Mol. Cells, Vol. 10, No. 2, pp. 193–198

Molecules and Cells

© Springer-Verlag 2000

Antisense Oligonucleotide of Clusterin mRNA Induces Apoptotic Cell Death and Prevents Adhesion of Rat ASC-17D Sertoli Cells

Sang Wook Kang, Sang Wook Lim, Sang Hyun Choi, Kyung Ho Shin, Boe Gwun Chun, In Sun Park[1], and Bon Hong Min*

Department of Pharmacology, College of Medicine, Korea University, Seoul 136-705, Korea;
[1] Department of Anatomy, College of Medicine, Inha University, Inchon 402-751, Korea.

(Received on December 13, 1999)

Clusterin has been known to play important roles in cell-cell and/or cell-substratum interactions. Recently we reported the transient expression of clusterin in pancreatic endocrine cells during the early developmental stages and suggested a role in aggregating the endocrine cells for islet formation. In the present study, we have investigated the involvement of clusterin in cell-substratum interaction by the inhibition of clusterin synthesis using antisense oligonucleotide. The expression of clusterin was transiently increased as early as 2–8 h after plating the ASC-17D Sertoli cells to the culture flask, which was the period of cell attachment. In addition, up-regulation of clusterin mRNA was so much greater when the Sertoli cells were plated on the petri dish for the bacterial culture instead of in a animal cell culture flask that therefore, the cells failed to attach to it. These findings suggested that interruption of cell to plate substratum interaction might lead to over-expression of clusterin from Sertoli cells to induce cell to cell aggregation or, perhaps, to re-establish attachment with the substratum. Transfection of ASC-17D Sertoli cells with a 20-base antisense oligonucleotide against clusterin mRNA resulted in extracellular release of LDH and DNA fragmentation. Sertoli cell death by antisense oligonucleotide of clusterin was sequence specific and dose dependent. Treatment of antisense oligonucleotide induced a marked reduction of synthesis for clusterin protein, but not for clusterin mRNA expression, suggesting the translational suppression of clusterin by antisense oligonucleotide. Further, microscopic observation showed that more noticeable cell death was induced by treating the antisense prior to plating the cells than by treating after cell attachment to the plate. From these results, we speculate that down-regulation of clusterin expression in the anchorage-dependent Sertoli cells prevents them from attaching to the plate, and therefore induces cell death.

Keywords: Antisense Oligonucleotide; Apototosis; Cell-substratum Interaction; Clusterin.

Introduction

Clusterin is an intriguing glycoprotein (75–80 kDa) consisting of two different subunits joined by disulfide bonds. This protein has been identified and cloned independently from many laboratories focusing on the diverse research areas. Thus, clusterin has many different names such as sulfated glycoprotein-2 (Griswold *et al.*, 1986), apolipoprotein-J (de Silva *et al.*, 1990), SP-40/40 (Murphy *et al.*, 1988), complement cytolysis inhibitor (Jenne and Tschopp, 1989), testosterone repressed prostate message-2 (Leger *et al.*, 1987), glycoprotein 80 (Hartmann *et al.*, 1991), and pADHC-9 (May *et al.*, 1989). Clusterin has been suggested to have roles in important biological processes, including cell adhesion, organogenesis, spermatogenesis, differentiation, complement regulation, lipid transport, and apoptosis based on its tissue expression pattern and/or biochemical properties (Fritz and Murphy, 1993; Jenne and Tschopp, 1992; Rosenberg and Silkensen, 1995; Tenniswood *et al.*, 1992). However, these proposed

* To whom correspondence should be addressed.
Tel: 82-2-920-6128; Fax: 82-2-927-0824
E-mail: bhmin@kuccnx.korea.ac.kr

Abbreviations: LDH, lactate dehydrogenase; PMSF, phenylmethylsulfonyl fluoride; POD, peroxidase; PVDF, polyvinylidene difluoride; TNF-alpha, tumor necrosis factor-alpha.

