Clusterin expression during regeneration of pancreatic islet cells in streptozotocin-induced diabetic rats

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Abstract

Aims/hypothesis. Beta-cell regeneration has been reported after islet injury in an animal model for diabetes. Recently, we showed up-regulation of clusterin after islet injury and suggested that clusterin might be involved in cytoprotection and in the regeneration of islet cells. The aim of this study was to investigate the correlation of clusterin expression with islet regeneration and its effect on islet cell replication.

Methods. Streptozotocin was administered to rats to induce various types of diabetes. Islet regeneration and clusterin expression were examined after islet injuries. Clusterin cDNA was transfected to MIN6 cells and their proliferation activity was measured by a [³H]thymidine-incorporation assay.

Results. A diabetogenic dose of streptozotocin injected in rats provoked an immediate degeneration of beta cells. In this model, islets showed increased clusterin expression with extensive proliferation of alpha cells but showed poor beta-cell replication. A subdia-

betogenic dose of streptozotocin, however, led to the proliferation of beta cells with clusterin up-regulation. In streptozotocin-treated neonatal rats, up-regulation of clusterin was noted during beta-cell proliferation. In all experimental models, clusterin was expressed in alpha cells in close correlation with islet cell proliferation, higher transcription of insulin mRNA and MAPKs activation. Cell replication was increased by 31 % in the MIN6 cells transfected by the clusterin cDNA.

Conclusion/interpretation. Up-regulation of clusterin in alpha cells might induce beta-cell proliferation and thus restore their population after islet injury. We suggest that clusterin could be considered as a growth factor-like molecule stimulating islet-cell proliferation by paracrine action. [Diabetologia (2001) 44: 2192–2202]

Keywords Clusterin, pancreas, islet, beta cell, alpha cell, regeneration, diabetes.

Clusterin, a heterodimeric glycoprotein, has been thought to be involved in important biological pro-

cesses, such as apoptosis and tissue remodelling in response to the cytotoxic injuries or to degenerative diseases [1, 2]. The cytoprotective effect of clusterin has been confirmed by studies showing that the protein is expressed in surviving cells, but not in dying cells of various tissues [3–7]. Induction of apoptotic cell death by the depletion of clusterin using antisense oligonucleotide against clusterin mRNA also supports the cytoprotective role of clusterin [8, 9]. It raises the question whether increased concentrations of clusterin could be involved in some biological actions. A recent report [10] showed that clusterin was up-regulated during the differentiation process induced by NGF as well as during proliferation induced

Received: 18 June 2001 and in revised form: 10 August 2001

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Abbreviations: NGF, Nerve growth factor; EGF, epidermal growth factor; TGF, transforming growth factor; AP-1, activator protein 1; PCNA, proliferating cell nuclear antigen; NBT, nitroblue tetrazolium; MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; rCLU, rat clusterin cDNA

by EGF. Another study [11] also showed that clusterin expression is regulated by B-MYB, a transcription factor expressed ubiquitously and involved in the regulation of cell survival, proliferation, and differentiation. We hypothesized that up-regulation of clusterin could induce cell proliferation and/or differentiation during tissue reconstruction after injury. This hypothesis is strongly supported in that clusterin is regulated by growth factors, such as TGF- β , NGF and EGF, and actually binds to the TGF- β receptor [12–17]. Interaction between clusterin and TGF- β receptor implies that clusterin could act as a member of the TGF- β receptor-associated protein regulating cell proliferation and differentiation of a wide variety of cell types [18, 19]. Some growth factors including TGF use the common Ras-extracellular signal-regulated kinase-AP1 signalling pathway to activate clusterin expression for cell proliferation or differentiation [10].

We report the expression of clusterin in the islet cells during development and in the diabetic condition in the pancreas. During development, it was transiently expressed in both alpha and beta cells [20]. Clusterin was also up-regulated in pancreatic cells in response to streptozotocin injection particularly in the surviving alpha cells, which reflected its cytoprotective role. Furthermore, we noted a higher expression of clusterin in islets at the initiation of islet reconstruction upon extensive destruction by streptozotocin treatment [7]. Therefore, besides being involved in cell protection, we postulated that clusterin might participate in islet reorganization during or after the onset of diabetes as a growth factor-like molecule.

