluted in 10 mM PBS, 0.3% Triton X-100, 3% bovine serum albumin, pH 7.2. A full serial dilution (neat, 1:10, 1:100, and 1:1000) was tested for each monoclonal antibody. Polyclonal antibodies were reacted at 1:10, 1:100, and 1:1000 dilutions. Optimal dilutions are reported.

RESULTS

TABLE I

| Classificationa | Antibody | Description | Type | Characteristics |
|-----------------|----------|-------------|------|-----------------|
| Group 1a | 2D9 | mouse monoclonal | IgG<sub>a</sub>,<sub>b</sub> | αβ-single epitope (higher affinity for de-glycosylated protein) |
| Group 1b | 1F8 | mouse monoclonal | IgG<sub>a</sub>,<sub>b</sub> | αβ-single epitope (higher affinity for de-glycosylated protein) |
| Group 1b | 2F6 | mouse monoclonal | IgG<sub>a</sub>,<sub>b</sub> | αβ-single epitope (higher affinity for de-glycosylated protein) |
| Group 2 | 6E9 | mouse monoclonal | IgG<sub>a</sub>,<sub>b</sub> | β-single epitope (higher affinity for de-glycosylated protein) |
| Group 2 | 7A8 | mouse monoclonal | IgG<sub>a</sub>,<sub>b</sub> | β-single epitope (higher affinity for de-glycosylated protein) |
| Group 3 | 301 | rabbit polyclonal | IgG<sub>a</sub>,<sub>b</sub> | α-multiple epitopes (higher affinity for de-glycosylated protein) |
| Group 4 | SGP2 | rabbit polyclonal | IgG<sub>a</sub>,<sub>b</sub> | α and β-multiple epitopes (higher affinity for glycosylated protein) |

a Group 1a, monoclonal antibodies recognizing full-length α and β recombinant clusterin constructs (specific for a single αβ clusterin epitope arising as a result of tertiary structure); group 1b, monoclonal α-chain-specific antibodies; group 2, α-chain-specific polyclonal antibodies; group 3, α-chain-specific polyclonal antibodies with multiple α epitopes; group 4, α- and β-chain polyclonal antibodies with multiple epitopes on both chains.

Characterization of Monoclonal and Polyclonal Clusterin-Specific Antibodies—Western analysis of E. coli recombinant MalE-clusterin fusion proteins was used to determine the specificity of each antibody for either the α-chain, the β-chain, or an epitope composed of amino acids from both the α- and β-chains, presumably arising as a result of tertiary structure in the recombinant immunogen (Fig. 2). As indicated in Table I, antibodies were classified into four main groups: monoclonal antibodies recognizing full-length α and β recombinant fusion proteins (group 1a, exemplified by 2D9); monoclonal antibodies recognizing a shortened α-chain resulting from proteolysis in E. coli and full-length β products (specific for a single αβ clusterin epitope arising as a result of tertiary structure); group 2, β-chain-specific monoclonal antibodies; group 3, α-chain-specific polyclonal antibodies with multiple α epitopes; group 4, α- and β-chain polyclonal antibodies with multiple epitopes on both chains.
Clusterin Biogenesis during Apoptosis

FIG. 2. Classification of antibodies for α-chain, β-chain, or mixed α/β epitopes in tertiary protein structure. Western analyses of clusterin proprotein, α-chain, or β-chain fused to the maltose-binding protein were performed as described under "Materials and Methods." F, proprotein fusion product; α, α-chain fusion product; β, β-chain fusion product; M, maltose-binding protein product. Based on reactivity with these fusion proteins, antibodies were classified into four main groups: group 1a, monoclonal antibodies recognizing full length α and β recombinant fusion proteins; group 1b, monoclonal antibodies recognizing a shortened α-chain resulting from proteolysis in E. coli and full-length β products; group 2, β-chain-specific monoclonal antibodies; group 3, α-chain-specific polyclonal antibody; group 4, polyclonal antibody with affinity for both α- and β-chains.

FIG. 3. Rat ventral prostate regression is associated with expression of a novel 42-kDa isoform of clusterin. Protein was extracted from normal prostate (day 0) and from regressing prostate on day 4 after androgen ablation. Western analysis was performed as described under "Materials and Methods." Day 0 and day 4 membranes were probed simultaneously and exposed for identical periods of time. Representative results from three replicate experiments are shown. A, fully glycosylated uncleaved mature proprotein (76 kDa); B, high mannos core precursor protein (64 kDa); C, low mannos core precursor protein (56 kDa); D, unglycosylated holoprotein (50 kDa); E, fully glycosylated cleaved β-chain; F, fully glycosylated cleaved α-chain.

