Clusterin Overexpression is Responsible for the Anti-apoptosis Effect in a Mouse Neuroblastoma Cell Line, B103

Kwan-Hee You[a], Young-Mi Ji[a], and O-Yu Kwon[b,*]

[a] Department of Biology, College of Natural Sciences, Chungnam National University, Taejon 305–764, Korea

[b] Department of Anatomy, College of Medicine, Chungnam National University, Taejon 301–747, Korea. Fax: 82-42-586-480. E-mail: oykwon@cnu.ac.kr

* Author for correspondence and reprint requests

Z. Naturforsch. **58 c**, 148–151 (2003);
received July 29/October 11, 2002

The functional role of clusterin in apoptosis was examined using flow cytometry. Clusterin cDNA was transfected into the mouse neuroblastoma cell line, B103, in order to determine if clusterin overexpression inhibits apoptosis. The increased clusterin expression level in the B103 cells tended to suppress the apoptotic index. This suggests an association of clusterin gene expression with apoptosis inhibition. These results support the conclusion that clusterin expression in B103 cells has an anti-apoptotic influence.

Key words: Clusterin, Apoptosis, Flow Cytometry

Introduction

Clusterin, which was first described as a secreted glycoprotein, is present in the rete testis fluid of rams. It was reported to elicit erythrocyte and Sertoli cell aggregation *in vitro* (Kissinger *et al.*, 1982). Although many homologs have been discovered in other species, its biological function is unclear. Clusterins isolated from different species have been assigned different names such as SGP-2 (sulfated glycoprotein-2), GP III (glycoprotein III), TRPM-2 (testosterone repressed message-2), CL I (cytolysis inhibitor) and Apo J (apoprotein J) (Rosenberg and Silkensen, 1995). However, since 1992, clusterin has been the name generally used (Jenne and Tschopp, 1992). Clusterin is a disulfide-linked heterodimeric glycoprotein (75 ~ 80 kDa) with 30% of its mass being a N-linked carbohydrate. Each form of clusterin consists of two 40 kDa subunits. It is encoded by a single gene and the translated product is internally cleaved to produce its α and β subunit prior to its secretion from the cell (Wilson and Easterbrook-Smith, 2000).

Early studies on the function of clusterin focused mainly on the reproductive tracts, the rete testis and the cauda epididymal fluids (Mattmueller and Hinton, 1991). Recent studies have revealed many functions of clusterin such as controlling the cell-cell interactions, regulating apoptosis, lipid transport, as a regulating complement and as a molecular chaperone (Wilson and Easterbrook-Smith, 2000; Humphreys *et al.*, 1999). In recent years, some researchers have suggested that clusterin affects neuronal apoptosis. However, the results vary. In neuronal cells, early studies have shown that clusterin is accumulated in apoptotic dying neurons in the CA1 hippocampal pyramidal cells, and clusterin gene expression is strongly related to apoptosis (Rozovsky *et al.*, 1994). On the other hand, other studies have reported that an exogenously infected SGP-2 gene does not induce apoptosis (D'Mello *et al.*, 1993). The biological function of clusterin associated with apoptosis in neurocytes is unclear. Therefore, as a first step toward understanding the role of clusterin in inducing apoptosis in the mouse neuroblastoma cell line, B103, an apoptotic cell population where exogenous clusterin was overexpressed was analyzed using flow cytometry.

Materials and Methods

The B103 cells derived from mouse neuroblastoma were maintained in Dulbecco's modified Eagle's medium (DMEM) (containing 4.5 g glucose per liter) supplemented with fetal bovine serum (10% final concentration), 2 mM L-glutamine, and penicillin-streptomycin (100 Unit/ml and 100 μg/ml, respectively) in a humidified 37 °C CO_2 incubator until the monolayers reached approximately 90% confluence (Cabral *et al.*, 1987). The B103 cells were grown on 6-well plates to a 50% confluence. The cells were then transfected with 0.5 μg of the different recombinant cDNAs (mouse clusterin and human Bcl-2) and 5 μg of lipofectin (Gibco BRL, CA), which were diluted separately in 100 μl Opti-Mem I (Gibco BRL), mixed for 15 min, added to cells, and removed the following day. The cells were subsequently transferred onto 10-cm plates, cultured for 48 h, and selected for 3 weeks with G418 (Geneticin)