Streptozotocin induces experimental diabetes by specific destruction of islet beta cells [21, 22]. Streptozotocin treatment of adult rats leads to Type I (insulin-dependent) diabetes mellitus, whereas Type II (non-insulin-dependent) diabetes mellitus could be induced by its administration to newborn rat pups. In adult rats, a single injection of a diabetogenic dose of streptozotocin provokes a prompt destruction of beta cells, whereas multiple administrations of subdiabetogenic doses can cause insulinitis with marked lymphocyte infiltration [23, 24]. In these models of diabetes, the pancreatic islet is markedly altered and reorganized in different ways according to the injuries induced by streptozotocin [22–25].

We examined clusterin expression in various models of diabetic animals undergoing islet reorganization after streptozotocin treatment. We also confirmed the proliferative action of clusterin on beta cells by transient transfection and over-expression of rat clusterin cDNA.

Materials and methods

Induction of pancreatic tissue reorganization by streptozotocin. Sprague-Dawley rats (Deahan Experimental Animals, Seoul, Korea) were used for induction of diabetes by streptozotocin. Streptozotocin (in 10 mmol/l sodium citrate, pH 4.5, Sigma, St. Louis, Mo., USA) was administered to adult and newborn rats to induce various types of diabetes as well as islet reorganization. To induce Type I diabetes, subdiabetogenic (group 1: 30 mg/kg body weight) and diabetogenic (group 2: 60 mg/kg) doses of streptozotocin were given to adult rats. For group 1, the subdiabetogenic dose was intraperitoneally injected to 5-week-old rats for 5 consecutive days. For group 2, a single intravenous injection of the diabetogenic dose was carried out on the jugular vein of 8-week-old rats. For the animal model of Type II diabetes, forming group 3, an intraperitoneal injection of streptozotocin (50 mg/kg) was given to the neonatal rats, 3 days after birth.

Animal care and tissue sampling. Experimental animals were allowed free access to standard diet and water after streptozotocin treatment, while the newborn rat pups were littered with their mother until weaning. Fasting glucose concentrations were examined in blood sampled at the tail vein using One Touch II Glucometer (Lifescan, Milpitas, Calif., USA). Upon anesthesia, the entire pancreas was sampled from the rats of groups 1 and 3 at 1, 2, 3, 4 and 5 weeks after the streptozotocin injection. For group 2, the pancreas was sampled at 3, 7 and 14 days after streptozotocin treatment. The tissues were fixed with Bouin's solution or 4% paraformaldehyde for immunocytochemistry and in situ hybridization, and embedded in paraffin. For western and northern blot analysis, tissue samples were rapidly frozen in liquid nitrogen.

Detection of apoptotic cell death. The tissue samples were homogenized with lysis solution and incubated at 65°C for 30 min. The lysates were treated with RNase (12 µg/ml) at 37°C and followed by proteinase K (60 µg/ml) at 55°C for 30 min. For agarose gel electrophoresis, 8 µl of extracted DNA was mixed with 2 µl loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol, H₂O). Samples were resolved on 1.8% agarose gel and visualized by ultraviolet light after EtBr staining. To examine the morphological alteration in the islets, including islet cell death and regeneration, the tissue sections were stained with hematoxylin and eosin for light microscopy.

Immunocytochemistry. Immunostaining was carried out by the technique of avidin-biotin-peroxidase complex [26]. The tissue sections were incubated with the goat anti-clusterin antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, Calif., USA) for 24 h at 4°C in a humid chamber. For detection of pancreatic hormones, tissue sections were incubated with mouse anti-insulin (1:1000, BioGenex, San Ramon, Calif., USA), rabbit anti-glucagon (1:2000, Dako, Carpinteria, Calif., USA) and anti-somatostatin (1:1000, Dako, Carpinteria, Calif., USA) for 24 h. After applying the biotinylated goat anti-mouse or goat anti-rabbit and ABC (Vector Labs., Burlingame, Calif., USA), a cytochemical reaction was applied and revealed by diaminobenzidine hydrochloride (Sigma, St. Louis, Mo., USA). To identify the proliferating cells to clusterin immunoreactivity, double immunostaining was carried out on the same sections using a mouse anti-PCNA (1:150, Zymed Lab. San Francisco, Calif., USA) and clusterin antibodies as described previously [20]. After immunostaining for PCNA, the tissue sections were treated for 90 s with vigorous agitation in a solution composed of 0.25% potassium permanganate (1 volume), 5%

