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

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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 β 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 β 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 steadystately 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.

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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 up-regulation in tissues undergoing degenerative changes in response to hormonal modulation or diseases (Jenne & Tschopp 1992, May & Finch 1992, Tenniswood *et al.* 1992). Recently, Michel *et al.* (1997) have shown that clusterin gene expression is correlated with 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

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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 polypeptide 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 buffer, pH 4·5) was injected into the jugular vein, while only the vehicle buffer was given to control animals. The rats were allowed free access to standard diet and water. No insulin treatment was carried out. Streptozotocin-treated rats were allocated to seven sub-groups according to the periods of time from streptozotocin injection to tissue sampling. After anesthesia with 4% chloral hydrate, whole pancreas was sampled at 1, 2, 3, 6, 12, 24 and 72 h after streptozotocin injection. Each experimental or control group comprised 10 animals. Pancreatic tissues were subjected to immunocytochemistry as well as to Western and Northern blot analysis for clusterin. Blood glucose was tested daily or at the time of tissue sampling on blood collected from the tail vein using the Glucometer with One Touch test strips (Lifescan Inc., Milpitas, CA, USA).

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 microscopy, small pieces of tissue were fixed in 4% paraformaldehyde 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 (NH₂-GDRIDSLMENDRQQS-COOH, amino acids 134–148) as described previously (Min *et al.* 1998). The antigen adsorption experiment was performed to assess the specificity of this clusterin antibody. Optimally diluted antibody was incubated overnight with an excess amount of synthetic peptide immunogen of clusterin α-subunit (20 μg/ml). For the control experiments, this preadsorbed antibody 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 (Incstar Corp., Stillwater, MN, USA). The reaction signal was detected by Western blotting.

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 sections. Thereafter, the immunocytochemical reaction was detected by diaminobenzidine hydrochloride (Sigma). To illustrate the co-localization of clusterin with the pancreatic 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 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

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-^{32}P]UTP-labeled cRNA probe was synthesized using T7 RNA polymerase as described previously (Min *et al.* 1998). Following hybridization with [α-^{32}P]UTP-labeled clusterin riboprobe (2 × 10^6 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 poly-vinylidene 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
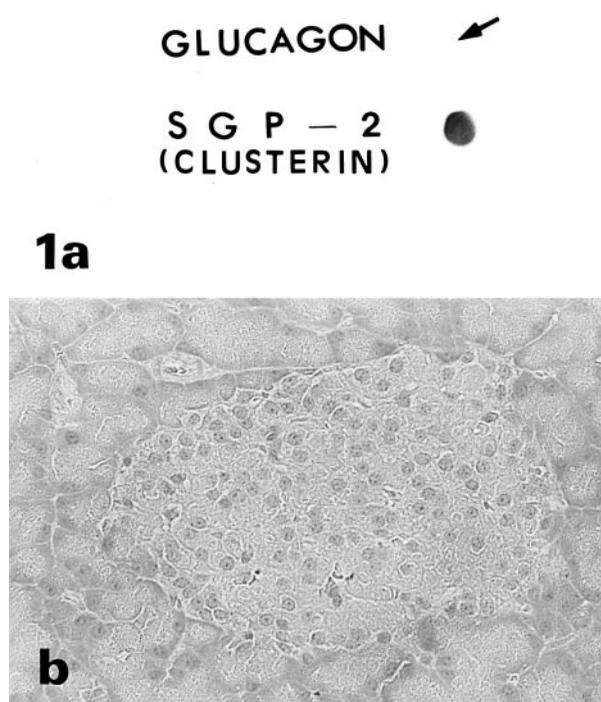

Figure 1 Control experiments for assessing the specificity of the clusterin (SGP-2) antibody. (a) Immunodot experiment to reveal absence of cross-reactivity between clusterin antibody and glucagon. Aliquots of clusterin and glucagon were applied to a nitrocellulose membrane, incubated overnight with the anti-clusterin antibody and revealed according to the method of Western blot. No clusterin immunoreaction was detected at the site of the glucagon antigen (arrow in 1a), while a specific signal was present at the site of the clusterin antigen. For the antigen-adsorption experiment (b), no positive immunoreaction was observed in the pancreatic tissue when the tissue sections were incubated with the clusterin antibody preadsorbed with its corresponding synthetic antigen. Magnification of b: × 200.

specifically with its corresponding clusterin peptide, and has no cross-immunoreactivity with other islet peptides including glucagon (Fig. 1*a*) 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 primary antibody preadsorbed with its corresponding synthetic antigen (Fig. 1*b*).