functions of clusterin need to be further characterized. The protein was originally identified as a cell-aggregating factor in ram rete testis fluid and named for its potential to induce clustering of suspensions of Sertoli cells and erythrocytes (Blaschuk *et al*., 1983; Fritz *et al*., 1983). Thereafter, many researches have been focused on the role of clusterin in the process of programmed cell death/apoptosis, since clusterin is markedly induced in tissues undergoing apoptotic cell death, such as mammary gland involution, neuro-degenerative diseases, renal injury and prostate regression by castration. Correlation between clusterin induction and cell death led to the possibility that clusterin could be directly involved in the process of apoptotic cell death. However, Silkensen *et al*. (1995) suggested that the function of clusterin is to enhance cell-cell and/or cell-substratum interactions which are perturbed in the renal injury, thereby preserving the integrity of the renal epithelial barrier and protecting the apoptotic cell death. Furthermore, Humphreys *et al*. (1997) showed that TNF-alpha induced-cytotoxicity was prevented by over-expression of clusterin in L929 murine fibrosarcoma cells. These reports suggested that clusterin expression is likely to be a cytoprotective response rather than a cause of cell death. Recently, we have also shown that clusterin was up-regulated in the surviving alpha-cells, but not in apoptotic beta-cells upon streptozotocin injection to rat and suggested that clusterin could play important cytoprotective roles by enhancing the cell interactions and membrane integrity of the pancreatic islet (Park *et al*., 1999). In this study, we have investigated the role of clusterin in apoptotic cell death and/or cell-plate substratum interaction by suppression of clusterin synthesis using the transfection of an antisense oligonucleotide. In addition, we have analyzed the levels of clusterin mRNA and protein suppressed by antisense oligonucleotide using Northern and Western blotting analysis.

Materials and Methods

Cell culture The rat ASC-17D Sertoli cells were kindly provided by Dr. Kenneth Roberts (University of Minnesota, Minneapolis, USA). ASC-17D cells were maintained in a humidified atmosphere of 5% CO_2 at 32°C in DMEM/F12 medium (Life Technologies, USA) that supplemented with 4% heat-inactivated fetal bovine serum and 1% antibiotics-antimycotics. The number of viable cells which excluded tryphan blue was counted.

Total RNA isolation and Northern blot analysis For the study of clusterin gene expression during cell attachment, ASC-17D Sertoli cells (5×10^5) were plated in 60 mm cell culture dish following detachment with 0.1% EDTA solution. RNA was isolated at various time points after re-plating. For the study of clusterin transcription from the attached or suspended-state of anchorage dependent cells, rat ASC-17D Sertoli cells were incubated in animal cell culture dish or bacteria culture dish for 2 d, respectively. Total RNA was isolated by the acid guanidium thiocyanate and phenol/chloroform extraction method as described by Chomczynski and Sacchi (1987). Five micrograms of total RNA were size fractionated on 1.2% agarose gel containing 0.67 M formaldehyde, and transferred to a Hybond N^+ nylon membrane (Amersham Pharmacia, USA). The membrane was baked at 120°C for 30 min and hybridized with [α-^{32}P]UTP labeled rat clusterin cRNA probe in the hybridization buffer (Min *et al*., 1998). After 14 h of hybridization, the membrane was washed twice in 0.5× SSC containing 0.1% SDS at 50°C for 15 min each, and more stringently washed twice in 0.1× SSC containing 0.1% SDS at 65°C for 30 min each. The membrane was then exposed to X-ray film with an intensifying screen at 80°C for 2 d.

Antisense oligonucleotide transfection Twenty bases of phosphothioate antisense oligonucleotide (5′-CACAGCAG-GAGAATCTTCAT-3′), which was designed to hybridize to the rat clusterin AUG translation initiation codon, was synthesized from SGS (Scandinavian Gene Synthesis AB, Sweden) and used to inhibit clusterin expression in rat ASC-17D Sertoli cells. Antisense oligonucleotide transfection was carried by the method of Bennett *et al*. (1992) using liposome to enhance the transfection efficiency. For the transfection of antisense oligonucleotide to the suspended ASC-17D Sertoli cells, subconfluent cells were detached using 0.1% EDTA and washed three times with Opti-MEM (Life Technologies, USA) prewarmed at 37°C. Five milliliters of ASC-17D rat Sertoli cells (1×10^5/ml) were distributed to 10 ml of polystyrene tube. Twenty five μg of Lipofectin (Life Technologies, USA) was added to 250 μl of Opti-MEM and mixed with twenty folds of the indicated concentration of oligonucleotide. Oligonucleotide/Lipofectin complexes (250 μl) were added to suspended-state of Sertoli cells and incubated for 4 h at 32°C. The medium was removed by centrifugation and replaced with 5 ml of normal growth medium. Transfected cell suspension (2 ml) was plated on a 6-well cluture dish for microscopic analysis. For the transfection of antisense oligonucleotide to attached ASC-17D Sertoli cells, cells (1×10^5/ml) were plated on 6-well cell culture plates. Antisense oligonucleotide was treated according to the same protocol described above at 1 d after plating.