(400 μg/ml). The resulting clones that showed a high expression level were tested by Northern blotting with a ^{32}P-labeled mouse clusterin cDNA probe for clusterin and by Western blotting with mouse anti-human antibodies for Bcl-2. Both methods were performed according to Sambrook's Molecular Cloning (Sambrook *et al.*, 1989). Unless otherwise stated, all other materials used in this study were purchased from Sigma Chemical Co. (St. Louis, MO). After treating the wild type cells, the clusterin infected cells and the Bcl-2 infected cells with various H_2O_2 concentrations, they were washed twice in cold PBS and fixed in a 70% ethanol solution in PBS on ice. The cells were then pelleted with RNase A (0.1 ng/ml) for 30 min at 37 °C, and then stained with a hypotonic fluorochrome solution containing 50 μg/ml propidium iodine. The cell cycle analysis of the B103 cells was measured by flow cytometry using a FACSort instrument (Becton Dickinson, NJ).

Results and Discussion

As shown in Fig. 1A, the clones that transfected and overexpressed clusterin gene were selected by Northern blotting using the ^{32}P-labeled mouse clusterin cDNA probe. As a control, the Bcl-2 overexpression stable transfectant was also analyzed by Western blotting with mouse anti-human Bcl-2 antibodies (Fig. 1B). Both clones were transfected and overexpressed by the infected genes, clusterin and Bcl-2, and were used in the following experiments on the H_2O_2 induced apoptosis. However, the possibility that clusterin overexpression enhances Bcl-2 expression, which also exhibits anti-apoptotic activity, was not ruled out, since the changes in Bcl-2 expression as a result of clusterin overexpression were the focus of this study. As shown in Fig. 1C, no significant amount of Bcl-2 expression was detected during clusterin overexpression. In addition, no up-regulated clusterin expression was detected during Bcl-2 overexpression (Fig. 1D). This suggests that stable clusterin overexpression is not directly involved in Bcl-2 expression.

The rate of the apoptotic cell population was analyzed by flow cytometry. As shown in Fig. 2, treatment of the wild type cells with various H_2O_2 concentrations (1; untreated, 2; 0.5 mM, 3; 1 mM and 4; 2 mM) enhanced the subdiploid G0 (Gap 0 phage)/G1 (Gap 1 phage) population in the cell

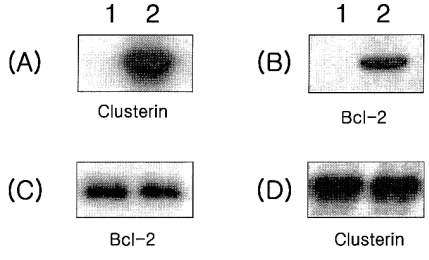

Fig. 1. Overexpression analysis of clusterin and Bcl-2. The cells were transfected with 0.5 μg of the different recombinant cDNAs [mouse clusterin (A, C) and human Bcl-2 (B, D)] and 5 μg lipofectin (Gibco BRL, CA). The resulting clones that showed a high expression level were selected with G418 (400 μg/ml) and tested using Northern blotting with a ^{32}P-labeled mouse clusterin cDNA probe for clusterin (A, D) and Western blotting with mouse anti-human antibodies for Bcl-2 (B, C). Lane 1; parental B103 cells, lane 2; transfected cells.