H₂SO₄ (1 volume) and distilled water (140 volume) to elute tissue-bound PCNA antibody. The sections were then immunostained with anti-clusterin antibody. The second immunoreaction was detected by nickel and diaminobenzidine substrate providing a black reaction product. In the same manner, clusterin immunolabelled sections were double stained with the mouse anti-insulin or rabbit anti-glucagon antibodies to identify the clusterin-positive cell type.

In-situ hybridization. The de-waxed and rehydrated tissue sections were digested with 150 mmol/l proteinase K (Roche, Mannheim, Germany) for 20 min at 37°C. After acetylation with 100 mmol/l triethanolamine (TEA), pH 8.0 and TEA-acetic anhydride, the sections were hybridized with digoxigenin labelled rat insulin cRNA in hybridization buffer (75% formamide, 2 X SSC, 5 X Denhardt's, 20 mmol/l sodium phosphate, pH 7.0, 1% dextran sulfate, 2 mg/ml yeast tRNA) for 16 h at 55°C. Rat insulin cDNA (220 bp) was cloned by reverse transcription-polymerase chain reaction (RT-PCR) and the cRNA probe was generated using the T7 RNA polymerase. For a control test, some sections were treated with hybridization buffer without the probe. After hybridization, the slides were treated with 40 µg/ml of RNase A for 45 min at 37°C. The detection of the digoxigenin-labelled hybrids was done by incubating the sections with the sheep anti-digoxigenin antibody conjugated with alkaline phosphatase (1:500, Roche, Mannheim, Germany). Revealing reaction was carried out by colour solution, which consisted of NBT and X-phosphate in 100 mmol/l Tris-HCL (pH 9.5).

Western blot analysis. Western blot analysis was done as described previously [7]. Briefly, pancreatic tissue samples were homogenized in lysis buffer (25 mmol/l Tris-HCL, pH 7.4, 1.0 mmol/l EDTA, 0.5% Triton X-100) with a Dounce homogenizer. After measuring the protein concentration using Bradford assay (Bio-Rad, Hercules, Calif., USA), 20 µg of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane in the buffer (39 mmol/l glycine, 48 mmol/l Tris base, 0.037% SDS, 20% methanol, pH 8.3) at 200 mA for 4 h. The membrane was pre-incubated with 1% blocking solution for 1 h at room temperature, and then incubated with rabbit anti-clusterin antibody (1:5000), goat anti-Erk 1, 2 antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and rabbit anti-pErk 1, 2 antibody (1:1000, New England Biolabs, Beverly, Mass., USA) for 24 h at 4°C. Detection was carried out with chemiluminescence substrate for 30 s.

Northern blot analysis. Total cellular RNA was isolated from the pancreatic tissue samples according to previously described protocols [27]. Ten µg of total RNA was separated by 1.0% of agarose gel containing 2.2 mol/l formaldehyde and transferred to a nylon membrane (Roche, Mannheim, Germany). The blot was baked at 120°C for 30 min and prehybridized in hybridization buffer (7% SDS, 50% formamide, 5 X SSC, 2% blocking reagent, 50 mmol/l sodium phosphate, pH 7.0, 0.1% N-lauroylsarcosine) at 60°C for 1 h. Hybridization was done with digoxigenin-labelled rat clusterin cRNA. After hybridization with the probe at 60°C for 16 h, the membrane was washed twice in 0.1 X SSC-0.1% SDS at 65°C for 15 min each and detected with CSPD (Roche, Mannheim, Germany) for 30 min to X-ray film.

Assessment of population of insulin and clusterin positive cells. The number of beta cells and clusterin-positive alpha cells was assessed on the immunocytochemically stained tissue sections by estimating the number of insulin or clusterin immu-

noreactive cells in each islet. All islets present were assessed in random sections obtained from at least five different rats in each experimental group. The actual number of insulin and clusterin cells is probably higher than the number estimated, because only the immunoreactive cells showing their nuclei were counted as positive cells.

