Up-regulation of clusterin (sulfated glycoprotein-2) in pancreatic islet cells upon streptozotocin injection to rats

I-S Park, Y-Z Che, M Bendayan1, S-W Kang2 and B-H Min2

Department of Anatomy, College of Medicine, Inha University, Inchon, 402–751, Korea
1Department of Pathology and Cell Biology, Faculty of Medicine, University of Montreal, Quebec, Canada H3C 3J7
2Department of Pharmacology, College of Medicine, Korea University, Seoul, 136–705, Korea

(Requests for offprints should be addressed to B-H Min, Department of Pharmacology, College of Medicine, Korea University, 5Ga 126–1, Anam-dong, Sungbuk-ku, Seoul, 136–705, Korea)

Abstract

Clusterin is a heterodimeric glycoprotein which has been shown to play important roles in programmed cell death and/or in tissue reorganization not only during embryonic development but also in damaged tissues. Recently, we reported the transient induction of clusterin in pancreatic endocrine cells during early developmental stages of islet formation. In the present study, we have investigated the expression of clusterin in pancreatic tissue of streptozotocin-treated rats which were undergoing extensive islet tissue reorganization due to degeneration of insulin ß cells. Clusterin was found in endocrine cells identified as glucagon-secreting α cells at the periphery of the islet. Using immunoelectron microscopy, clusterin-positive cells showed the typical ultrastructural features of pancreatic α cells. In addition, colocalization of clusterin and glucagon in the same secretory granules was shown by double immunogold labeling. These results imply that clusterin is a secretory molecule having endocrine and/or paracrine actions in parallel with glucagon. Further, we noted that clusterin expression was increased in pancreatic α cells during the process of ß cell death upon streptozotocin injection. The increase was significant as early as 1–3 h after streptozotocin treatment prior to any morphological alteration of islet β cell and any manifestation of hyperglycemia. The expression of clusterin was steadily up-regulated during the process of islet reorganization caused by streptozotocin-induced cytotoxic injury. Therefore, we suggest that clusterin might be considered as a molecule induced by both embryonic development and drug-induced reorganization of the endocrine pancreas. Since clusterin expression is up-regulated in α cells, but not in β cells undergoing degeneration, it may play a protective role against the cytotoxic insult.

Introduction

Clusterin or sulfated glycoprotein-2 (SGP-2) has been identified as a secretory molecule participating in the process of aggregation and maturation of sperm cells in the reproductive system (Blaschuk et al. 1983, Fritz et al. 1983, Sylvester et al. 1991, Tung et al. 1992). However, more emphasis has been given to its role in the process of programmed cell death/apoptosis, because of its emphasis has been given to its role in the process of programmed cell death/apoptosis, because of its involvement in neuronal apoptosis in lesioned olfactory mucosa, suggesting that clusterin could be directly involved in the process of apoptosis. In contrast, there are also many reports suggesting a protective role of clusterin in tissues bounded by epithelial lining (Griswold et al. 1986, Aronow et al. 1993).

Sensibar et al. (1995) reported that transfection of LNCaP human metastatic prostate adenocarcinoma cells with clusterin antisense results in significant cell death, supporting the concept that clusterin depletion, rather than its expression, is associated with cell death. Humphreys et al. (1997) showed that tumor necrosis factor-α (TNF-α)-induced cytotoxicity was suppressed by over-expression of clusterin in L929 murine fibrosarcoma cells. They suggested that clusterin expression could be a stress-associated response for cell protection rather than an inducer of cell death.

In a previous study, we reported the transient expression of clusterin in the early developmental process of pancreatic islets (Min et al. 1998). Therefore, we hypothesized that clusterin could play a role in the endocrine pancreas undergoing intensive cell reorganization. It is well known that insulin-secreting β cells are selectively vulnerable to autoimmune reaction and to some particular chemicals such as streptozotocin. The aim of the present study was to
examine clusterin expression in pancreatic tissue at cellular
and sub-cellular levels in normal rats, and its modification
in relation to cell injury and cell reorganization after streptozotocin treatment. Using a specific antibody against
clusterin, we have revealed its localization in islet cells of
normal and streptozotocin-injected animals by light and
electron microscopic immunocytochemistry. In addition,
we have analyzed the levels of pancreatic clusterin poly-
peptide and transcript by Western and Northern blotting
respectively.

