J. Microbiol. Biotechnol. (2004), **14**(6), 1333–1337

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

The Effect of Overexpression of Rat Clusterin in L929 Fibroblasts

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Received: December 12, 2003
Accepted: February 11, 2004

Abstract Oxidants such as hydrogen peroxide are powerful inducers of cell damage, ageing, and apoptosis. Since clusterin, a 75–80 kDa mammalian glycoprotein, is frequently found to be inducible in apoptotic cells and tissues, this study inquired into whether this would be a protective mechanism against further cell death. The aim was to find out whether overexpression of clusterin could protect cells from oxidant-induced stress and apoptosis. To clarify this issue, we generated and analyzed stable cell lines expressing fusion proteins of a rat clusterin with an enhanced green fluorescent protein (EGFP). When treated with varying concentrations of hydrogen peroxides, clusterin transfectants indeed showed increased resistance to apoptosis and exhibited a much higher survival rate than mock-transfected cells. On the other hand, neither intracellular re-distribution nor local concentration of clusterin-EGFP was observed, which leaves the question open about its anti-apoptotic mechanism. In conclusion, the overexpression of clusterin provides a means for protecting cells against oxidative stress and subsequent cell death.

Key words: Apoptosis, clusterin, green fluorescent protein, oxidants

Although described for the first time around 20 years ago in apoptotic tissues [6], clusterin is still a largely unknown protein. Some putative functions are suggested such as a lipid transport molecule, a regulator of the complement system, or a controller of cell-cell interactions, *etc.* [21], but none of these have been definitively confirmed. Nonetheless, there is a rising consensus that clusterin might act as a heat-shock protein (HSP)-like chaperone that is possibly also involved in apoptotic cell death and in the protection of cells against various stress signals [9, 15]. Indeed, clusterin has been associated for a long time with apoptosis, in that clusterin mRNA expression was shown to be upregulated in apoptotic cells [1, 12], and in that clusterin protein expression was induced after tissue or cell injury and in regressing tissues [2]. Additionally, it was further shown that purified or transfected clusterin was able to protect cells from exogenous stress signals including tumor necrosis factor-α (TNF-α) or transforming growth factor-β (TGF-β)-induced programmed cell death [8, 18]. In reference to these reports, in the present study, it is questioned whether the overexpression of clusterin would also confer resistance to oxidant-induced stress and might possibly counteract the subsequent induction of apoptosis in cultured cells.

The exposure to reactive oxygen species as well as other oxidants represents a major stress factor on the living organism, inducing not only damage or ageing in individual cells but also in lipids, proteins, or DNA [3]. Intrinsic antioxidant compounds such as vitamins C, E, and ubiquinone, as well as antioxidant enzymes like superoxide dismutase and glutathione peroxidase, protect cells when exposed to such hazardous molecules. Nevertheless, a high level of oxidative stress can eventually lead to programmed cell death or even to necrosis [4]. Interestingly, in connection with clusterin, it was previously shown that oxidative stress does result in the induction of clusterin mRNA expression in A431 human epidermoid cells [20]. Furthermore, in that particular study, A431 transfectants expressing an antisense-clusterin construct displayed increased rates of cell death upon exposure to hydrogen peroxide, suggesting that under normal circumstances, clusterin plays a protective role against oxidants. Surprisingly, however, the transfection and overexpression of human clusterin cDNA could not increase the survival rate of oxidants-treated cells. One possible explanation was that endogenous clusterin would already suffice by increasing maximum cell survival so that the effect of any overexpressed clusterin would be

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negligible. Interestingly, there are also other reports where, for example, the addition of purified human clusterin *in vitro* induced the aggregation of LLC-PK1 pig kidney epithelial cells and thereby protected these cells against oxidative stress [18]. Regarding these contradictory reports, it was seen as quite evident that further studies are necessary to get a more clear answer about the possible role of clusterin in oxidative stress. Additionally, it was also necessary to clarify another issue that addresses the cellular re-distribution of clusterin upon stress induction. While clusterin is known primarily as a secreted protein [21], several studies also reported clusterin expression in the cytoplasm and even in the nucleus [8]. The biological function of this intracellular clusterin is not clear and its presence is also not quite in agreement with its putative role as a secreted HSP-like chaperone [21]. In this context, the intracellular re-distribution of clusterin has also been linked to a change in biological function by clusterin. Therefore, it was necessary to examine 1) whether the overexpression of clusterin protects against oxidant-induced apoptosis, and 2) the extent to which such oxidant-induced stress affects the intracellular re-distribution and expression of clusterin. Using L929 fibroblasts that overexpressed rat clusterin which was fused with EGFP, it was possible to determine the effects of oxidants such as hydrogen peroxide on apoptosis and on the intracellular distribution of clusterin.

