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Adenovirus-Mediated Expression of the Anticoagulant Hirudin in Human Islets: A Tool to Make the Islets Biocompatible to Blood

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Human islets induce an injurious clotting reaction at the time of transplantation. A potential strategy to counteract this reaction would be to allow the islets to express hirudin, a protein with direct anticoagulative activity. Human islets were transduced with an adenoviral vector encoding hirudin, an empty corresponding vector, or left untreated. Islet culture supernatants were analyzed for hirudin using an ELISA, a chromogenic substrate assay based on the thrombin-binding properties of hirudin and in a whole blood viscosimetry assay. Immunohistochemical evaluation and determination of hirudin content revealed an abundant expression of hirudin after transduction. Hirudin content in transduced islets was in the range of the insulin content levels. A delay in human whole blood clotting time could be observed after addition of supernatants taken from islet cultures expressing hirudin. However, transduced islets showed an impaired glucose-stimulated insulin release, but could readily be retrieved 6 weeks after transplantation to athymic mice. A marked expression and secretion of hirudin with functional capacity can be induced in human islets using an adenoviral vector. The impairment in glucose-stimulated insulin release in hirudin-secreting islets, compared to controls, indicates that the additional protein synthesis affects the functional capacity of the islets.

Key words: Transplantation; Coagulation; Human islets of Langerhans; Hirudin; Adenoviral vector

INTRODUCTION

We have previously reported that isolated human islets, when exposed to whole human blood in vitro, elicit an instant blood-mediated inflammatory reaction (IBMIR) (2). The effects of this acute inflammatory reaction could provide a reasonable explanation for early loss of islet mass after islet transplantation and thereby the need of islets from more than one donor. IBMIR is characterized by platelet consumption and activation of the coagulation and complement systems. Recent studies have shown that IBMIR is triggered by tissue factor (TF), produced and secreted by the endocrine cells of the islets of Langerhans (7). Considering the current deficit of pancreata available for islet isolation, preventing IBMIR may prove a valuable strategy in the effort to reduce the number of islets needed to achieve insulin independence, and thereby allowing for an increased number of patients to benefit from this therapy. Because coagulation is central in IBMIR, a conceivable strategy to prevent this process would be to induce islet expression of suitable transgenes using viral vectors. Gene transfer of different proteins using adenoviral vectors to human islets has been successful in several reports, but to date few studies have included functional proteins (1,4,11).

Naturally occurring hirudin is a low molecular weight (~7 kDa) polypeptide produced in the salivary glands of the leech *Hirudo medicinalis*. Its anticoagulant activity comes from the chemical ability to inhibit thrombin, a serine protease catalyzing the final step in the blood coagulation cascade. Hirudin has many advantages over the commonly used anticoagulants such as heparin (e.g., hirudin does not interact with other blood proteins and, unlike heparin, can act upon bound thrombin). Hirudin's small size and lack of necessary posttranslation modifications makes it a simple protein to express in adenoviral vectors, in comparison to heparin whose polysaccharide structure makes it impossible for cloning into the viral vectors.

As a means to reduce IBMIR, we have in this study examined the possibility to induce synthesis and secretion of biologically active anticoagulant hirudin in human islets using an adenoviral vector. We demonstrate that human islet expression and secretion of hirudin results in a delay in clotting time in human blood in vitro, although the induced protein synthesis appeared to, at

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least transiently, to reduce the capacity of the islets to respond to glucose stimulation.

MATERIALS AND METHODS

Human Islet Isolation and Culture

The pancreata were obtained from 10 normoglycemic human donors, after appropriate consent for multiorgan donation. Human islets of Langerhans were isolated at the Division of Clinical Immunology at the University of Uppsala, using a modification of previously described semiautomated digestion–filtration method (3,6,8), followed by purification on a continuous density gradient in a refrigerated COBE 2991 centrifuge (COBE Blood Component Technology, Lakewood, CO, USA).

The islet preparations were of good quality but were available for experimental use because the total islet volume was too low for clinical transplantation. Islet volume and purity were determined by microscopic sizing on a grid after staining with diphenylthiocarbazone. The purities of the islet preparations varied between 70% and 90%.