Materials and Methods

Experimental animals

Adult male rats (Sprague-Dawley, 180–200 g, Daehan
Experimental Animal Research Center, Seoul, Korea)
displaying normal blood glucose levels in the fasting state
were used. A single dose of streptozotocin (Boehringer-
Mannheim, Mannheim, Germany, 60 mg/kg body
weight in 10 mM sodium citrate bu
er and embedded in Epon or
Lowicryl semi-thin sections. The
expression of clusterin antibody and glucagon. For this, the
demonstration of clusterin and glucagon. For this, the
subcellular identification of clusterin in pancreatic tissues, electron
microscopic immunogold labeling was applied to the
ultra-thin sections. After incubation with the clusterin
antibody for 24 h at 4 °C, the grids were rinsed in
phosphate-buffered saline (PBS) and transferred onto a
drop of protein A-gold complex formed by 15 nm gold
particles for 30 min at room temperature (Bendayan
1995). A double immunogold labeling technique
(Bendayan 1982) was performed for the simultaneous
demonstration of clusterin and glucagon. For this, the
ultra-thin sections were mounted on uncoated nickel grids in order to expose the two faces of the sections. One face of the tissue sections was labeled on drops of the clusterin antibody and protein A-gold (particle size=15 nm) as mentioned previously. Then, the opposite face of the sections was incubated with glucagon antibody

Immunocytochemistry

Immunocytochemical staining was carried out at the light
and electron microscope levels using the specific antibodies against clusterin and pancreatic peptide
hormones as described previously (Min et al. 1998). For
light microscopic immunostaining, the avidin-biotin-
peroxidase complex technique (ABC, Hsu et al. 1981) was
carried out on paraffin and Epon semi-thin sections. The
tissue sections were incubated with the diluted primary
antibodies for 48 h at 4 °C. Biotinylated goat anti-rabbit
immunoglobulin and ABC (Vector Labs, Burlingame,
CA, USA) were subsequently applied to the tissue sec-
tions. Thereafter, the immunocytochemical reaction was
detected by diaminobenzidine hydrochloride (Sigma). To
illustrate the co-localization of clusterin with the pancre-
atic hormones at light microscopic level, adjacent
semi-thin sections were immunostained with mouse
monoclonal anti-insulin (BioGenex, San Ramon, CA,
USA), rabbit anti-glucagon (Dako, Carpiteria, CA, USA)
and rabbit anti-clusterin respectively. For the subcellular
identification of clusterin in pancreatic tissues, electron
microscopic immunogold labeling was applied to the
ultra-thin sections. After incubation with the clusterin
antibody for 24 h at 4 °C, the grids were rinsed in
phosphate-buffered saline (PBS) and transferred onto a
drop of protein A-gold complex formed by 15 nm gold
particles for 30 min at room temperature (Bendayan
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Tissue preparation for immunocytochemistry

For light microscopy, tissue samples were fixed with
Bouin’s solution for 12 h, and embedded in paraffin
according to standard procedures. For electron micro-
scopy, small pieces of tissue were fixed in 4% paraformal-
dehyde or 1% glutaraldehyde-4% paraformaldehyde in 0·1 M phosphate buffer and embedded in Epon or
Lowicryl K4M (Bendayan 1995). Thick paraffin (5 µm)
and semi-thin Epon sections (1 µm) were mounted on
poly-L-lysine coated slides. The ultra-thin sections
obtained from Lowicryl embedded tissues were mounted
on nickel grids.

Preparation and assessment of clusterin antibody

Anti-clusterin antibody was raised in rabbit using a
synthetic peptide of the clusterin α-subunit (NH_2-
GDRIDSLMENDRQQS-COOH, amino acids 134–
148) as described previously (Min et al. 1998). The antigen
adsorption experiment was performed to assess the specific-
ity of this clusterin antibody. Optimally diluted antibody
was incubated overnight with an excess amount of syn-
thetic peptide immunogen of clusterin α-subunit (20 µg/
ml). For the control experiments, this preadsorbed anti-
body was substituted for the untreated primary antibody in
the labeling protocol (Min et al. 1998). An immunodot
experiment was performed on a nitrocellulose membrane
in order to confirm the absence of cross-reactivity
between the clusterin antibody and glucagon. Clusterin
and glucagon (Sigma, St Louis, MO, USA) were applied
to a nitrocellulose membrane and incubated overnight
with the anti-clusterin or an anti-glucagon antibody
(Inctar Corp., Stillwater, MN, USA). The reaction signal
was detected by Western blotting.
and transferred to the protein A-gold solution (particle size=5 nm).