Over-expression of clusterin and analysis of [3H]-thymidine incorporation. Clusterin over-expression vector pcDNA3-rCLU was delivered into cells by lipofection. Briefly, solutions for lipofection were made by mixing 10 µg/ml of Lipofectin reagent in Opti-MEM (Life Technologies, Grand Island, N. Y., USA) media with 1.5 µg of plasmid DNA and the mixture was incubated at room temperature for 15 min. Mouse insulinoma cells (MIN6) were transfected with lipofectin-plasmid mixture for 12 h and washed once with serum-free media and cultured in complete growth media. A day before transfection, exponentially proliferating MIN6 cells were plated on 6-well plates at a density of 3×10^5 per well and transfected with pcDNA3 or pcDNA3-rCLU plasmid construct. The medium was changed with normal growth medium at 12 h after transfection. The cells were labelled with 2 µCi of [³H]thymidine (NEN, Boston, Mass., USA) for 6 h, washed three times with PBS and fixed with ice-cold 10% trichloroacetic acid for 10 min. The cells were lysed with 0.2 N NaOH for 30 min at 37°C, and the radioactivity from 300 µl of the cell lysate in 4 ml scintillation fluid was assessed by beta-emission counter. Secreted clusterin was confirmed by western blot analysis using 10 µl of culture media either from pcDNA3 or pcDNA3-rCLU transfected MIN6 cells.

Statistical analysis. Results were statistically analysed by ANOVA and Student's *t* test. We considered *p* values of less than 0.05 (ANOVA) and 0.01 (Student's *t* test) as significant. All results are expressed as mean values ± SEM and analysed using SAS software (version 6.12; SAS Institute, Cary, N. C., USA).

Results

Changes of body weight and blood glucose concentrations. Growth retardation or weight loss of the rats in all the experimental groups was noted and was particularly significant in group 1 and 2 (Fig. 1A, B). The rat pups in group 3 showed normal weight gain during the period of suckling until 3 weeks after birth but showed retarded growth after weaning (Fig. 1C). Severe hyperglycaemia developed within 1 to 3 days after injection of streptozotocin in groups 1 and 2 (Fig. 1A, B). Blood glucose in the group 1 rats gradually decreased to 11.1–16.7 mmol/l from 2 weeks after the last injection of streptozotocin, whereas the group 2 rats manifested a prolonged hyperglycaemia and died within 2 weeks after the streptozotocin injection. The group 3 rats showed mild hyperglycaemia during the experimental period. A recovery from hyperglycaemia to control concentrations was observed between 3 to 4 weeks after streptozotocin treatment (Fig. 1A–C).

Islet remodelling by cell death and proliferation. In the rats of group 1 (multiple injection of streptozotocin

Fig.1A-C. Changes in blood glucose concentration (thick line) and body weight (thin line) of the experimental rats (open circle) in group 1 (A), 2 (B), 3 (C) and their corresponding control rats (solid circle). Data are expressed as mg/dl (for blood glucose concentrations) and g (for body weight) Mean values $\pm$ SE of 25 animals in group 1, 41 in group 2, and 25 in group 3 respectively

with subdiabetogenic dose), beta-cell death with cells showing characteristic apoptotic figures was induced within 2 to 4 days after the first injection of streptozotocin and lasted for an additional 2 to 3 days. During these periods, pancreatic islets showed various degrees of insulitis and apoptotic bodies (Fig.2A, B), while DNA fragmentation was well detected by electrophoresis of nuclear DNA. Thereafter, beta cells regenerated for the duration of the experiment. Regeneration of islet cells was assessed by the presence of PCNA positive nuclei, specific molecular signals for cell proliferation in the central portion of islets. In particular, we observed extensive budding and branching of the pancreatic ducts and PCNA labelled nuclei on their epithelial linings 3 weeks after streptozotocin treatment, implying the neogenesis of pancreatic parenchyma from the duct lining. Although a small number of beta cells (3.75 ± 1.92 SEM per islet) survived 1 week after streptozotocin treatment, they increased to 9.38 ± 5.85 SEM and 15.43 ± 10.53 SEM at 3 and 5 weeks, respectively (Fig.3A, Fig.4A, B). In this group, insulin mRNA was detected by in situ hybridization, indicating insulin synthesis in regenerating beta cells (Fig.4A, B). Multiple injections of a lower dose of streptozotocin did not seem to injure glucagon cells. They were rather proliferative during the experimental period, even at the phase of beta-cell death (Fig.3A, Fig.4C, D).