LDH assay Cell death was examined by extracellular LDH release. ASC-17D Sertoli cells were transfected with various concentrations of antisense or random oligonucleotides for 24 h. Culture supernatant (50 μl) was incubated with an equal volume of LDH substrate solution (Promega, USA) for 30 min. Enzyme reaction was then terminated by the addition of 50 μl of 1.0 M acetic acid and the optical density of each reaction was determined by a microplate reader (Uniskan II 3.0) at a wavelength of 492 nm.

DNA fragmentation ASC-17D Sertoli cells were plated on 60 mm dishes and transfected with 0.5 μM of antisense or random oligonucleotides. Following exposure to antisense or random oligonucleotides for 24 h, adherent and floating cells were lysed with lysis buffer and treated with RNase A (20 μg/ml) at 37°C for 30 min and proteinase K (10 μg/ml) at 55°C for another 30 min (Ahn *et al*., 1999). The DNA was then extracted with phenol-chloroform, precipitated with isopropanol, washed with ethanol, and air-dried. DNA samples (10 μg) were separated

by electrophoresis in 2.0% agarose gel and visualized by short wave UV transilluminator.

Western blot analysis Transfected ASC-17D Sertoli cells were harvested at the time indicated, washed twice with cold PBS and lysed with lysis buffer (150 mM NaCl, 50 mM Tris·Cl, pH 8.0, 0.1% SDS, 1% NP-40, 100 μg/ml PMSF). After quantification of total protein extract, protein sample (25 μg) was loaded on each lane and resolved by electrophoresis on 7.5% SDS-polyacrylamide gel. Clusterin immunoreactive bands were detected with the ECL Western Blotting Substrate (POD) (Amersham Pharmacia, USA) using polyclonal sheep anti-rat clusterin antibody (Quidel, USA).

Results and Discussion

Expression of clusterin during cell attachment Because cell-substratum interactions are often disturbed in the tissues undergoing apoptotic cell death with the increased expression of clusterin (Park *et al*., 1999; Rosenberg and Silkensen, 1995), we examined the clusterin expression at various time points after plating rat ASC-17D Sertoli cells to the culture plate. Clusterin expression was transiently increased as early as 2–8 h after plating ASC-17D Sertoli cells on the culture flask, and then decreased at 24 h (Fig. 1). Up-regulation of clusterin occurred at the time of cell attachment after re-plating, suggesting the involvement of clusterin in cell adhesion. Podophyllotoxin treatment to porcine renal epithelial cell (LLC-PK1) resulted in disruption of microtubules and induced clusterin expression. Disruption of microfilaments by cytochalasin B also resulted in the increase of clusterin transcription, indicating the disruption of microtubule or microfilament was associated with clusterin expression (Silkensen *et al*., 1995). Since cell harvest using 0.1% EDTA and re-plating might also disrupt cytoskeleton structure, clusterin induction during attachment after re-plating needs to be further characterized whether it is for cell adhesion or remodeling of cytoskeleton. However, significant increase of LLC-PK1 cell adhesion on clusterin-coated plate (Silkensen *et al*., 1995) supports our suggestion that clusterin expression could be induced to enhance the cell attachment to plate substratum.

Clusterin induction by disruption of cell-substratum ASC-17D Sertoli cells cultured on normal culture plate for animal cell expressed low level of clusterin. When cell-substratum interactions were disrupted by culturing on bacterial culture plate, clusterin was highly expressed in the anchorage dependent ASC-17D Sertoli cells (Fig. 2). In addition, we observed that most cells were suspended and aggregated each other in the media instead of attaching to the plate and still survived for 48 h when the Sertoli cells were plated on the petridish for bacterial culture. These findings imply that Sertoli cells being disrupted in the cell-plate substratum interactions express clusterin to induce cell to cell aggregation, or perhaps, to re-establish attachment with the substratum. When porcine renal epithelial cells were plated on the flask coated with agarose to inhibit cell adhesion, up-regulation of clusterin was observed (Silkensen *et al*., 1995). Such up-regulation of clusterin indicates that clusterin may play an important role in the cell aggregation as originally suggested by Fritz *et al*. (1983). In order to investigate if clusterin has the ability of enhancing cell-substratum interaction, clusterin antisense was transfected to Sertoli cells before or after plating the cells to the culture flask. We found that more cell death was induced by antisense treatment prior to plating the cells (A panel in Fig. 3) than by treatment after cell attachment to the plate (B panel in Fig. 3). From these