Firstly, to clarify whether recombinant rat clusterin does protect cells from oxidant-induced cell death, L929 mouse fibrosarcoma cells (ATCC, U.S.A.) were transfected with

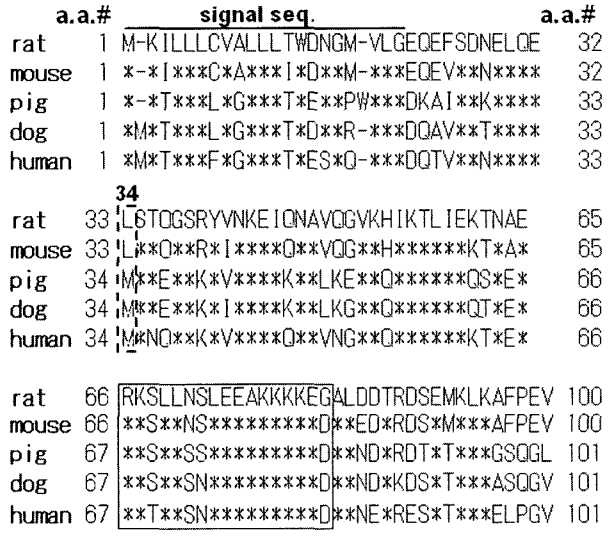

Fig. 1. Alignment and analysis of the primary amino acid sequence of rat clusterin in comparison to that of other species.
The N-terminal amino acid sequences of the clusterin precursor proteins are compared among different species. The first N-terminal 100 amino acid sequences of each species are shown. The predicted signal sequence is underlined, and the putative nuclear localization region is shown in a box. The alternative start codon at a.a. 34 is indicated with a dotted box.

an expression vector that encodes for a fusion protein of EGFP and rat clusterin. The rat clusterin cDNA was cloned using the primer pair 5'-atcgaattcgatgaagattctcctgc-3' (*Eco*RI site is underlined) and 5'-atcggatccttccgcacggcttttcc-3' (*Bam*HI site is underlined). The *Eco*RI/*Bam*HI-digested clusterin PCR fragment was subcloned into the corresponding sites of the pEGFP-N3 vector (Clontech, U.S.A.). The cDNA was placed in a frame at the N-terminal end of the EGFP because the expression of clusterin as a C-terminal fusion protein is known to show a significant loss of its biological activity, at least in the human system [22]. Stable transfectants were selected with neomycin (G418) for more than 2 weeks. Surviving cells were pooled and electronically sorted using a FACSCalibur cell sorter (Becton Dickinson Flow Cytometry Systems, U.S.A.) based on the green fluorescence of the expressed clusterin-EGFP fusion protein transfected cells [11]. Starting from a 5% positively transfected population, clusterin-EGFP positive cells were enriched up to 96% purity. Finally, single clones of the transfectants were obtained by end-point limiting dilution as previously described [14]. The successful expression of transfected proteins was confirmed by RT-PCR using rat clusterin-specific oligonucleotide primers as well as by probing the cell lysate of transfectants using EGFP- and clusterin-specific antibodies in Western blots (data not shown). Figure 2A shows this process in summary, and in all the following studies, only transfectants from 100% clusterin-EGFP positive clones were used. While two different independent clones were generated by these procedures, both clones showed identical characteristics in all experiments.