In the rats of group 2 (single diabetogenic dose of streptozotocin), islet cell death was observed at 6 h after the streptozotocin injection. Most beta cells showed chromatin condensation and disruption of cell-to-cell adhesion in the core of islets. At 12 to 48 h after streptozotocin treatment, cell death was observed in beta cells and also in some alpha cells at the periphery of the islets, resulting in a shrinkage of islet mass. At 72 h, PCNA immunoreaction was present in the remaining islet cells (data not shown). Seven days after the streptozotocin injection, extensive proliferation was seen in alpha cells, but not in beta cells, of which small numbers remained during the rest of the experiment (Fig.4E-H). The morphometric analysis showed restructuring and reorganization of the islets as a result of considerable depopulation of beta cells and the proliferation of alpha cells (Fig.3B).

Streptozotocin treatment to newborn rat pups (group 3) induced less severe damage and an active regeneration of islet cells. Both alpha and beta cells showed a similar pattern of cell death and regeneration in this group (Fig.3C, Fig.4I-L). During the first 3 weeks of the experiment, the number of alpha and beta cells decreased to the same extent. After weaning, however, a gradual regeneration of beta cells led to a noticeable restoration of its population 5 weeks after streptozotocin treatment. The regenerated islet cells were easily detected as single cells or as cell clusters near the small pancreatic ducts, implying islet cell

Fig. 2A–H. Pancreatic islets examined by routine hematoxylin-eosin (**A**, **B**) and double immunostaining experiments for glucagon-clusterin (**C**, **D**), PCNA-insulin (**E**, **F**) and PCNA-clusterin (**G**, **H**). Insulitis was seen after second (**B**, higher magnification) and third injection (**A**, lower magnification) of streptozotocin in group 1. An islet shows infiltration of lymphocytes (arrowheads) with apoptotic bodies (arrows) among the islet cells (asterisks). Double immunostaining experiment

(**C**, **D**) illustrated the co-expression of glucagon and clusterin (arrowheads in **C**, **D**), while a glucagon cell lacked clusterin (arrows in **C**, **D**). Although most PCNA-labelled proliferating cells do not show their specific cell types, an insulin-immunoreactive beta cell (arrow in **E**, **F**) and some clusterin-immunoreactive alpha cells (arrows in **G**, **H**) showed PCNA labelling in their nuclei. Bars = 50 μ m

Fig. 3A–C. Changes in islet cells accessed by the number of beta and clusterin immunoreactive alpha cells in islet of group 1 (A), 2 (B) and 3 (C). Mean values $\pm$ SEM of nine animals in group 1, 10 in group 2, and 14 in group 3. Statistical significance was determined by ANOVA. * $p < 0.05$ vs the corresponding normal control rats in each group

regeneration by neogenesis. The alpha cells decreased in number in the normal control rats after weaning but not in streptozotocin treated rats (Fig. 3C).

Changes of clusterin expression in islet cells. Clusterin expression was evaluated by immunocytochemistry, western and northern blots analyses. Clusterin immunoreactivity was confined to alpha cells. As we previ-

Fig. 4A–L. Expression of insulin (A, B, E, F, I, J) and clusterin (C, D, G, H, K, L) was detected by in situ hybridization (A, B, I, J) and immunocytochemistry (C–H, K, L) in group 1 (A–D), group 2 (E–H) and group 3 (I–L). In group 1 and 3, cell population and intensity of reaction signals for insulin and clusterin showed a remarkable increase at 5 weeks in most cases (B, D, J, L) when compared with those of normal control rats (C) or at 1 week after streptozotocin treatment (A, I, K). Although a small number of surviving beta cells were found at 3 (E) and 7 (F) days, proliferation of clusterin positive cells was evident at 3 (G) and 7 (H) days after treatment of streptozotocin. Bars = 50 μ m

ously reported [7], it was weakly detected in tissues of normal adult rats but increased in islets upon streptozotocin treatment in the early stage of the experi-

