N-Acetylcysteine derivative inhibits procoagulant activity of human islet cells

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Abstract

Aims/hypothesis The early loss of beta cells after islet cell transplantation has been attributed in part to blood coagulation at the implant site. Tissue factor expressed by beta cells and contaminating duct cells is considered to activate this process. Here, we investigated the ability of *N*-acetyl-L-cysteine to suppress the in vitro procoagulant activity of duct cells and human islet cell preparations.

Materials and methods The effects of Nacystelyn, a salt derivative of *N*-acetyl-L-cysteine, were first assessed on

procoagulant activity induced in human plasma by recombinant tissue factor, human primary duct cells or human islet cell preparations. The influence of Nacystelyn on clot formation, platelet counts and D-dimers were measured in a whole blood tubing loop model. Human beta cell viability and insulin synthesis after Nacystelyn treatment were assessed to exclude cytotoxicity of Nacystelyn.

Results Nacystelyn efficiently inhibited the procoagulant activity of human recombinant tissue factor, primary duct cells and human islet cell preparations at clinically relevant concentrations without cellular toxicity.

Conclusions/interpretation Nacystelyn is a pharmaceutical candidate to reduce early beta cell loss related to tissue factor-dependent coagulation after islet transplantation.

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Introduction

Activation of the coagulation cascade is thought to play an important role in early beta cell loss after islet cell transplantation, thus interfering with metabolic outcome [1]. The expression of tissue factor by beta cells and by contaminating duct cells has been shown to trigger coagulation events [1, 2]. Pharmacological inhibition of tissue factor activity exerted by human islet cells is therefore expected to improve beta cell survival following transplantation. *N*-acetyl-L-cysteine, an anti-inflammatory and antioxidant drug, has been shown previously to inhibit activation of coagulation by downregulating translation of tissue factor mRNA [3] and by interfering directly with coagulation factors [4]. In addition, we have recently shown that a salt derivative of *N*-acetyl-L-cysteine (Nacys-

telyn), which has the advantage over the parental compound of having a neutral pH whereas *N*-acetyl-L-cysteine is acidic in solution, might promote the emergence of regulatory T cells and could favour graft acceptance [5]. In this study we assessed the effects of Nacystelyn on tissue factor-dependent procoagulant activity of human islet cell preparations similar to those used in transplantation trials [6].

Materials and methods

Cell preparations Human primary duct or islet cell preparations were isolated from 14 human donor organs (age of donors 38 ± 18 years; eight male and six female) and cultured as described previously [2, 6]. The organs were procured by European hospitals affiliated with the Eurotransplant Foundation (Leiden, the Netherlands) and processed by the Beta Cell Bank in Brussels within the framework of a multicentre programme on beta cell transplantation in diabetes undertaken by the Juvenile Diabetes Research Foundation Center for Beta Cell Therapy in Diabetes. Collagenase digests were separated by Ficoll gradient purification into an islet fraction and an exocrine fraction. The exocrine fraction was cultured in serum-free medium for at least 4 days, leading to a preparation enriched in duct cells. The purity of these duct cell preparations was routinely above 90–95% [2]. The islet cell preparations were cultured for 13 ± 4 days as described previously [2, 6]. The cellular composition of the preparations used in the present experiments was comparable with that of human islet cell grafts used in the transplantation trial, with 15–58% insulin-positive cells and 16–66% cytokeratin 19-positive duct cells.

Procoagulant activity assay Procoagulant activity was determined in duplicate samples by a single-stage clotting assay on cell extracts obtained by repetitive thawing-freezing or in solutions of human recombinant tissue factor (Dade Behring, Marburg, Germany). A portion of each sample (100 μ l) was incubated at 37°C for 1 min with 100 μ l of normal citrated plasma before the initiation of clotting by addition of 100 μ l of 25 mmol/l CaCl_2 . Clotting time was recorded with a KC10 apparatus (Amelung, Lemgo, Germany) and procoagulant activity in mU/ml was determined by reference to a standard curve generated with rabbit thromboplastin (BioMérieux, Marcy l'Etoile, France). The amount of thromboplastin that yielded a clotting time of 12.4 s was assigned a value of one unit.