After establishing stable monoclonal cell lines, the intracellular distribution of the recombinant fusion proteins was analyzed. Although clusterin is known primarily as a secreted protein [9], recent studies have also observed intracellular expression and have shown that there exists a truncated form of human clusterin that specifically accumulates in the nucleus and leads to the induction of apoptosis [16, 22]. This truncated form can supposedly be generated using an alternative translation start codon, which is located 99 nucleotides (or 33 amino acids) downstream of the regular start codon (Fig. 1). Interestingly, while this second start codon is highly conserved in humans (GenBank Accession Number A41386), dog (A40018), and pig (A42108), in the case of the rat, this alternative second start codon is missing. As shown in Fig. 1, the conserved methionine residue (dotted box), which generates the truncated form without a signal peptide for extracellular secretion, is not present in rat nor in mouse clusterin. This intriguing observation led to the question of whether rat or mouse clusterin would also be able to be translocated into the nucleus as in human and other species.

To do this, stable transfectants were harvested the day before analysis and seeded into 6-well plates (NUNC,

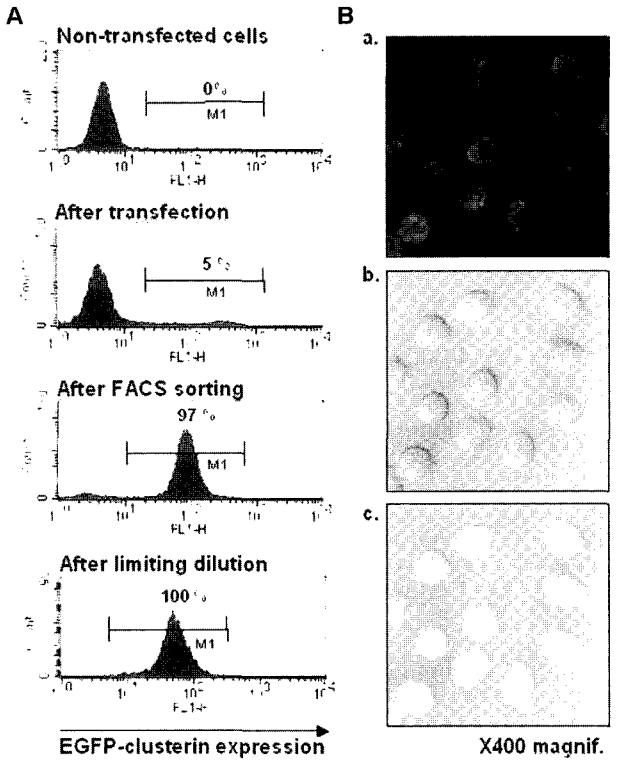

Fig. 2. Analysis of clusterin-transfected L929 cells.
A. Flow cytometric analysis of the selection process for the generation of stable clusterin transfectants. Cells expressing the recombinant clusterin-EGFP fusion proteins in a constitutive manner were enriched and cloned using fluorescence-activated cell sorting and end-point limiting dilution, respectively. The process was monitored by flow cytometry based on the fluorescence signal of the EGFP. B. Confocal laser scanning microscopy of transfected EGFP-clusterin expression. Stable transfected cells were analyzed upon recombinant EGFP-clusterin expression using a confocal laser scanning microscopy. a. Laser-activated fluorescent image of clusterin transfectants (Optical plane is a mid-section through the cells). b. Transmitting light image of the same cells. c. Combined image of Figures a and b.

Denmark) containing sterile coverslips, at a concentration of 4×10^5 cells/ml. Cells were then treated with hydrogen peroxide or other agents as described in the individual experiments. After incubation, the coverslips with the attached cells were carefully taken from the media, washed in PBS, and fixed [10]. Analysis of the coverslips was then performed using a confocal laser scanning microscope system (Leica Lasertech GmbH, Germany). Surprisingly, when analyzing the subcellular localization of clusterin-EGFP fusion proteins by confocal laser scanning microscopy (CLSM), it was observed that clusterin was not only in the cytoplasm but was also expressed in the nucleus (Fig. 2B). Furthermore, since CLSM enables the optical sectioning of the transfectants and thereby indicates the precise intracellular location of the tagged proteins, it was concluded from the readings of mid-optical section through the cells (Fig. 2B) that the recombinant clusterin is expressed throughout the cell without a subcellular localization or local accumulation.