Fig. 5 A–C. Clusterin mRNA expression analysed by northern blot in group 1 (**A**), 2 (**B**) and 3 (**C**). Up-regulation of clusterin expression in group 1 (**A**) was detected at 3 (lane 3) and 5 weeks (lane 4) compared with normal control rats (lane 1) as well as at 1 week after streptozotocin treatment (lane 2). Clusterin mRNA was also highly up-regulated at 3 (lane 2) and 7 (lane 3) days after streptozotocin injection in group 2 (**B**), compared with the normal control rats (lane 1). In group 3 (**C**), it was up-regulated at 3 (lane 4) and 5 (lane 6) weeks after streptozotocin treatment compared with age-matched control rats in lanes 3 and 5 respectively, whereas it was lower at 1 week after streptozotocin treatment (lane 2) than the corresponding control rats (lane 1). The blots shown in **A**, **B** and **C** are representative for four similar experiments respectively

ments. In all experimental conditions, colocalization of clusterin with glucagon in the alpha cells was shown in the islet alpha cells by double immunolabelling (Fig. 2C, D). No immunoreactivity for clusterin was seen in the insulin-secreting beta cells during the experimental period. We also carried out double immunolabelling experiments to show the replicating activity of insulin and clusterin producing cells. Small populations of both insulin (Fig. 2E, F) and clusterin cells (Fig. 2G, H) were double-labelled with PCNA in their nuclei. We found a remarkable increase of clusterin expression in the period of islet cell proliferation rather than at the time of injury (Fig. 3, 4). In group 1, the number of clusterin immunoreactive alpha cells was noticeably increased in the period of islet-cell regeneration particularly at 3 and 5 weeks after streptozotocin treatment (Fig. 3A, Fig. 4C, D, Fig. 5A, Fig. 6A). In group 2, numerous clusterin immunoreactive cells occupied not only the periphery of the islets, but also the central area at 3 and 7 days after streptozotocin treatment. Clusterin expression,

Fig. 6 A–C. Clusterin expression assessed by western blot analysis in pancreatic tissue of group 1 (**A**), 2 (**B**) and 3 (**C**). The order of the lanes is identical to those given in Figure 5. The clusterin detected as the 42000 M_r protein (arrows) in the immunoblots, showed a similar pattern to its mRNA expression in Figure 5. The blots shown in **A**, **B** and **C** are representative for five similar experiments respectively

the concentrations of protein and its mRNA, increased in parallel with the increase of clusterin immunoreactive cells in this group (Fig. 3B, Fig. 4G, H, Fig. 5B, Fig. 6B). In group 3, a remarkable up-regulation of clusterin in the pancreas of streptozotocin treated rats was detected at 3 and 5 weeks after the streptozotocin injection (Fig. 3C, Fig. 4K, L, Fig. 5C, Fig. 6C). However, clusterin expression in islet cells was higher in control rats than in the streptozotocin treated ones at the early suckling age of up to 10 days after birth (Fig. 5C, Fig. 6C), the period corresponding to the postnatal growth of endocrine cells and formation of mature islet [28].

Modification of MAPKs activity. Activation of Ras-MAPKs cascade is required to pass the G1 restriction checkpoint to progress into the cell cycle [29]. In our experiments, the activation of p42 and 44 MAPKs was assessed by western blot analysis using antibodies specific for both phosphorylated and non-phosphorylated forms of the enzymes. We found a correlation between the increase of phosphorylated p42 and 44 MAPKs and expression of clusterin during the regeneration of islet cells in all experimental models. The activation of p42 and 44 MAPKs occurred at the period of islet cell proliferation between 3 and 5 weeks after streptozotocin injections in group 1 and 3 (Fig. 7A, C). However, higher degrees of phosphorylation were observed in the control rats than in the streptozotocin treated ones at 1 week in group 3 (Fig. 7C). In group 2, a marked activation of p42 and 44 was seen in the period of alpha cell proliferation, 3 days and 1 week after streptozotocin injection (Fig. 7B). It could indicate that islet-cell regeneration was predominant in streptozotocin treated rats at 3 and 5 weeks and that stimulation in replication was

Fig. 7 A–C. Erks expression in the pancreas of group 1 (A), 2 (B) and 3 (C) was shown in each experimental group in the same order of lanes as presented in Figures 5 and 6. The upper panels represent the expression of phosphorylated p44 and p42 and the non-phosphorylated ones (lower panels). In each group, Erks (p44 and 42) are activated along with the expression of clusterin shown in Figures 5 and 6, while the inactivated proteins remain unchanged. The blots shown in A, B and C are representative for five similar experiments respectively

not initiated at 1 week when the islet cells were normally replicating in untreated rats. Phosphorylation of p42 and 44 was in parallel with the expression pattern of clusterin which was up-regulated at the time of proliferation or regeneration of islet cells.