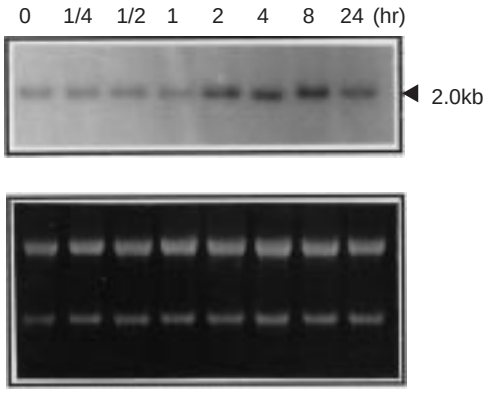

Fig. 1. Northern blot analysis of clusterin in rat ASC-17D Sertoli cells during cell attachment. Total RNA was isolated at various time points after re-plating, following detachment with 0.1% EDTA solution. Five micrograms of total RNA were size fractionated by 1.2% denatured-agarose gel electrophoresis and blotted onto nylon membrane. Blot was hybridized with [α-^{32}P]UTP labeled-clusterin cRNA probe (310 bp). Equal loading of RNA was confirmed by ethidium-bromide gel staining.

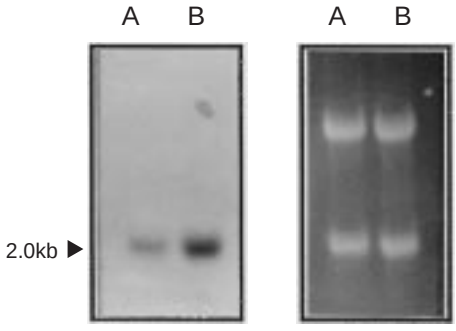

Fig. 2. Transcription of clusterin gene in attached- or suspended-state of rat ASC-17D Sertoli cells. Cells were plated on cell culture plate (**A**) or petridish for bacteria culture (**B**) for 2 d and then total RNA was isolated from each plate. Five micrograms of total RNA were fractionated in 1.2% denatured agarose gel and transferred to nylon membrane with capillary action. Blot was hybridized with [α-^{32}P]UTP labeled clusterin cRNA probe. Equal loading of RNA was confirmed by ethidium-bromide gel staining.

results, we speculate that clusterin suppression by antisense oligonucleotide in anchorage-dependent Sertoli cells prevents them from attaching to the plate, and therefore induces cell death.

Cell death induced by transfection of clusterin antisense Clusterin up-regulation during cell attachment and by the disruption of cell-substratum interaction led us to test whether it is involved in adhesion and/or survival of anchorage-dependent Sertoli cells. To suppress the expression of clusterin, we synthesized an antisense oligonucleotide designed to hybridize to the rat clusterin mRNA covering AUG translation initiation codon. Transfection of ASC-17D Sertoli cells with a 20-base antisense oligonucleotide to clusterin mRNA resulted in cell death in a dose-dependent manner. The induced cell death by clusterin antisense was sequence specific because the oligonucleotide of random sequences with the same size to antisense had no cytotoxic effect to Sertoli cells at the same molar concentration (Fig. 4). The fact that clusterin depletion, rather than clusterin induction, could directly induce cell death was coincident with LNCaP prostate cancer cell death induced by high concentration of clusterin antisense oligonucleotide (Sensibar *et al.*, 1995). For efficient transfection, we tested several liposome carriers and found that Lipofectin (Life Technologies, USA) yielded greater transfection efficiency as carrier.