The change in Western reactivity on day 4 of prostastic regression is illustrated in the lower panel of Fig. 3. Group 1 α/β-specific antibodies demonstrate a highly restricted pattern of reactivity. The group 1a antibody 2D9 only reacts with a 42-kDa band, whereas group 1b antibodies (1F8 and 2F6) fail to detect clusterin on Western blot. In contrast, the group 2, β-specific antibodies 6E9 and 7A8 react strongly with the 64- and 56-kDa species but no longer recognize the other species. 6E9 also identifies the 42-kDa band detected by 2D9. The polyclonal group 3 and group 4 antibodies identify an increase in all of the clusterin glycoforms identified in normal prostate (76, 64, 56, 50, 48 (anti-SGP2 only), and 32 kDa) but do not detect the 42-kDa glyco/isoform identified by group 1a and 2 antibodies. The specificity of these analyses was confirmed by competition of antibody with excess recombinant clusterin and by immunoblotting in the absence of primary antibodies. As with proteins extracted from the normal prostate protein, signal was not observed using pre-adsorbed primary antibodies or secondary and tertiary reagents in isolation indicating that the bands (including the novel 42-kDa species) are clusterin iso/glycoforms (data not shown).

Alterations in the Biogenesis of Clusterin during Apoptosis in Regressing Rat Ventral Prostate—These data demonstrate changes in clusterin mobility following androgen depletion and cell death. To establish whether the 42-kDa apoptosis-associated clusterin species are the result of changes in proteolyis or glycosylation, normal prostate and day 4 regressing prostate protein extracts were deglycosylated by N-glycosidase F and subjected to Western analysis (Fig. 4A). Control reactions included protein incubated under identical conditions in the absence of enzyme. Western analysis demonstrated that the clusterin glyco/isoforms in the control reactions were identical to data shown in Fig. 3. As shown in Fig. 4, incubation with an excess of N-glycosidase F shifted the mobility of the 76-kDa proprotein, 48-kDa β-chain, and 32-kDa α-chain to calculated sizes of 60 kDa (recognized by all four groups of antibodies), 28 kDa (recognized by 6E9 and 7A8), and 26 kDa (recognized by 301 and anti-SGP-2) (Fig. 4A). A 97-kDa nonspecific band, which cannot be competed out with excess recombinant clusterin, is detected by all the monoclonals but not by the polyclonal antibodies (Fig. 4B). 2D9 and 1F8, the group 1 monoclonal antibodies, fail to recognize cleaved α and β clusterin chains in isolation. The mannos core intermediate forms of clusterin (58 and 56 kDa) appear to be resistant to N-glycosidase F digestion, suggesting that either the core sugars are α-1–3-linked core fucose chains rather than mannos residues or that deglycosylation is incomplete. Because we have not detected core fucose residues on human clusterin (37), it appears more likely that clusterin is not completely deglycosylated by standard enzymatic treatment. We have, however, been unable to completely strip these mannos core intermediates of their sugar with higher concentrations of enzyme (data not shown).

The increase in clusterin during prostastic regression observed in the absence of experimental deglycosylation (Fig. 3) is...
also seen in the experimentally deglycosylated samples (Fig. 4). An increase in the relative levels of the 60- and 56-kDa species is detected on day 4 relative to normal tissue. The faint staining associated with the putative apoptosis-associated 42-kDa and a β-reactive 32-kDa species is present in the deglycosylated samples of protein isolated from intact animals (Fig. 4, upper panel) and is significantly increased 4 days after castration (Fig. 4, lower panel). In addition a 37-kDa band is detectable with group 1b antibodies (1F8 and 2F6) and group 2 (6E9 and 7A8) monoclonals. Both the 42- and 37-kDa species are detected by all of the monoclonal antibodies that recognize the β subunit after deglycosylation but not by the α-specific polyclonal antibody 301 (Fig. 3, lower panel), suggesting that this glycoisofrom contains significant portions of the β-chain and that the epitopes for the group 1 antibodies are normally masked by glycosylation. Because the 42-kDa species is only observed on day 4 in untreated samples, these data also suggest that the 50-kDa holoprotein is not glycosylated and is subsequently cleaved into these lower molecular mass species or that aberrant deglycosylation and proteolytic processing of one or more glycoforms of clusterin (60 kDa, 56 kDa, or mature 76 kDa) occurs during prostate regression. It is, however, apparent that the 42-kDa glycoisofrom is not cleaved at Arg<sup>205</sup> as part of the post-translational processing because α- and β-chains smaller than the expected full-length chains are not detected in experimentally deglycosylated samples.