Effect of clusterin over-expression on proliferation of MIN6 cells. To investigate regulatory effect of clusterin on beta cells, we assessed the DNA synthesis rate of proliferating MIN6 insulinoma cells using [^{3} H]thymidine incorporation assay, after a transient transfection of pcDNA3-rCLU. MIN6 cells transfected with pcDNA3-rCLU highly expressed clusterin, whereas cells transfected with the control plasmid pcDNA3 did not express clusterin (Fig. 8A). Over-expression of clusterin enhanced the incorporation of [^{3} H]thymidine into DNA synthesis about 31 % when compared with the control vector transfected cells, at 48 h after transfection (Fig. 8B). This result indicated that clusterin over-expression stimulated cell proliferation in MIN6 cells.

Discussion

Evidence of islet-cell regeneration has been reported in animal models [25, 30–34]. It has been suggested that dynamic changes during the process of beta-cell

Fig. 8 A, B. Clusterin over-expression and [^{3} H]thymidine incorporation in MIN6 cells. MIN6 cells were transiently transfected with clusterin over-expression plasmid, pcDNA3-rCLU by Lipofectin (Life Technologies, Grand Island, N. Y., USA). The vector plasmid pcDNA3 was used as a control. [^{3} H]thymidine incorporation assay was done 48 h after the transient transfection. Clusterin over-expression increased [^{3} H]thymidine incorporation by MIN6 cells by 31 % (pcDNA3-rCLU in B). Over-expression of rat clusterin in MIN6 cells was shown by western blot analysis 48 h after transfection. A higher concentration of clusterin was identified in culture media of clusterin cDNA transfected cells (pcDNA3-rCLU in A), whereas no signal was seen in the control (pcDNA3 in A). Mean values $\pm$ SEM. Data were analysed using the Student *t* test from six different experiments. *p < 0.01 vs control

replication could accommodate insulin production to reduce hyperglycaemia [35–37]. However, the mechanism for islet cell regeneration has not been shown. Clusterin is known to be up-regulated at sites of tissue remodelling through differentiation and proliferation after apoptosis [10, 14, 16, 41]. We have reported the

expression of clusterin in the developing pancreas and in the pancreatic tissue of the streptozotocin treated rats and suggested that this protein must play important roles [7, 20]. Therefore, we examined whether the expression of clusterin is associated with islet-cell replication and with islet reorganization in diabetic animal models. Expression of clusterin was assessed by immunocytochemistry as well as by western and northern blots. In general the isolated islets can provide a precise evaluation of the islet proteins in western and northern blot analysis. However, we used whole pancreas samples instead of isolated islets, because the process of islet isolation, particularly in diabetic animals, could give rise to the loss of the mantle area of islets considered to be a major expression site of clusterin in pancreatic tissue. We observed two phases in islet cell reorganization, a phase of cell death followed by a period of renewal, though the features of islet cell death and regeneration differed among experimental groups. Remarkable up-regulation of clusterin expression was shown not only during the late stage of islet-cell injury but also during islet regeneration in all cases. The murine diabetic model induced by a subdiabetogenic dose of streptozotocin matches the development of human Type I diabetes in many aspects [24]. In our study, the subdiabetogenic dose of streptozotocin induced insulinitis with lymphocyte infiltration and apoptotic cell death in the early stages. Afterwards, the remaining beta cells proliferate and increase in number, expressing a higher expression of insulin mRNA than in the control rats. This reflects feedback mechanisms that reactivate islet-cell regeneration to compensate for their loss or dysfunction [31, 38]. Beta-cell proliferation with increased insulin synthesis in this model is able to reduce blood glucose concentrations and to improve survival. Intravenous injection of a diabetogenic dose of streptozotocin provoked immediate cell death. The animals showed limited beta-cell regeneration, whereas proliferation of alpha cells was pronounced. It is likely that the high cytotoxic effect could induce a decrease in replication capability in remaining beta cells. In contrast, partial damage in alpha cells seemed to trigger their replication. Thus, we suggest that the nature and degree of islet-cell damage should be considered as an important factor for beta-cell regeneration. In these models, clusterin was highly expressed in the surviving alpha cells but not in beta cells during the phase of islet damage. This supports the hypothesis that clusterin is expressed in surviving cells rather than being a molecule related to cell death [5, 7, 42]. After the phase of islet destruction, clusterin expression was enhanced along with islet cells proliferation, indicating that clusterin is involved in regeneration. Rats treated with streptozotocin at neonatal stage have been used as a model for Type II diabetes with insulin resistance and glucose intolerance [22, 39, 40]. It has been suggested