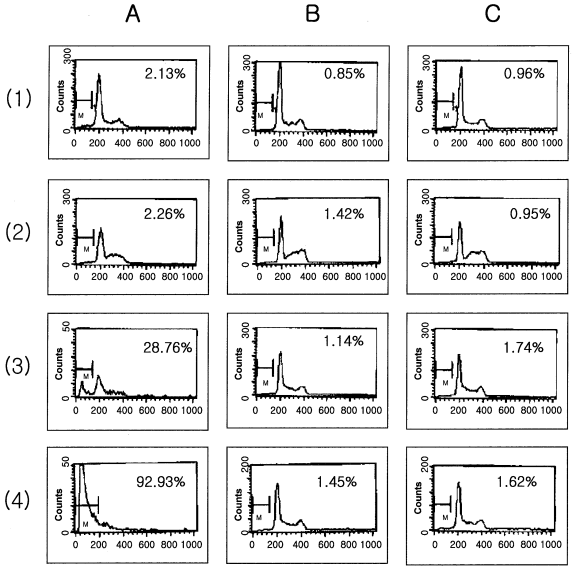

Fig. 2. Flow cytometric analysis. Cell cycle analysis of the B103 cells after treating the wild type cells (A), the clusterin infected cells (B) and the Bcl-2 infected cells (C) with various H_2O_2 concentrations (untreated, 0.5 mM, 1 mM, and 2 mM; indicated vertically in Fig. by 1–4) for 12 h, respectively. The distribution of cells in the cell cycle phases was measured using a FACSort instrument (Becton Dickinson, NJ). The vertical axis represents the relative number of events and the horizontal axis the fluorescence intensity. The indicated percentages represent the subdiploid population of the cells and M indicates mitosis phage, eventually that means apoptosis index.

cycle after 12 h of exposure, and reached approximately 2% 29% and 93% of the total cells, respectively (A). In contrast, after treatment at the highest H_2O_2 concentration for 12 h, the clusterin transfectant showed a strongly suppressed G0/G1 population of < 2% (B). A similar anti-apoptotic effect of < 2% subdiploid population through the various H_2O_2 concentrations was obtained when the cells were transfected with Bcl-2 (C). Since several studies have demonstrated that Bcl-2 strictly inhibits apoptosis, Bcl-2 was used as a negative control of the apoptotic effect (Liu *et al.*, 2001). These results demonstrate that an overexpressed clusterin is a powerful inhibitor of apoptosis (Fig. 2A). Therefore, clusterin plays a key role in the anti-apoptotic effect in mouse neuroblastoma cells (B103).

The striking relationship between clusterin expression and the onset of apoptosis have raised the hypothesis that clusterin might play a direct role in apoptosis (Norman *et al.*, 1995). The molecular relationships between clusterin and apoptosis are unclear. It has been reported that in non-nerve cells, up-regulated clusterin is involved in human A431 cell and prostate cancer cell apoptosis and the down-regulation of clusterin expression induces apoptosis in Sertoli cells originated from mouse testis (Kalka *et al.*, 2000; Kang *et al.*, 2000; Steinberg *et al.*, 1997). In recent years, some researchers have suggested that clusterin affects neuron apoptosis. However, the results vary. In neuronal cells, early studies have shown that clusterin is accumulated in the apoptotic dying neurons in CA1 hippocampal pyramidal cells, and clusterin gene expression is strongly correlated with apoptosis (Rozovsky *et al.*, 1994). Other studies have shown that an exogenously infected SGP-2 gene, does not induce apoptosis and that ApoJ expression is strongly associated with neuronal apoptosis in the olfactory mucosa (D'Mello *et al.*, 1993; Michel *et al.*, 1997). On the other hand, in the several photoreceptor neurons, clusterin is not causally involved in apoptosis, but may have cytoprotective functions (Jomary *et al.*, 1999). Although the biological function of clusterin associated with apoptosis including a detailed mechanism of apoptosis induction is unclear in neuroblastoma cells, the results in this study do show a close link between the induction of apoptosis and clusterin expression. Therefore, the expression of the clusterin gene may be a useful indicator of the presence of apoptosis in neurons. Furthermore, clusterin overexpression may offer a biochemical therapy through the inhibition of apoptosis in neurons.