Although the data discussed above provide evidence for either an apoptotic-specific failure to glycosylate or deglycosylation accompanied by proteolysis (generating the 42- and 37-kDa glyco/isoforms), there is also evidence for altered glycosylation following androgen ablation. The ability of group 1b antibodies 1F8 and 2F6 to detect the 60-, 56-, 50-, 42-, and 31-kDa species of clusterin in deglycosylated samples on day 4 (Fig. 4A) but not in untreated protein (Fig. 3) indicates that their epitopes are masked during prostate regression; group 1 antibodies are capable of detecting the intermediate forms of clusterin in untreated samples in normal prostate samples. Loss of reactivity only occurs following prostate regression. Furthermore, it is important to note that group 1b antibodies detect the 42-kDa glyco/isoform following epitope “unmasking” by experimental deglycosylation. Given that these reagents do not detect cleaved α- and β-chains (Figs. 3 and 4A), this observation further supports the assertion that the apoptosis-specific 42-kDa species is not cleaved at Arg<sup>205</sup>.

Characterization of Serum Clusterin—To determine whether the alterations in clusterin biogenesis observed the regressing rat ventral prostate are the result of normal secretory processes, Western analyses of normal and regressing prostate samples (Fig. 2) were compared with that of normally secreted serum clusterin (Fig. 5). Group 1 antibodies (2D9 and 1F8) fail to react with serum clusterin. Group 2 β-specific antibodies (6E9 and 7A8) detect the cleaved 48-kDa β-chain, whereas the group 3, α-specific antibody, 301, detects only the 76- and 64-kDa proproteins. Finally, group 4 anti-SGP2 detects the 76-kDa proprotein, the 64-kDa mannose core protein, and fully glycosylated, cleaved α- and β-chains (48 and 32 kDa). These data indicate that serum clusterin is highly glycosylated given the strong affinity of anti-SGP2 and weak affinities of the other antibodies for the serum protein. Significantly, none of the bands unique to the regressing prostate are seen in serum protein.

Differential Unmasking of β Clusterin Chain during Regression of the Rat Ventral Prostate—Western analysis indicates that hormone-dependent apoptosis in the rat ventral prostate is characterized by changes in clusterin processing. Immunohistochemistry of normal and regressing rat ventral prostate was performed to determine the relevance of these changes to protein localization. Representative photomicrographs are shown in Fig. 6. Only α-reactive antibodies (group 1, group 3, and group 4) detect clusterin in normal prostate (Fig. 6, A, E, and G). The intensity of the α-specific immunoreaction is directly proportional to the avidity of these antibodies for glycosylated α-chain with anti-SGP2 showing the strongest signal, followed by 301, and only weak immunoreactivity demonstrated by the group 1 monoclonal antibody 2D9. The immunoreactivity is predominantly localized to the cytoplasm in both stromal and epithelial cells. Group 2 antibodies, as exemplified by 6E9, failed to detect clusterin in the normal prostate (Fig.
Because the β-chain of clusterin can be detected by β-specific group 2 antibodies in normal prostate by Western analysis (Fig. 3) but not by immunohistochemistry (Fig. 6C), these data indicate that a large portion of the β-chain of the molecule is selectively masked in vivo under normal conditions, most likely as a result of glycosylation-dependent tertiary conformation.

All antibodies demonstrate an increase in clusterin labeling on day 4 after castration with protein localized primarily to perinuclear regions of epithelial cells by the two monoclonal antibodies 6E9 and 2D9 (Fig. 6, B and D) and to cytoplasm and apical region of the epithelial cells by 301 and anti-SGP-2 (Fig. 6, F and H). The punctate staining evident using the latter two antibodies is suggestive of vesicular localization and is restricted to columnar epithelial cells. No change in protein expression or localization is noted in stromal cells. Antibodies capable of reacting with α-specific epitopes continue to detect cytoplasmic staining in stroma at equivalent levels in normal and regressing prostatic samples. This labeling is directly proportional to the affinity of the antibody for glycosylated α-chain.