Tubing loop model A whole-blood experiment protocol was adapted from a model described previously [1, 2]. Loops

made of polyvinyl chloride tubing (inner diameter 6.3 mm, length 390 mm) and treated to give a Corline heparin surface were purchased from Corline (Uppsala, Sweden). Loops were supplemented with cell samples suspended in PBS in the absence or presence of either Nacystelyn or nicotinamide. Five millilitres of non-anticoagulated blood from healthy volunteers was then added to each loop. To generate a blood flow of about 45 ml/min, loop devices were placed on a platform rocker inside an incubator at 37°C. Blood samples were collected into EDTA (final concentration 4.1 mmol/l) and citrate (final concentration 12.9 mmol/l) tubes before and 30 min after the start of perfusion. Platelets were counted with a CellDyn 4000 (Abbott Laboratories, Abbott Park, IL, USA) and fibrinogen was determined using a Behring Coagulation System (Dade Behring).

Viability assays The percentages of viable, apoptotic or necrotic cells were determined after 6–24 h of exposure to Nacystelyn by cellular staining with propidium iodide and Hoechst 33342, as described previously for beta cell enriched human preparations [7]. Exact quantification of cell death in whole islets, as performed in the human islets in the present study, is difficult because of superposition of cells. The data obtained in whole islets thus provide an approximate percentage of dead cells [8]. There was >90% agreement between the two observers (one of them was unaware of sample identity) in the different assessments of viability, confirming the reproducibility of the method.

Insulin biosynthesis After preculture for 13–17 days, human islet cells were cultured for 24 h with and without 5 mmol/l Nacystelyn. At the end of culture, insulin synthesis was measured during 2 h of incubation without drug and in the absence or presence of 10 mmol/l glucose, as described previously [9].

Statistical analysis Data were compared using the non-parametric paired *t* test.

Results

Human primary duct cells preincubated with Nacystelyn show decreased procoagulant activity We observed previously that human primary duct cells exert procoagulant activity as a result of their tissue factor expression [2]. Here, we first assessed whether Nacystelyn could inhibit procoagulant activity of duct cells after an incubation period of 24 h. Nicotinamide was chosen as the benchmark because it has recently been shown to downregulate tissue factor concentration in islet preparations [10]. In this

setting, Nacystelyn and nicotinamide both dose-dependently inhibited procoagulant activity of duct cells assessed in human plasma (Fig. 1a). Real-time RT-PCR experiments allowed us to show that Nacystelyn had no significant effect on the expression of tissue factor mRNA, whereas nicotinamide decreased it (Fig. 1b), as described previously by others [10].

Nacystelyn directly inhibits coagulation induced by recombinant tissue factor; human primary duct cells or human islet cell preparations We then compared the direct effects of Nacystelyn and nicotinamide on procoagulant activity of human recombinant human tissue factor. Therefore, both recombinant human tissue factor and Nacystelyn or nicotinamide were added simultaneously to plasma. We found that Nacystelyn dose-dependently inhibited the procoagulant activity of recombinant human tissue factor, whereas nicotinamide had no significant effect (Fig. 2a). Nacystelyn was then assessed for its effects on procoagulant activity induced by either primary duct cells (Fig. 2b) or islet cell preparations isolated from human donor organs (Fig. 2c). Nacystelyn dose-dependently and significantly inhibited procoagulant activity when drugs were added at the time of contact of duct cells or islet cells with plasma (Fig. 2b,c). No effect of nicotinamide on the procoagulant activity of duct cells or islet cells was observed in this experimental approach (Fig. 2b,c).