Acknowledgments

This work was supported by grant No. R05-2001-000-00464-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

Cabral G. A., McNerney P. J., and Mishkin E. M. (1987), Interaction of delta-9-tetrahydrocannabinol with rat B103 neuroblastoma cells. Arch. Toxicol. **60**, 438–449.

D'Mello S. R., and Galli C. (1993), SGP2, ubiquitin, 14K lectin and RP8 mRNAs are not induced in neuronal apoptosis. Neuroreport **4**, 355–358.

Humphreys D. T., Carver J. A., Easterbrook-Smith S. B., and Wilson M. R. (1999), Clusterin has chaperone-like activity similar to that of small heat shock proteins. J. Biol. Chem. **274**, 6875–6881.

Jenne D. E., and Tschopp J. (1992), Clusterin: the intriguing guises of a widely expressed glycoprotein. Trends Biochem. Sci. **17**, 154–159.

Jomary C., Darrow R. M., Wong P., Organisciak D. T., Neal M. J., and Jones S. E. (1999), Lack of causal relationship between clusterin expression and photoreceptor apoptosis in light-induced retinal degeneration. J. Neurochem. **72**, 1923–1929.

Kalka K., Ahmad N., Criswell T., Boothman D., and Mukhtar H. (2000), Up-regulation of clusterin during phthalocyanine 4 photodynamic therapy-mediated apoptosis of tumor cells and ablation of mouse skin tumors. Cancer Res. **60**, 5984–5987.

Kang S. W., Lim S. W., Choi S. H., Shin K. H., Chun B. G., Park I. S., and Min B. H. (2000), Antisense oligonucleotide of clusterin mRNA induces apoptotic cell death and prevents adhesion of rat ASC-17D Sertoli cells. Mol. Cells **10**, 193–198.

Kissinger C., Skinner M. K., and Griswold M. D. (1982), Analysis of Sertoli cell-secreted proteins by two-dimensional gel electrophoresis. Biol. Reprod. **27**, 233–240.

Liu D., and Huang Z. (2001), Synthetic peptides and non-peptidic molecules as probes of structure and function of Bcl-2 family proteins and modulators of apoptosis. Apoptosis **6**, 453–462.

Mattmueller D. R., and Hinton B. T. (1991), *In vivo* secretion and association of clusterin (SGP-2) in luminal fluid with spermatozoa in the rat testis and epididymis. Mol. Reprod. Dev. **30**, 62–69.

Michel D., Moyse E., Trembleau A., Jourdan F., and Brun G. (1997), Clusterin/ApoJ expression is associated with neuronal apoptosis in the olfactory mucosa of the adult mouse. J. Cell Sci. **110**, 1635–1645.

Norman D. J., Feng L., Cheng S. S., Gubbay J., Chan E., and Heintz N. (1995), The lurcher gene induces apoptotic death in cerebellar Purkinje cells. Development **121**, 1183–1193.

Rosenberg M. E., and Silkensen J. (1995), Clusterin: physiologic and pathophysiologic considerations. Int. J. Biochem. Cell Biol. **27**, 633–645.

Rozovsky I., Morgan T. E., Willoughby D. A., Dugichi-Djordjevich M. M., Pasinetti G. M., Johnson S. A., and Finch C. E. (1994), Selective expression of clusterin (SGP-2) and complement C1qB and C4 during responses to neurotoxins *in vivo* and *in vitro*. Neuroscience **62**, 741–758.

Sambrook J., Fritsch E. F., and Maniatis T. M. (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Press.

Steinberg J., Oyasu R., Lang S., Sintich S., Rademaker A., Lee C., Kozlowski J. M., and Sensibar J. A. (1997), Intracellular levels of SGP-2 (Clusterin) correlate with tumor grade in prostate cancer. Clin. Cancer Res. **3**, 1707–1711.

Wilson M. R., and Easterbrook-Smith S. B. (2000), Clusterin is a secreted mammalian chaperone. Trends Biochem. Sci. **25**, 95–98.

Satz und Druck: AZ Druck und Datentechnik GmbH, Kempten