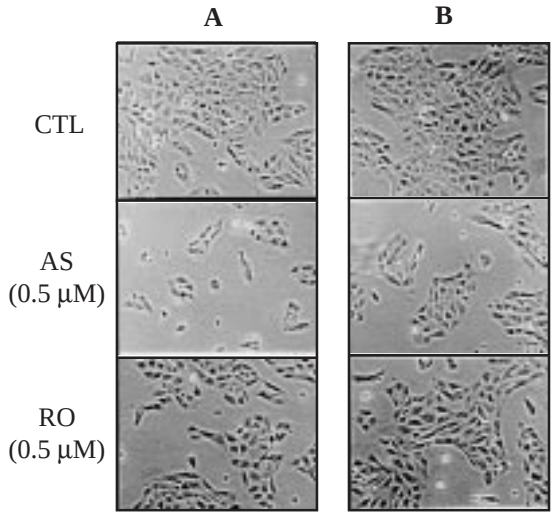

Fig. 3. Light microscopic photograph of rat ASC-17D Sertoli cells following transfection with antisense oligonucleotide. Cells were transfected with 0.5 μM of antisense or random oligonucleotides before (**A**) or after (**B**) cell attachment. The transfected cells by antisense oligonucleotide, not the random oligonucleotide, underwent cell death and were scarcely seen on the plate. In case of transfection prior to cell attachment, more cell death was observed.

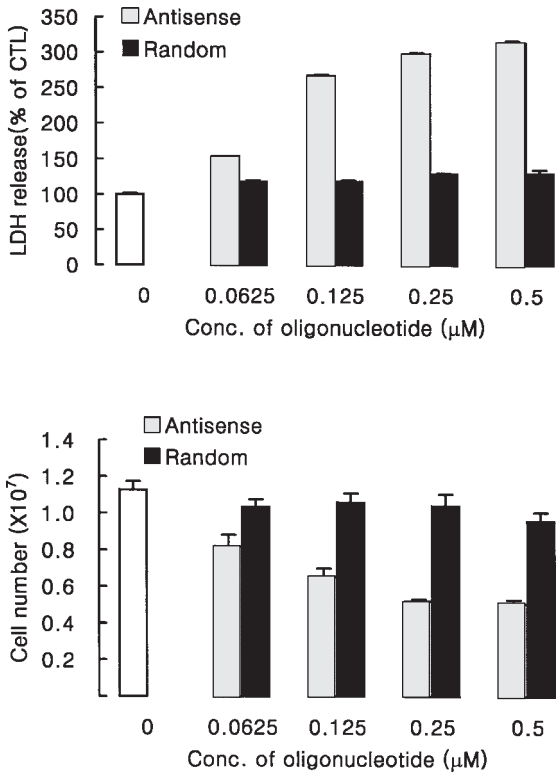

Fig. 4. Cell death induced by clusterin antisense oligonucleotide. ASC-17D Sertoli cells were transfected with various concentrations of clusterin antisense or random oligonucleotides for 24 h. Cell death was examined by extracellular release of LDH (**A**) and by counting the viable cells (**B**). Cell death by clusterin antisense was induced in a dose-dependent manner and showed sequence specificity.

Without the liposome carrier, cell death or growth inhibition was not detected up to 10 μM (data not shown). However, ASC-17D Sertoli cell death could be induced at as low a level as 0.125 μM using liposome carrier (Fig. 4). Further, we also found that clusterin antisense oligonucleotide induced DNA ladder formation, one of the biochemical features of apoptosis (Fig. 5). However, the mechanism of apoptotic cell death by clusterin depletion needs to be further characterized.

Translational suppression by clusterin antisense Inhibition of gene expression by transfection of antisense oligonucleotide could be explained by several mechanisms including inhibition of the new protein synthesis by translational arrest, promotion of mRNA degradation by RNase H-dependent mechanism and transcriptional repression by forming a triple helix structure (Chiang *et al.*, 1991; Landgraf, 1996; Stein and Cohen, 1988). In order to identify the mechanism of clusterin suppression by antisense, we analyzed the levels of clusterin mRNA and protein in ASC-17D Sertoli cells transfected with antisense oligonucleotide by northern and western blotting, respectively. Treatment of antisense oligonucleotide did