Fig. 1 Modulation of tissue factor (TF)-dependent procoagulant activity (PCA) and mRNA expression by Nacystelyn (NAL). **a** Procoagulant activity of cell extracts of human duct cells (10^6 cells/ml) in human plasma. Duct cells were incubated with graded concentrations of Nacystelyn or nicotinamide (NAM) for 24 h. Cell extracts were obtained and procoagulant activity was measured in human plasma. Data are mean $\pm$ SEM of five independent experiments ($n=2$ for nicotinamide). * $p<0.05$, *** $p<0.001$ vs control non-treated cells. **b** Duct cells were incubated with graded concentrations of Nacystelyn or nicotinamide for 6 h. Messenger RNA was extracted from primary duct cells and was analysed by RT-PCR for tissue factor and β -actin. Data are mean $\pm$ SEM of four independent experiments done with duct cells purified from three donors. Results are expressed per 10^6 mRNA copies of β -actin. * $p<0.05$ vs control non-treated cells

Nacystelyn inhibits clotting induced by human islet cell preparations in whole blood The tubing loop model is based on the injection of non-anticoagulated blood into plastic loops coated inside with heparin to prevent contact-dependent blood coagulation, allowing the investigation of coagulation in the presence of platelets, i.e. in a more

Fig. 2 Direct inhibition by Nacystelyn (NAL) of coagulation induced by recombinant tissue factor, primary duct cells and islet cells. **a** Procoagulant activity (PCA) of recombinant human tissue factor in human plasma. Graded amounts of Nacystelyn or 30 mmol/l nicotinamide (NAM) were added to 90 μ l of recombinant human tissue factor immediately before procoagulant activity was measured. Data are mean $\pm$ SEM of five independent experiments. ** $p<0.01$, *** $p<0.001$ vs control non-treated cells. **b** Procoagulant activity of cell extracts of human primary duct cells (0.25×10^6 cells/ml) in human plasma. Graded concentrations of Nacystelyn ($n=4$) or nicotinamide ($n=3$) were added to 90 μ l of cell extract immediately before measurement of procoagulant activity. Data are mean $\pm$ SEM of four independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs control non-treated cells. **c** Procoagulant activity of cell extracts of human islet cells (10^6 cells/ml) in human plasma. Graded concentrations of Nacystelyn were added to 90 μ l of cell extract immediately before measurement of procoagulant activity. Data are mean $\pm$ SEM of five independent experiments. ** $p<0.01$, *** $p<0.001$ vs control non-treated cells

physiological context [1, 2]. Blood coagulation was assessed after 30 min incubation by macroscopic examination (presence of clots) and by decreased platelet counts and increased concentrations of D-dimers. In this system, 10^5 human islet cells were sufficient to induce clot formation, a major decrease in the platelet count and an increase in the level of D-dimers. As shown in Table 1, the addition of 5 mmol/l Nacystelyn to islet cell suspensions significantly inhibited the coagulation process in five independent experiments. Two additional experiments using 10 mmol/l Nacystelyn indicated that this concentration completely prevented activation of coagulation induced by islet preparations.

Nacystelyn inhibits procoagulant activity of human islet cell preparations without altering their viability and beta cell insulin synthesis We verified that Nacystelyn did not affect the viability and function of human islet cells at concentrations up to 10 mmol/l. Using propidium iodide and Hoechst 33342 to discriminate between living and dead cells, we observed that Nacystelyn did not affect the viability of human islet cells after 6 h. After 24 h and only at 10 mmol/l, we observed a reproducible but very modest (14%) effect on cell viability (Fig. 3a).

We then assessed a potential functional toxic effect of Nacystelyn on human beta cells in concentration conditions that are achievable in patients. We observed that exposure to 5 mmol/l Nacystelyn, although it strongly inhibited coagulation in a whole-blood model (Table 1), did not affect insulin biosynthesis at all after 24 h (Fig. 3b).