that neonatal streptozotocin treatment stimulates neogenic proliferation of islet cells [31, 32, 34]. Indeed in streptozotocin-treated rat pups a remarkable regeneration of beta cells was recorded together with a noticeable reduction of blood glucose. Enhanced expression of insulin mRNA in the regenerating beta cells shown in this study could account for the transient remission of the hyperglycaemic state in the streptozotocin treated rat pups [22, 32–34]. This regeneration appears, however, incomplete leading to the establishment of a mild hyperglycaemic state in the adult animals. As for other animal models of Type I diabetes, clusterin expression was enhanced with cell proliferation after the phase of islet destruction, indicating again the involvement of clusterin in cell regeneration activity. Many studies have shown that the growth factors, including TGF-, beta-NGF and EGF, stimulate clusterin expression [10, 12–15]. Together, these data suggest that clusterin must be involved in differentiation- and proliferation- signalling pathways. Further, it was found that NGF and EGF use the common Ras-extracellular signal-regulated kinase-AP1 (activator protein 1, a transcription factor of clusterin) signalling pathway to activate clusterin gene expression [10]. We also found simultaneous activation of MAPKs and an increase of clusterin expression during islet-cell regeneration. Stimulation of the Ras-ERK signalling cascade could activate clusterin expression during islet cell regeneration. Among the different growth factors, NGF is relevant in the increase of clusterin expression. Islet cell injury in diabetes was reported to induce an increased expression of NGF and a reactivation of the peri-islet Schwann cells enveloping pancreatic islets [17]. It was suggested that the growth factor must play an important role in the processes leading to islet cell repair. Furthermore, [16] a parallel increase of beta-NGF and clusterin in islet cells of streptozotocin-treated animals was reported, suggesting that they stimulate cell growth and remodelling. We, therefore, speculate that an increase of beta-NGF in the peri-islet Schwann cells in response to islet injury could cause the up-regulation of clusterin.

It has not been defined whether the clusterin is directly involved in cell proliferation and differentiation. It could function as a cell signalling molecule involved in proliferation and differentiation after being expressed from surviving islet cells [7, 20]. Clusterin was found within the secretory granules of the various cell types including pancreatic alpha cells [7]. As a secretory molecule, clusterin could exert paracrine action. Recent studies showed that clusterin interacts with the intracellular portion of the TGF- receptor and thereafter induces autophosphorylation of the receptor [15]. We have shown stimulatory effects of clusterin on cell proliferation by over-expressing the protein in a beta cell line. Since cell replication increased in the MIN6 cells transfected by the cDNA

of the clusterin gene, it is probably caused by the increase of clusterin molecules in the cultured media secreted by transfected cells.

In summary, our studies indicate that clusterin could induce cell growth not only on the alpha cells, but also on remaining beta cells of islet streptozotocin-treated rats by paracrine action during islet remodelling. In this process, clusterin could act as a signal molecule for the Ras-Erks signalling cascade of cell proliferation and differentiation. We suggest that clusterin is a growth factor-like molecule stimulating islet cell replication and plays important roles in maintaining beta-cell population after the onset of the various types of diabetes.

Acknowledgements. This study was supported by a Science Research Center grant from Korea Science and Engineering Foundation (KOSEF) to the Nitric Oxide Radical Toxicology Research Center (NORTReC).

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