The changes in clusterin immunolocalization in normal and apoptotic tissue indicate that β-chain epitopes are only unmasked in hormonally dependent epithelial cells of the regressing rat ventral prostate; in cells triggered to undergo apoptosis following androgen ablation this unmasking is most likely the result of changes in tertiary conformation as a consequence of the apoptotic-specific changes in clusterin biogenesis described above, particularly the novel proteolytic cleavage that produces the 42-kDa glyco/isoform. The stromal cells continue to express basal levels of normal clusterin following castration with cytoplasmic localization and masking of the vast majority of β-specific epitopes.

DISCUSSION

Clusterin was first characterized as an apoptosis-associated transcript after it was identified in apoptotic epithelial cells of the regressing rat ventral prostate following castration (8, 11). Although clusterin mRNA is consistently up-regulated in dying cells during injury-induced apoptosis (8–11, 14–17, 19), localization of protein has proven controversial. In a variety of experimental cell death models, clusterin protein has been identified in dying cells, surviving cells, invading phagocytic cells, and extracellular deposits located in the vicinity of damaged tissue (9, 14, 19, 24–28). This controversy has made it difficult to establish a role for the protein in apoptosis.

Biogenesis of Clusterin in Normal Tissue—Our results indicate that in normal prostate clusterin has at least five different glyco/isoforms: fully glycosylated mature pro-protein (76 kDa), cleaved fully glycosylated α- and β-chains (32 and 48 kDa), two intermediate uncleaved processing forms of proprotein (presumably the high mannose (64 kDa) and low mannose species of clusterin (56 kDa)), and full-length unglycosylated holoprotein (50 kDa). Identification of these different glycoforms requires several antibodies. No single antibody (α-specific, β-specific, tertiary structure-specific, or glycosylation-specific) is capable of detecting all products simultaneously. This characterization is consistent with the literature. The predicted size of holoclusterin is 50 kDa. Mature glycosylated protein and cleaved clusterin have been reported to range in size from 75 to 80 kDa ( uncleaved protein), 32 to 35 kDa (α-chain), and 45 to 48 kDa (β-chain) depending upon tissue and species (9, 19, 32, 38–41). The 64-kDa proprotein is probably the same product as the 61–68-kDa high mannose form of clusterin identified in...
endoplasmic reticulum in canine kidney epithelial cells, rat Sertoli cells, and murine limb bud epiblasta (19, 32). Although the smaller species of uncleaved clusterin (56 kDa) have not been explicitly characterized, comparably sized fragments have been observed by Western analysis of protein extracted from Madin-Darby kidney cells, murine limb-bud, human recombinant protein synthesized by hamster kidney fibroblasts, and human seminal fluids (19, 38).

Our analysis of normally secreted protein in rat serum is also consistent with previous reports (32, 39, 41). Circulating clusterin is present in only three configurations: fully glycosylated mature proprotein (76 kDa), cleaved fully glycosylated α- and β-chains (32 and 48 kDa), and high mannose core proprotein (64 kDa). Glycosylated product (both mature uncleaved and cleaved protein) predominate over the mannose core species. The finding that the high mannose form of clusterin can be secreted has been previously demonstrated experimentally (32). Although N-linked carbohydrate modification has been shown to be essential to ensure the “bulk” passage of newly synthesized protein from Golgi to plasma membrane through the apical transport vesicles for constitutive protein secretion, regulated clusterin release depends upon the selective sorting of protein into immature secretory granules and subsequent vesicular aggregation in the trans-Golgi network for exocytotic release (32). These authors have also demonstrated that fully glycosylated clusterin is more efficiently processed for constitutive secretion, whereas “under-glycosylated” clusterin is more efficiently packaged for regulated secretion.

A hypothetical biogenesis pathway for clusterin synthesized and secreted under normal conditions is shown in Fig. 7. In this model, clusterin precursor protein is translated on bound ribosomes associated with the endoplasmic reticulum. The signal sequence is removed, and 50-kDa holoprotein is translocated into the lumen of the endoplasmic reticulum (Fig. 7A). Folding and disulfide bond formation occurs (Fig. 7B), and core sugars are added in the endoplasmic reticulum generating the 64-kDa high mannose form of clusterin (Fig. 7C), which is partially modified by glycosidase and mannosidase I (Fig. 7D) prior to vesicular transport to the cis-Golgi, where the intermediate mannose form of clusterin can undergo one of two possible fates. In the constitutive secretion pathway, the core sugars are trimmed to the 56-kDa inner mannose core in the Golgi apparatus (Fig. 7E), and the low mannose species is then processed for secretion by enzymatic addition of complex carbohydrate side chains generating the 76-kDa proprotein (Fig. 7F). Following glycosylation, the full-length clusterin protein can then be cleaved into distinct disulfide-linked α- and β-chains (Fig. 7G). Both the full-length and cleaved products are then bulk pro-
cessed into transport vesicles and released. In the regulated secretion pathway, the intermediate-mannose form (Fig. 7D) can be packaged in secretory granules, aggregated in the trans-Golgi network, and stored until a putative signal elicits exocytotic release.