It was then necessary to find in which way the recombinant rat clusterin was directed to the nucleus in the absence of the alternative start codon. To address this issue, the rat clusterin amino acid sequence was analyzed using the PSORT (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences; http://psort.ims.u-tokyo.ac.jp) software, which predicts possible nuclear localization signals (NLS) as well as other sorting and intracellular transport associated motifs of a given protein. As a result of this analysis, it was found that rat clusterin contains a highly conserved NLS, starting from a.a. 66, which corresponds to the sequence "**RKSLLNSLEEAKKKKEG**" (Fig. 1). This NLS motif possibly enables the active transport of this protein into the nucleus. In this study, it was not discovered whether the motif is constitutively exposed and accordingly accessible to the intracellular transport machinery. But obviously, at least for rat clusterin, the generation of a truncated form of clusterin is not a prerequisite for the presence of nuclear clusterin. Another interesting aspect of the nuclear rat clusterin is that its expression in the nucleus is neither cytotoxic nor induces apoptosis in the transfected overexpressing cell. This is in contrast to the human nuclear clusterin [22], and currently, it can only be speculated as to whether the presence of rat clusterin in the nucleus and its causing of no further hazardous effect is an observation specific to rats, or whether only the human nuclear clusterin is an apoptosis-inducing molecule.

After the phenotypic characterization of a rat clusterin transfected cell line, it was investigated whether clusterin overexpression would further protect cells from oxidant-induced programmed cell death. As shown in Fig. 3A, when treating both clusterin-transfected and mock vector-transfected cells with increasing concentrations of hydrogen peroxide, clusterin transfected cells exhibited a much better survival rate than mock-transfected cells. Cell viability was determined by the trypan blue exclusion method as previously described [19], where cells were resuspended in a 0.04% (end concentration) trypan blue solution in PBS, and counted after 1 min incubation using an improved Neubauer-type hemocytometer (Sigma) under a microscope. Only cells without intracellular trypan blue staining were counted as alive, and these were counted and the ratio of live cells determined by comparison with the overall cell numbers. As described previously, an increasing concentration of hydrogen peroxide represents a stress condition that commits cells to programmed cell death, but in this experiment, in the presence of clusterin overexpression, individual cells showed a higher resistance to stress as well as to the induction of apoptosis.

To further confirm and extend the effect of clusterin overexpression, the proliferation rate of clusterin transfected cells under oxidants-treated conditions was determined by employing the MTT method as previously described

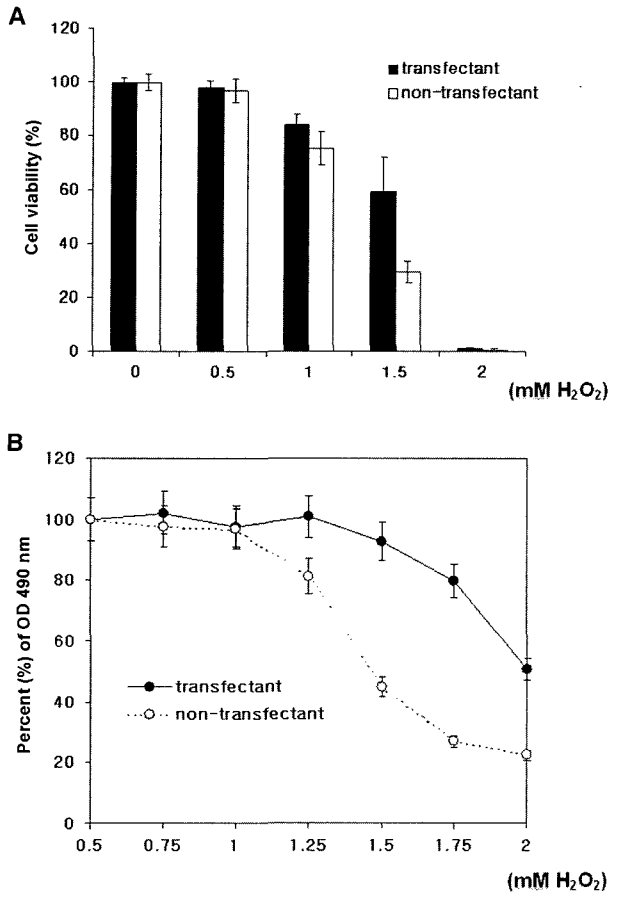

Fig. 3. Determination of cell viability in clusterin-transfected and mock-transfected cells after induction of apoptosis.
A. Cell viability was determined by trypan blue exclusion after overnight incubation with hydrogen peroxide-supplemented media in concentrations as indicated. B. Analysis of the relative proliferation of clusterin- or mock-transfected cells after incubation with hydrogen peroxide using the MTT-assay. Results are shown as means +/- S.D. from three independently performed experiments.