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
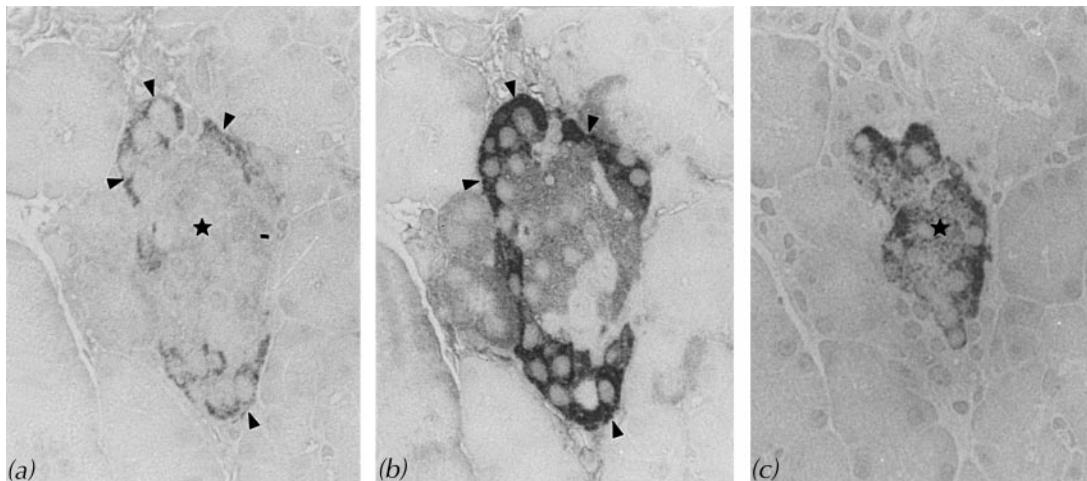
Figure 2 Three adjacent semi-thin sections of pancreatic tissue obtained from a rat 1 h after streptozotocin injection. The sections were immunostained with antibodies against (a) clusterin, (b) glucagon and (c) insulin. Clusterin immunoreactive cells at the periphery of the islet (arrowheads in a) were simultaneously immunoreactive for glucagon (arrowheads in b), while insulin cells (asterisk in c) showed no immunoreaction for clusterin (asterisk in a). Magnification × 400.
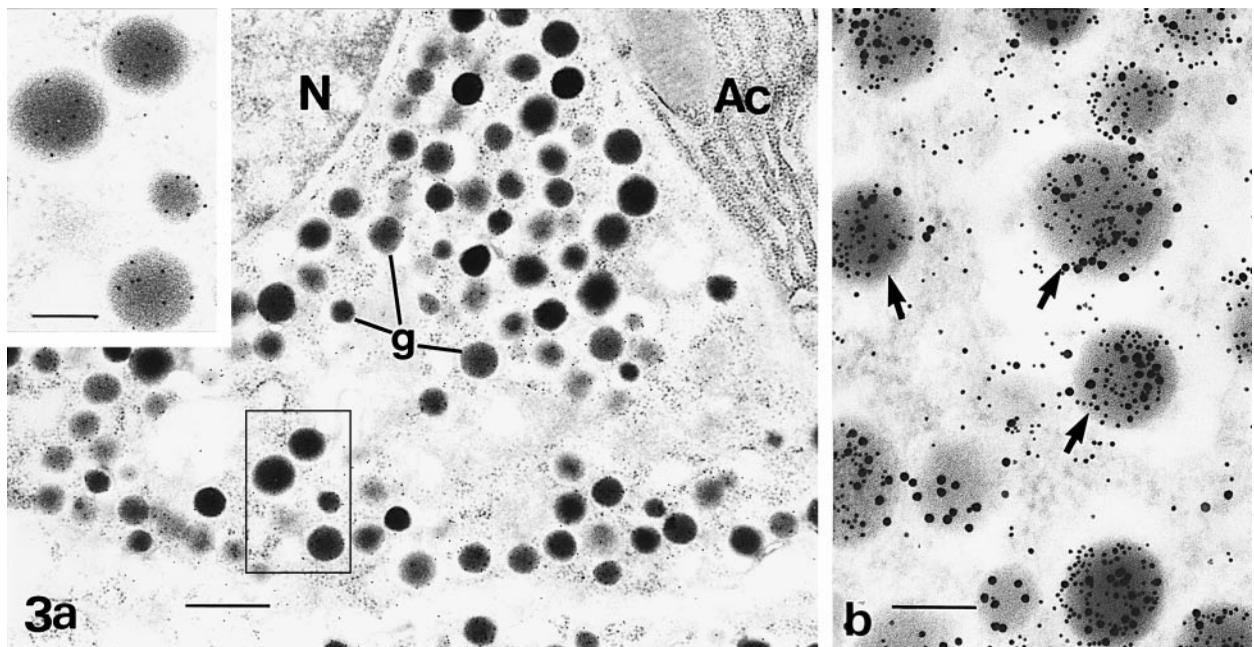