**Altered Biogenesis of Clusterin during Active Cell Death**—The data presented in this manuscript demonstrate that clusterin biogenesis is fundamentally altered by hormonal ablation in rat ven tral prostate. Three regression-associated modifications have been identified. First, induction of active cell death in the prostatic epithelial cells results in an increase in clusterin synthesis with enhanced levels of the fully glycosylated proprotein (cleaved and uncleaved), unglycosylated holoprotein, and mannose core intermediates.

Second, a novel species of clusterin has been detected that migrates with an electrophoretic mobilities of 42 kDa. This isoform of clusterin is not derived from the cleavage of the proprotein into discrete α and β during processing. Although the 42-kDa isoform is only identified in protein samples following hormonal ablation, low levels of the 42-kDa protein can be detected in experimentally deglycosylated protein extracts in both normal and regressing prostate. This observation suggests that these species are probably products of an endogenous but under-utilized synthetic pathway in normal cells, possibly due to cleavage at a cryptic Arg/Ser cleavage site in the α-chain at position Arg108/Ser109. Cleavage at this point would produce a protein with a predicted molecular mass of 42 kDa, containing 97 amino acids derived from the α-chain and all 221 amino acids of the β-chain but lacking the disulfide linkages to the α-chain and the α-chain epitopes used to raise the 301 antibody. The origin of the 37-kDa glyco/isoform seen after deglycosylation, however, remains a mystery.

Third, hormonal ablation results in a conformational change in clusterin protein. This structural alteration unmasks β-chain-specific epitopes detectable in tertiary structure by immunohistochemistry. Under normal conditions, clusterin is only identified in situ using α-chain-specific antibodies with affinity for the glycosylated protein (301 and anti-SGP-2). These reagents localize clusterin throughout cytoplasm in both epithelial and stromal compartments. Following castration, clusterin can be identified using β-chain-specific antibodies (2D9 and 6E9) and is localized in the perinuclear region of the columnar epithelial cells that are susceptible to apoptosis after hormonal ablation. Using α-chain-specific antibodies, basal levels of clusterin are still present in the cytoplasm of normal stroma. Enhanced immunoreactivity is only evident in columnar epithelial cells using the 301 antibody that recognizes unglycosylated α epitopes. These data indicate that although clusterin is present in both surviving and apoptotic cells during regression of the ventral prostate, the increase in protein synthesis observed by Western analysis is restricted to dying epithelial cells and is accompanied by a conformational change in the α-chain.

Fig. 7 illustrates possible changes in clusterin biogenesis associated with apoptosis. Following induction of active cell death, one of two possible events occurs. Glycosylation of the 50-kDa holoprotein is not initiated in the endoplasmic reticulum, and the unglycosylated protein undergoes proteolysis at the cryptic Arg/Ser site on the α-chain generating the 42-kDa species detected in regressing ventral prostate (Fig. 7H). Alternatively, but less likely, deglycosylation of clusterin intermediate followed by proteolysis may be responsible for the apoptosis-associated 42-kDa glyco/isoform (Fig. 7H). This novel species may be packaged into secretory vesicles evidenced by the shift in immunohistochemical localization or may be retained intracellularly, possibly in the nucleus. It is possible that a disulfide bridging between adjacent cysteine residues on the β-chain of the molecule in the absence of α-specific residues is the conformational signal that results in the unmasking of β-specific immunoreactivity, but this has not been demonstrated experimentally. The function of these apoptosis-associated forms of clusterin is unknown. However, these data and our hypothetical model reconcile the apparent controversies in the literature concerning clusterin protein expression during apoptosis. Clusterin synthesis is increased during active cell death, and the newly synthesized protein is localized to apoptotic cells. The α-chain-specific, β-chain-specific, tertiary structure-specific, and glycosylation-sensitive antibodies described in this manuscript should prove invaluable for the elucidation of the biogenesis of the different glyco/isoforms of clusterin and their role(s) in apoptosis and other physiological functions.
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