Northern blot analysis

Total RNA was isolated from pancreatic tissue of normal and streptozotocin-injected rats by the acid guanidinium thiocyanate and phenol/chloroform extraction method as described previously (Chomczynski & Sacchi 1987). Total RNA samples (10 µg) were size-fractionated in a 1·0% agarose-formaldehyde gel and transferred to a Hybond-N+ nylon membrane (Amersham, Arlington Heights, IL, USA). The membrane was baked at 120 °C for 30 min and prehybridized in hybridization buffer (50% formamide, 1·5 × SSC, 5 × Denhardt’s reagent, 1·0% SDS, 20 mM sodium phosphate pH 7·0, and 100 µg/ml yeast RNA) at 55 °C for 2 h. Rat clusterin cDNA was cloned by reverse transcriptase-PCR and the [α-32P]UTP-labeled cRNA probe was synthesized using T7 RNA polymerase as described previously (Min et al. 1998). Following hybridization with [α-32P]UTP-labeled clusterin riboprobe (2 × 10⁶ c.p.m./ml) at 55 °C for 16 h, the membrane was washed twice in 0·5 × SSC and 0·1% SDS for 30 min, and more stringently in 0·2 × SSC and 0·1% SDS at 65 °C for 30–45 min. The membrane was then exposed to X-ray film with an intensifying screen at −80 °C for 2 days.

Western blot analysis

Protein samples were extracted from pancreatic tissue of normal and streptozotocin-injected rats as described previously (Min et al. 1998). Protein samples (50 µg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Richmond, CA, USA). The membrane was blocked with 1% blocking solution for 1 h and incubated with the anti-clusterin antibody (1:5000 dilution) in 0·5% blocking solution for 24 h at 4 °C. The blot was then incubated with horseradish peroxidase conjugated to an anti-rabbit immunoglobulin G in 0·5% blocking solution. Detection was carried out using the BM Chemiluminescence Western Blotting Kit (Boehringer Mannheim).

Results

In the present study, we evaluated the expression of clusterin in pancreatic tissue of normal rats and of rats undergoing islet cell reorganization induced by streptozotocin. Changes in clusterin expression were examined by immunocytochemistry, Western and Northern blot analysis using a specific antibody and a cRNA probe for clusterin. The control experiments demonstrated that the anti-clusterin antibody used in the present study reacts specifically with its corresponding clusterin peptide, and has no cross-immunoreactivity with other islet peptides including glucagon (Fig. 1a) which turned out to be colocalized with clusterin in the same cells. In addition, no positive immunoreaction was observed when the tissue sections were incubated with the clusterin antibody preadsorbed with its corresponding synthetic antigen. Magnification of b: × 200.

Cellular localization of clusterin

Clusterin was found mainly in the endocrine portion of the pancreas, the islets of Langerhans, in normal as well as in streptozotocin-treated rats. Even though few clusterin-positive cells were localized in the epithelial lining of the pancreatic ducts, clusterin was not detected in the acinar cells, or in the central portion of the islet. In the pancreas of normal rats, a weak immunoreaction for clusterin was found in endocrine cells displaying a distribution similar to that seen in the islets of Langerhans.
that of glucagon-secreting α cells at the periphery of the islets. The consecutive semi-thin section staining experiment revealed that clusterin cells also demonstrated glucagon immunoreactivity, whereas the insulin cells lacked clusterin immunoreactivity (Fig. 2).

**Ultrastructural localization of clusterin**

To determine subcellular localization of clusterin, protein A-immunogold labeling was performed on the Lowicryl embedded pancreatic tissues. Clusterin antigenic sites
were detected mainly in the secretory granules of α cells displaying high electron density with round profile. No labeling was found in insulin-secreting β cells, or in somatostatin δ cells. Double immunogold labeling demonstrated the colocalization of clusterin (15 nm in gold particle size) with glucagon (5 nm) in the same granules, even though some granules were labeled for clusterin or glucagon only (Fig. 3).

Changes in clusterin expression

By means of Northern blot analysis, we observed higher expression of clusterin transcripts in the pancreas of the streptozotocin-treated rats, while little expression was detected in the pancreas of the normal rats. The level of clusterin mRNA was significantly increased as early as 3 h after streptozotocin injection, prior to any morphological changes of islet cells or hyperglycemia. It reached about fivefold induction at 72 h after streptozotocin treatment (Fig. 4A). A 70 kDa clusterin polypeptide was detected by Western blot analysis using the specific antibody against the synthetic peptide of clusterin α-subunit. This pancreatic protein was considerably increased in the streptozotocin-treated rats compared with the normal rats, in accordance with clusterin mRNA expression (Fig. 4B).

Changes in blood glucose levels

Blood glucose levels were examined at 1, 2, 3, 6, 12, 24, 48 and 72 h after streptozotocin injection, and the results demonstrated that rats maintain their normal level (80–150 mg/dl) for at least 12 h after streptozotocin injection. A steep increase in blood glucose (>300 mg/dl) took place in the streptozotocin-treated rats from 12 to 24 h after streptozotocin injection, and persisted thereafter.