Figure 3 Ultrastructural localization of clusterin in pancreatic islet of normal rat (a), and its co-localization with glucagon in an islet A cell of a streptozotocin-treated rat 1 h after treatment (b). In normal pancreas (a), a typical A cell was seen at the periphery of the islet. The cell showed clusterin/protein A-gold (10 nm) labeling on the secretory granules at a higher magnification (inset, an enlarged representation of the boxed area). Double immunogold labeling of clusterin/protein A-gold (15 nm) and glucagon/protein A-gold (5 nm) revealed the coexistence of clusterin with glucagon in most of the secretory granules (arrows in b). N, nucleus; g, secretory granules; Ac, acinar cell. Scale bar in a=0·5 μm. Scale bars in b and inset=0·2 μm.

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
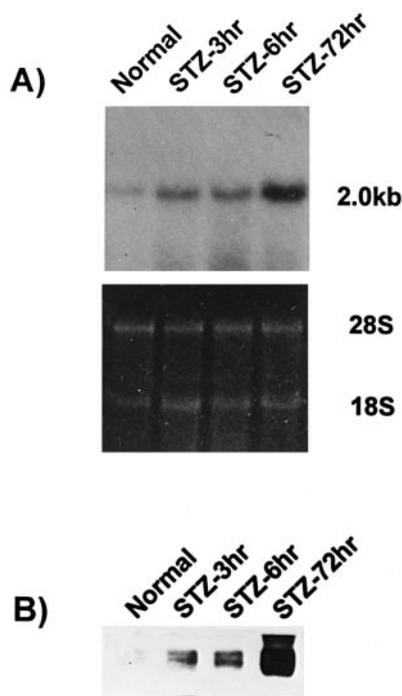
Figure 4 Expression of clusterin in rat pancreas following streptozotocin (STZ) treatment. (A) Northern blot analysis of clusterin mRNA from pancreatic tissues of normal and STZ-treated rats for 3, 6, and 72 h. Ten micrograms total pancreas RNA were loaded in each lane. Equivalent loading of RNA was confirmed by ethidium bromide staining of 18S and 28S rRNA. (B) Western blot analysis of clusterin in pancreas at various time points after injection of STZ.