[13]. Again, clusterin overexpressing cells showed a much higher activity than the control group when both groups of cells were exposed to oxidant-induced stress conditions (Fig. 3B). This shows that clusterin-overexpressing cells are not only more viable but also actively proliferate and are physiologically active. In conclusion, it is evident that clusterin overexpression confers resistance to oxidant-induced apoptosis. Why there is only a rather narrow range of hydrogen peroxide concentration (1 mM- 1.5 mM) in which rat clusterin can confer resistance is currently not clear. A possibility is that endogenous clusterin acts as a buffer in lower concentrations of oxidants, while in higher concentrations of peroxide, the overexpressed clusterin shows the additional effect of cytoprotection. This possibility could be tested when genetically engineered clusterin-deficient mice [7] are available. Consequently, in the case of using clusterin-deficient cells from such animals, one would expect to see that overexpressed clusterin performed a protective role in a much wider range of hydrogen peroxide concentrations.

The mechanism by which clusterin actually protects the cells from reactive oxygen species is not known. Since clusterin-transfected cells exhibited no cell clumping or aggregation properties (Fig. 2B), a simple mechanical explanation such as that due to aggregation, during which only the cells at the outer area of a cell clump are exposed to the oxidants, is not applicable. Recent reports that propose clusterin as an extracellular chaperone might provide some hints about an effect of clusterin in neutralizing or stabilizing/recovering proteins that have been damaged by oxidants. However, how such a property would inhibit apoptosis signaling that is induced by oxidants can only be speculated. Interestingly, in the system observed in this study, the treatment with hydrogen peroxide did not involve an intracellular re-distribution and/or local accumulation of the recombinantly expressed clusterin. Nuclear accumulation, however, has been observed in other studies [22] when using other apoptosis-inducing factors such as TGF-β or TNF-α [17]. To reproduce these observations, rat clusterin transfected cells were treated with recombinant TGF-β, or as a control with fibroblast growth factors (FGF) which has no stress-inducing effect on L929 cells, and the intracellular distribution of the EGFP-clusterin was examined afterwards. Similar to the oxidant-induced experiment, no specific relocalization of EGFP-clusterin was observed under these conditions, and the evenly distributed expression of rat clusterin was preserved (data not shown). Thus, at least for rat clusterin, the various cellular stress conditions do not seem to act as extracellular signals that will lead to a selective accumulation in the nucleus. Rather, it seems that in the absence of any particular intracellular re-distribution, rat clusterin is able to neutralize the triggering signals for apoptosis by oxidant, because it is already present in the nucleus.

The discrepancy of such physiological functions between rat and human clusterin could possibly be accounted for by the difference in their N-terminal primary structure (Fig. 1). Accordingly, single amino acid exchange or deletion mutants of the rat or human clusterin precursor protein will provide some explanations about this observation in future studies. Encouragingly, some newer studies have also been reported to have similar cell protective effects with recombinantly expressed human clusterin [5], including the prevention of premature senescence after ter-butylhydroperoxide (t-BHP) or ethanol treatment in human fibroblasts, so that the protective effect of clusterin seems to be extendable to other species. It can be concluded that understanding the mechanism by which clusterin overexpression can protect cells from such reactive oxygen species will provide further clues to the development of biological reagents, and procedures for repairing or

protecting cell and tissue damage induced by hazardous oxidants. In particular, such findings will bring in new tools for the generation of preservatives or stabilizer proteins in medical, cosmetic, or other biotechnological cell and protein products.

Acknowledgment

This work was supported by grant No. R01-2003-000-10762-0 from the Basic Research Program of the Korea Science and Engineering Foundation.

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