Table 1 Nacystelyn inhibits activation of coagulation induced by islet cell preparations in whole blood

Islet cells	Nacystelyn (mmol/l)	No. of experiments	Clot	Platelets ($\times 10^3/\mu\text{l}$)	D-Dimers (ng/ml)
–	0	5	No	276 $\pm$ 9	<400
+	0	5	Yes	36 $\pm$ 21	2403 $\pm$ 818
+	5	5	No	203 $\pm$ 58 ^a	1286 $\pm$ 755 ^a
+	10	Expt 1	No	267	<400
+	10	Expt 2	No	268	<400

Islet cells (10^5) from four human islet cell preparations containing 42 $\pm$ 7% (mean $\pm$ SEM) beta cells were added to 5 ml whole blood from five donors and incubated in tubing loops under agitation at 37°C. Nacystelyn was added to the loops extemporaneously. Clot formation, platelet counts and levels of D-dimers were monitored after 30 min of incubation. D-Dimers are expressed as fibrinogen equivalent units (FEU) in ng/ml. Data are mean $\pm$ SEM of five independent experiments, except for the condition with Nacystelyn 10 mmol/l, for which two independent experiments are shown (Expt 1 and Expt 2)

^a $p<0.05$ compared with levels measured in absence of Nacystelyn

Fig. 3 Effect of Nacystelyn (NAL) on cell viability and insulin biosynthesis in human islet preparations. **a** Viability in whole human islets was estimated after 6 h (open bars) and 24 h (grey bars) of treatment with graded concentrations of Nacystelyn. Data are mean $\pm$ SEM of four determinations based on two human islet preparations. * $p<0.05$ vs control. **b** Human islet cells were cultured for 24 h with 5 mmol/l Nacystelyn. After 24 h, half of the cells were challenged with 10 mmol/l glucose (G10; grey bars) and the remaining half (control) were not challenged with glucose (G0; open bars). Insulin biosynthesis was measured after 2 h. Data are mean $\pm$ SEM of three independent experiments

Discussion

The present study demonstrates that Nacystelyn prevents the activation of coagulation induced by human islet cell preparations similar to those used in transplant trials. This effect was also observed in human primary duct cells, which are known to constitute a substantial proportion of islet cell preparations and to contribute to tissue factor-mediated coagulation activation [2]. As described previously for nicotinamide [10], Nacystelyn was able to inhibit duct cell-induced procoagulant activity when cells were preincubated with the drug before addition of plasma. However, in contrast with nicotinamide, which was shown to affect tissue factor gene transcription, Nacystelyn exerted no inhibition of tissue factor mRNA expression. This is consistent with the observation of Brisseau et al. [3], who demonstrated that *N*-acetyl-cysteine, from which Nacystelyn derives, inhibits expression of tissue factor in activated peripheral blood mononuclear cells at a post-translational level.

In addition Nacystelyn, but not nicotinamide, exerted very potent direct inhibition of coagulation induced by recombinant tissue factor or by duct or islet cells, i.e. inhibition was also observed when tissue factor or cells were added together with Nacystelyn to plasma. This might be important for potential therapeutic application as there would be no need for islet manipulation before grafting. This direct effect is probably mediated by a modification of the structure and/or function of one or several factors of the coagulation pathway [11]. Indeed, these factors are mosaic proteins that share highly similar

structures and might all present various degrees of sensitivity to Nacystelyn [11].

It is interesting to note that 50% of islet cell procoagulant activity in plasma was already inhibited with only 5 mmol/l of Nacystelyn. In addition, when we used the tubing loop model to assess the inhibitory effects of Nacystelyn on coagulation induced by islet cells, we observed nearly total inhibition of coagulation at the concentration of 5 mmol/l. The tubing loop model mimics more closely the in vivo situation, in which inhibitors of coagulation are expressed by the vessel walls, than procoagulant activity assessed in isolated plasma. In relation to the prospect of therapeutic application, 5 mmol/l of Nacystelyn is in the range of *N*-acetyl-L-cysteine concentrations measured after in vivo administration in patients with paracetamol intoxication [12]. Intravenous injection of *N*-acetyl-L-cysteine was usually well tolerated in both patients and healthy volunteers, although allergic reactions were observed occasionally [4]. Moreover, we observed no adverse effect on the vitality or insulin biosynthesis of Nacystelyn in human beta cells. We therefore propose *N*-acetyl-L-cysteine or its salt derivative Nacystelyn as adjunct therapy at the time of islet cell transplantation in order to reduce beta cell loss caused by tissue factor-mediated coagulation and immune-mediated events.

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