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. 5*f*). 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
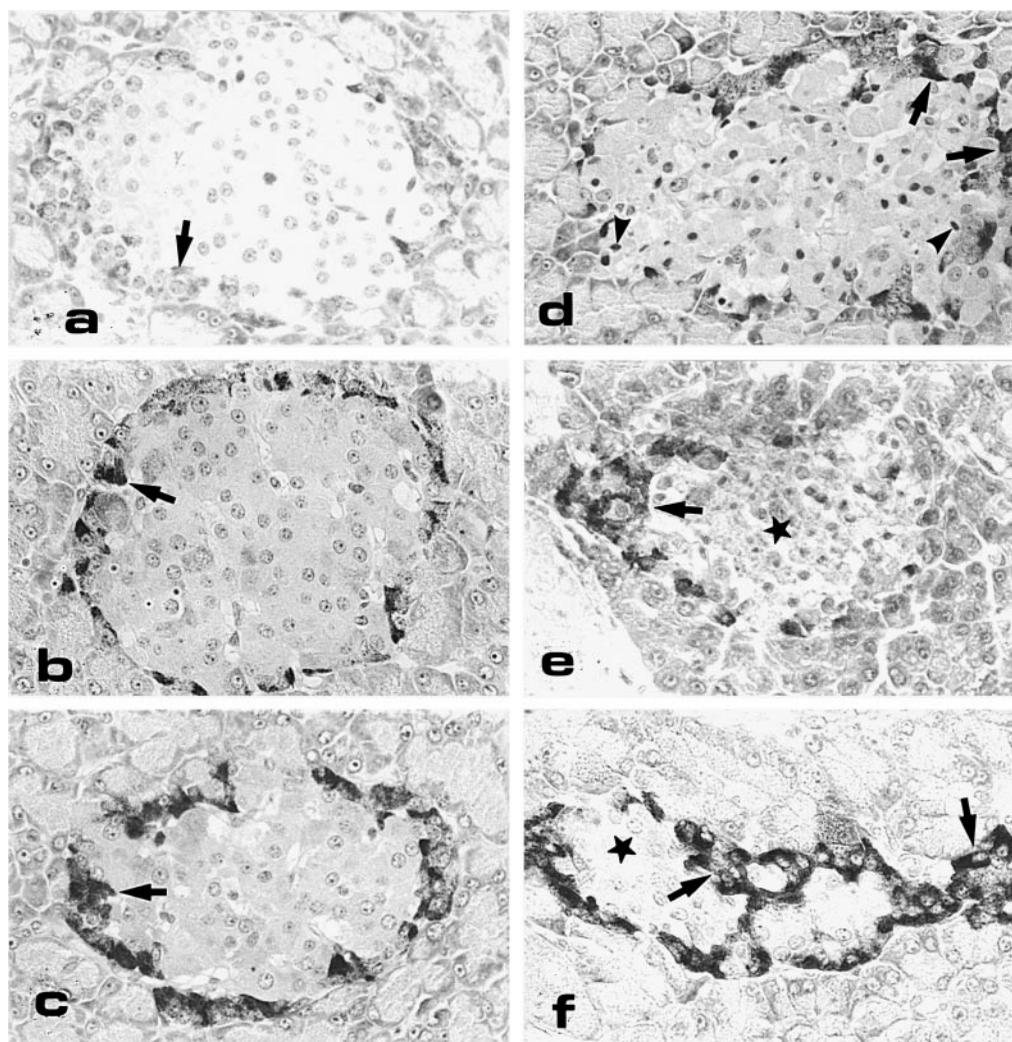
Figure 5 Clusterin immunoreactivity in the pancreatic islet of normal (a), and streptozotocin-treated rats at 1 (b), 3 (c), 6 (d), 12 (e) and 72 h (f) after treatment. As compared with normal rat (arrow in a), a considerable increase in clusterin immunoreactivity is seen as early as at 1 and 3 h after 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.