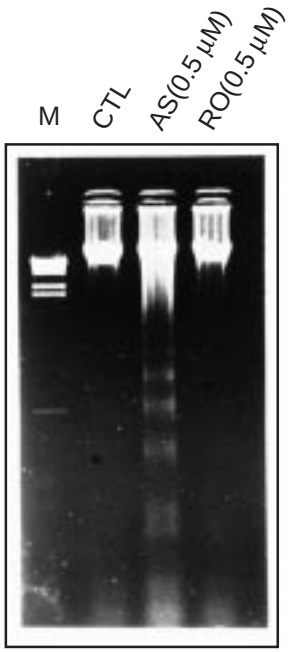

Fig. 5. DNA fragmentation induced by clusterin antisense oligonucleotide. ASC-17D Sertoli cells were plated on 60 mm dishes and transfected with 0.5 μM of antisense or random oligonucleotides. Following exposure to antisense or random oligonucleotides for 24 h, DNA was then extracted from adherent and floating cells. DNA samples (10 μg) were separated by electrophoresis in 2.0% agarose gel and visualized by short wave UV transilluminator.

not change the level of clusterin mRNA, but markedly reduced the synthesis of clusterin protein (Fig. 6), indicating the translational suppression of clusterin by antisense oligonucleotide.

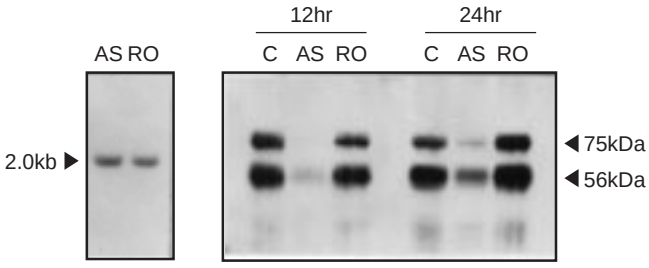

Fig. 6. Translational suppression of clusterin in ASC-17D rat Sertoli cells by antisense transfection. Cells were transfected with antisense or random oligonucleotides and incubated for 24 h. Total RNA and protein were isolated for Northern and Western blot analysis, respectively. Five micrograms of total RNA were fractionated in 1.2% denatured agarose gel and then blotted onto nylon membrane. The blot was hybridized with ^{32}P-labeled rat clusterin cRNA probe (left panel). Twenty five micrograms of cellular protein were fractionated by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. Clusterin immunoreactive bands were detected by the ECL western blotting substrate (POD) using polyclonal sheep anti-rat clusterin antibody (right panel).

In summary, we showed here that clusterin could be repressed in ASC-17D Sertoli cells by transfection with an antisense oligonucleotide of 20 bases to clusterin mRNA. Clusterin suppression by antisense prevented the adhesion of the anchorage-dependent Sertoli cells to plate substratum, thereby inducing cell death. From these results, we speculate that clusterin could play important roles in preventing cell death by enhancing cell-cell and/or cell-substratum interactions.

Acknowledgments This study was supported by the academic research fund of Ministry of Education, Republic of Korea to B. H. Min (GE97-40).

References

Ahn, J. H., Ko, Y. G., Park, W. Y., Kang, Y. S., Chung, H. Y., and Seo, J. S. (1999) Suppression of ceramide-mediated apoptosis by HSP70. *Mol. Cells* **9**, 200–206.

Bennett, C. F., Chiang, M. Y., Chan, H., Shoemaker, J. E., and Mirabelli, C. K. (1992) Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. *Mol. Pharmacol.* **41**, 1023–1033.

Blaschuk, O., Burdzy, K., and Fritz, I. B. (1983) Purification and characterization of a cell-aggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. *J. Biol. Chem.* **258**, 7714–7720.

Chiang, M. Y., Chan, H., Zounes, M. A., Freier, S. M., Lima, W. F., and Bennett, C. F. (1991) Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms. *J. Biol. Chem.* **266**, 18162–18171.

Chomczynski, P. and Sacchi, N. (1987) Single step method for RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.

de Silva, H. V., Stuart, W. D., Duvic, C. R., Wetterau, J. R., Ray, M. J., Ferguson, D. G., Albers, H. W., Smith, W. R., and Harmony, J. A. (1990) A 70-kDa apolipoprotein designated ApoJ is a marker for subclasses of human plasma high density lipoproteins. *J. Biol. Chem.* **265**, 13240–13247.

Fritz, I. B. and Murphy, B. (1993) Clusterin: insights into multifunctional protein. *Trends Endocrinol. Metab.* **4**, 41–45.