Histopathological changes of the islets

Streptozotocin treatment led to cellular alterations from 6 h after injection. At this stage, the outlines of the islets became irregular, and many islet β cells showed condensation of their nuclear chromatin. At 12–24 h after treatment, histopathological changes of the islets were evident with karyolysis and dissociation of the intercellular junctions in most β cells. However, at 72 h after treatment, the remaining islet cells showed a normal appearance. As islet cells underwent progressive cell death, the volume of the islets gradually decreased until 48 h after streptozotocin injection.

Changes in clusterin immunoreactivity

Our time course experiments after streptozotocin injection demonstrated that an increase in clusterin immunoreactivity preceded degenerative changes of the islets and increased blood glucose levels (Fig. 5). A considerable increase in clusterin expression was seen in the peripheral α cells of the islets as early as 1 and 3 h after streptozotocin treatment. For some islets this was also evident at 6 and 12 h after treatment in spite of an extensive cellular degeneration in their central portion. In particular, the α cells occupied most of the islet 7 days after streptozotocin treatment (data not shown) and displayed more intense immunoreactivity for clusterin at 72 h (Fig. 5f). On the other hand, the β cells displayed normal insulin immunoreactivity in the central portion of the islets until initiation of their degeneration 6 h after streptozotocin treatment. From that time on, however, a marked degenerative change took place in the β cells in parallel with a decrease in insulin immunoreactivity. Many β cells were lost due to cell death at 12–24 h, and no proliferation of β cells was observed (Fig. 6). In contrast, streptozotocin treatment did not seem to affect somatostatin cells. In the late stage of the experiment, somatostatin cells were found between clusterin-positive cells.

Discussion

In our previous study, we found a transient high expression of clusterin in pancreatic endocrine cells during
pre- and perinatal stages (Min et al. 1998). In the process of pancreatic development, it was suggested that clusterin is an important molecule in the overall elaboration of the pancreatic islets of Langerhans. Although clusterin was down-regulated after formation of the islets in the postnatal period, we observed that low levels of clusterin were steadily expressed in some islet cells. Thus, we speculated that clusterin in islet cells of the adult pancreas could serve as a molecule responding to various cellular insults. Islet β cells are susceptible to chemical and autoimmune insults, and destruction or impairment of insulin secretion leads to insulin-dependent diabetes mellitus (Portha et al. 1974, Crisa et al. 1992, Atkinson et al. 1994). One of the reasons for such susceptibility could be the absence of clusterin in these cells. We could hypothesize that clusterin might be involved in the biological processes of islet cell death and/or reorganization upon streptozotocin injection, since this protein has been shown to be involved in cell death (Buttyan et al. 1989, Jenne & Tschopp 1989, Danik et al. 1991), as well as in cell protection and tissue

Figure 5 Clusterin immunoreactivity in the pancreatic islet of normal (a), and streptozotocin-treated rats at 1 (b), 3 (c), 6 (d), 12 (e) and 72 h (f) after treatment. As compared with normal rat (arrow in a), a considerable increase in clusterin immunoreactivity is seen as early as at 1 and 3 h after treatment (arrows in b and c), and lasted during the experimental period (arrows in b–f), even though some islet cells showed distinct degenerative changes with condensation of chromatin (arrowheads in d) and dissociation of cell-to-cell adhesion (asterisk in e) at 6 and 12 h after treatment. At 72 h after treatment, numerous cells demonstrated high clusterin immunoreaction (arrows in f), and encompassed clusters of islet cells showing no degenerative change (asterisk in f). Magnification × 400.

As early as 1 to 3 h after streptozotocin treatment, a remarkable increase in clusterin was registered in pancreatic islets. This time point precedes alterations of islet cell structure and the raising of blood glucose levels. It coincides with the reported prompt up-regulation of clusterin in rat thymocytes undergoing dexamethasone-induced cell death prior to DNA fragmentation (Bettuzzi et al. 1991). As such, high expression or accumulation of clusterin might be taken as an early indication of tissue injury. Our data, however, showed that the prompt up-regulation of clusterin occurred in $\alpha$ cells at the periphery, and not in the $\beta$ cells in the central region of the islet of the streptozotocin-treated rats. Higher expression of clusterin in cells surviving the drug insult indicates that this protein may exert a protective action, rather than leading to cell death. This is supported by the fact that clusterin expression has not been correlated with the programmed or teratogen-induced cell death in the post-implantation rat embryo (Little & Mirkes 1995). Sensibar et al. (1995) also reported that clusterin depletion by antisense oligonucleotide, rather than its expression, was associated with cell death in LNCaP cells, an androgen-sensitive human prostatic cancer cell line. Moreover, they revealed that stable transfection and subsequent overexpression of clusterin resulted in resistance to the