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 post-natal period, we observed that low levels of clusterin were steadily expressed in some islet α cells. Thus, we specu-lated 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 clus-terin 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
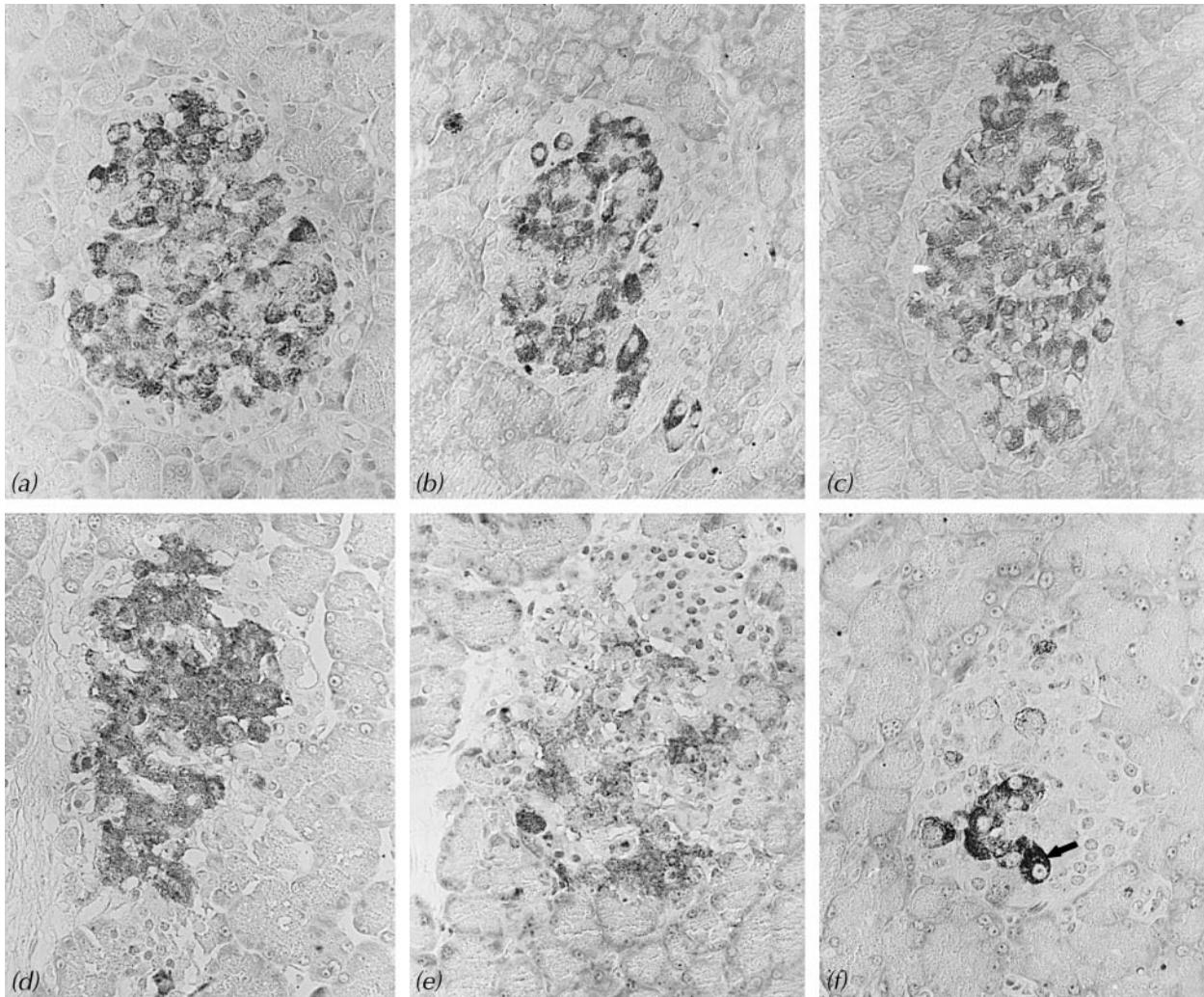
Figure 6 Insulin immunoreactivity in the pancreatic islets of normal (a), and streptozotocin-treated rats at 1 (b), 3 (c), 6 (d), 24 (e) and 72 h (f) after treatment. The β cells displayed normal insulin immunoreactivity in the central portion of the islets until 6 h after streptozotocin treatment (a–d), whereas decreased immunoreactivity for insulin with marked degenerative changes were seen at 12–24 h after streptozotocin treatment. After degradation of most of the β 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 × 200.

remodeling (Harding *et al.* 1991, French *et al.* 1994, Little & Mirkes 1995, Sensibar *et al.* 1995, Humphreys *et al.* 1997, Silkensen *et al.* 1997).

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 α cells at the periphery, and not in the β 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 over-expression of clusterin resulted in resistance to the

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 efferent 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-to-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.

Acknowledgements

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