Fritz, I. B., Burdzy, K., Setchell, B., and Blaschuk, O. (1983) Ram testis fluid contains a protein(clusterin) which influences cell-cell interactions *in vitro*. *Biol. Reprod.* **28**, 1173–1188.

Griswold, M. D., Roberts, K., and Bishop, P. (1986) Purification and characterization of a sulfated glycoprotein secreted by Sertoli cells. *Biochemistry* **25**, 7265–7270.

Hartmann, K., Rauch, J., Urban, J., Parczyk, K., Diel, P., Pilarsky, C., Appel, D., Haase, W., Mann, K., Weller, A., and Kosh-Brandt, C. (1991) Molecular cloning of gp80, a glycoprotein complex secreted by kidney cells *in vitro* and *in vivo*. A link to the reproductive system and to the complement cascade. *J. Biol. Chem.* **266**, 9924–9931.

Humphreys, D., Hochgrebe, T. T., Easterbrook-Smith, S. B., Tenniswood, M. P., and Wilson, M. R. (1997) Effects of clusterin overexpression on TNFalpha- and TGFbeta-mediated death of L929 cells. *Biochemistry* **36**, 15233–15243.

Jenne, D. E. and Tschopp, J. (1989) Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: identity to sulfated glycoprotein 2, a constituent of rat testis fluid. *Proc. Natl. Acad. Sci. USA* **86**, 7123–7127.

Jenne, D. E. and Tschopp, J. (1992) Clusterin: the intriguing

guises of a widely expressed glycoprotein. *Trends Biochem. Sci.* **17**, 154–159.

Landgraf, R. (1996) Antisense targeting in behavioural neuroendocrinology. *J. Endocrinol.* **151**, 333–340.

Leger, J. G., Montpetit, M. L., and Tenniswood, M. P. (1987) Charaterization and cloning of androgen-repressed mRNAs from rat ventral prostate. *Biochem. Biophys. Res. Commun.* **147**, 196–203.

May, P. C., Johnson, S. A., Poirier, J., Lampert-Etchells, M., and Finch, C. E. (1989) Altered gene expression in Alzheimer's disease brain tissue. *Can. J. Neurol. Sci.* **16**, 473–476.

Min, B. H., Jeong, S. Y., Kang, S. W., Crabo, B. G., Foster, D. N., Chun, B. G., Bendayan, M., and Park, I. S. (1998) Transient expression of clusterin (Sulfated glycoprotein-2) during development of rat pancreas. *J. Endocrinol.* **158**, 43–52.

Murphy, B. F., Kirszbaum, L., Walker, I. D., and d'Apice, A. J. (1988) SP-40, 40, a newly identified normal human serum protein found in the SC5b-9 complex of complement and in the immune deposits in glomerulonephritis. *J. Clin. Invest.* **81**, 1858–1864.

Park, I. S., Che, Y. Z., Bendayan, M., Kang, S. W., and Min, B. H. (1999) Up-regulation of clusterin (sulfated glycoprotein-2) in pancreatic islet cells upon streptozotocin injection to rats. *J. Endocrinol.* **162**, 57–65.

Rosenberg, M. E. and Silkensen, J. R. (1995) Clusterin and the kidney. *Exp. Nephrol.* **3**, 9–14.

Sensibar, J. A., Sutkowski, D. M., Raffo, A., Buttyan, R., Griswold, M. D., Sylvester, S. R., Kozlowski, J. M., and Lee, C. (1995) Prevention of cell death induced by tumor necrosis factor alpha in LNCaP cells by overexpression of sulfated glycoprotein-2 (clusterin). *Cancer Res.* **55**, 2431–2437.

Silkensen, J. R., Skubitz, K. M., Skubitz, A. P., Chmielewski, D. H., Manivel, J. C., Dvergsten, J. A., and Rosenberg, M. E. (1995) Clusterin promotes the aggregation and adhesion of renal porcine epithelial cells. *J. Clin. Invest.* **96**, 2646–2653.

Stein, C. A. and Cohen, J. S. (1988) Oligodeoxynucleotides as inhibitors of gene expression: a review. *Cancer Res.* **48**, 2659–2668

Tenniswood, M. P., Guenette, R. S., Lakins, J., Mooibroek, M., Wong, P., and Welsh, J. E. (1992) Active cell death in hormone-dependent tissues. *Cancer Metastasis Rev.* **11**, 197–220.