Figure 6 Insulin immunoreactivity in the pancreatic islets of normal (a), and streptozotocin-treated rats at 1 (b), 3 (c), 6 (d), 24 (e) and 72 h (f) after treatment. The $\beta$ cells displayed normal insulin immunoreactivity in the central portion of the islets until 6 h after streptozotocin treatment (a–d), whereas decreased immunoreactivity for insulin with marked degenerative changes were seen at 12–24 h after streptozotocin treatment. After degradation of most of the $\beta$ cells, a few insulin cells presenting relatively higher immunoreactivity (arrow in f) were observed in the central region of the islet at 72 h after streptozotocin treatment. Magnification $\times$ 200.
cytotoxic effect of TNF-α in LNCaP cells. They have provided evidence that clusterin does indeed play a role in preventing cell death.

Cells exposed to a harsh environment are likely to require substances displaying hydrophobicity, strong propensity to aggregation and promoting interaction with other molecules (Tsuruta et al. 1990, Jenne et al. 1991, Jenne & Tschopp 1992). The cyto-protecting effect of clusterin seems to be associated with its biochemical properties and physiological nature. Clusterin has a highly acidic isoelectric point (3·6), is highly glycosylated, hydrophobic and aggregates easily with itself to form dimers and tetramers (Blaschuk et al. 1983). Carbohydrates in clusterin molecules seem to be important moieties in eliciting cell–cell interactions which are mediated via carbohydrate-binding protein located on the plasma membrane, since deglycosylation of clusterin renders this protein unable to elicit cell aggregation (Cheng et al. 1988). In addition, Aronow et al. (1993) suggested that clusterin could neutralize surface active hydrophobic or other deleterious compounds by formation of soluble complexes for protecting cellular membranes exposed to potentially toxic hydrophobic agents. In pancreatic tissue, an increased secretion of clusterin from pancreatic α cells could enhance cell–cell interaction and membrane stability among neighboring cells, and could prevent them from being dissociated. It may account for the undamaged cell adhesion properties between clusterin-containing α cells in spite of streptozotocin treatment, whereas clusterin-lacking β cells are prone to intercellular dissociation, an initial feature in the onset of cell death. Therefore, we suggest that enhanced cell–cell interactions by secreted clusterin primarily protect α cells. In addition, clusterin has been shown to be a potent inhibitor of the terminal complement cascade. It suppresses cytolytic potential of complement membrane-attack complex by binding to one of the lytic complement complex, C5b–9 (Hansch 1992, May & Finch 1992). In this manner, clusterin secreted from islet cells could also protect them from cytolytic attack by complement complex, as clusterin in body fluids stabilizes epithelial cell membranes at diverse fluid–tissue interfaces (Jenne & Tschopp 1989, Aronow et al. 1993).

Clusterin appeared, however, not to provide such cytoprotective effects for insulin secreting β cells in the central part of the islets. Owing to the intact barrier of cell junctions between α cells, the clusterin molecules secreted into the intercellular spaces would cohere around α cells, otherwise clusterin reaches the nearby capillaries connected with the effenter vessels which do not pass across the β cell area. A few β cells, which we could assume have direct contact with clusterin secreting cells, would survive despite streptozotocin treatment. This is further supported by the fact that most of the somatostatin secreting δ cells, which are usually in contact with α cells, remain intact after streptozotocin-induced injury.

Upon severe damage, tissue reorganization would occur in pancreatic islets. In our experiment, we observed numerous clusterin-positive cells, these representing a major population in the islet 7 days after streptozotocin treatment. At this stage, clusterin cells were no longer confined in the outer mantle area of the islets. This may result from the disappearance of insulin cells, leading to islets mainly composed by glucagon/clusterin cells. Taken together with our recent study demonstrating the role of clusterin in the process of islet formation (Min et al. 1998), these results suggest that clusterin plays an important role in the remodeling of pancreatic islets involving cell–cell aggregation during embryonic development, and preventing cell–to–cell dissociation.

In summary, this study showed that clusterin could play an important cytoprotective role against streptozotocin-induced injury by enhancing its expression in α cells. In early stages of streptozotocin cytotoxicity, an up-regulation of clusterin could keep the α cells aggregated and could enhance their membrane integrity. It may account for the reason why insulin secreting β cells, which are devoid of clusterin, are selectively susceptible to certain types of injury.

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