The islet preparations were placed in untreated petri dishes Sterilin (Tamro Med. Lab. AB) and kept at 37°C in a atmosphere of 5% CO_2 in humidified air in culture medium, CMRL 1066 (GIBCO BRL, Invitrogen) supplemented with 10 nM nicotinamide (Sigma Chemicals), 10 mM HEPES buffer (GIBCO BRL, Invitrogen), 0.25 μg/ml fungizone (GIBCO BRL, Invitrogen), 50 μg/ml gentamicin (GIBCO BRL, Invitrogen), 2 mM L-glutamine (GIBCO BRL, Invitrogen), 10 μg/ml Ciprofloxacin (Bayer), and 10% (v/v) heat-inactivated human serum. After transduction the islets were cultured for 14–16 days. In five isolations (culture supernatant assays) samples of 1 ml islet supernatant were collected on days 1–7, 9, 12, and 14. Additional medium of 1 ml supplemented with serum was added after each sampling, except days 7 and 14, when medium was completely changed. In another five isolations (immunohistochemistry, protein/DNA content experiments) medium was changed every second day.

Adenoviral Vectors and Transduction Procedures

We used a replication defective E1, E2a, and E3 region-deleted adenoviral vector Av3HHV1, coding for secretory hirudin cDNA, or Av3null vector carrying no transgene, generously provided by Novartis Pharma Research (Basel, Switzerland). The hirudin gene is inserted into the deleted E1 region under the control of Rous sarcoma virus promoter (RSV) as previously described (5). At the time of transduction, the islets were sedimented by light centrifugation (1000 rpm for 60 s) and then washed in serum-free culture medium, M199 Earl's salt (GIBCO BRL, Life Technologies). Then 10 μl of islets (1 μl = 500 IEQ) was transduced with 10–20 μl of adenoviral vector Av3HHV1 (2.6×10^{10} pfu/ml; $n = 10$) or Av3null (6.5×10^{10} pfu/ml; $n = 3$), and incubated for 1 h in 500 μl of serum-free medium before medium supplemented with human AB serum was added. During the different experiments, control islets not exposed to adenoviral vector were treated in the same way as the corresponding transduced islets.

Quality Test

To examine whether the transduction of the islets with Av3HHV1 had any adverse effect on islet function, the quality of the islets was assessed in terms of insulin secretion in response to a glucose stimulation in a dynamic perfusion system between days 4 and 7 and days 14 and 16 after transduction ($n = 5$). To evaluate the effects of the transduction procedure itself, islets were also incubated with Av3null vector carrying no transgene ($n = 3$; evaluation on days 6 and 9 after transduction). Islets were challenged with two glucose concentrations (initially in 1.67 mmol/L, thereafter in 16.7 mmol/L glucose, and finally back to 1.67 mmol/L). Fractions were collected with 6-min intervals during 120 min. Concentrations of insulin were analyzed with a commercial ELISA kit (Mercodia). Values are given as pmol/L.

Measurement of Soluble Hirudin Using ELISA

Blocking solution (1% BSA in PBS) of 250 μl was incubated for 1 h at room temperature in micro-test wells that were precoated with 150 μl sheep-anti-hirudin polyclonal antibody (Affinity Biologicals Inc.) for antigen capture. Collected islet culture supernatants (100 μl) were added and incubated for 1 h followed by an incubation with 100 μl of the same sheep-anti-hirudin polyclonal antibody conjugated to biotin. The subsequent incubation for 15 min with 100 μl streptavidin-conjugated horseradish peroxidase (HRP; Amersham Biosciences, UK) completed the formation of the antibody–enzyme complex, which was developed by adding 100 μl TMB substrate. The reaction was stopped after 5 min with 100 μl 1 M H_2SO_4. After each incubation time the wells were washed three times with wash buffer (PBS, 0.05% Tween 20). The hirudin levels were determined by measuring absorbance at a wavelength of 450 nm. A serial dilution of known amount of recombinant hirudin (Refludan®, Aventis Pharma) was run simultaneously to create a standard curve. The background average of the blanks was deducted from the standards and sample readings.

Chromogenic Substrate Assay

For the chromogenic hirudin determination a thrombin binding-based assay was used (Chromogenix, Mölndal, Sweden). Incubation of collected samples of islet culture supernatants with an excess of human thrombin

results in rapid complex formation between hirudin present in the supernatant and thrombin. The inhibited thrombin activity is proportional to the amount of hirudin present in the sample. The remaining amount of thrombin hydrolyses the chromogenic substrate S-2366, thus liberating the chromophoric group, pNA. The color is then read photometrically at 405 nm. Islet culture supernatant (50 µl) sample was incubated for 2–3 min at 37°C with 100 µl human thrombin. Subsequently, 100 µl of the chromogenic substrate S-2366 was added and incubated for exactly 3 min. The reaction was stopped with 20% citric acid, and the change in absorbance was measured at 405 nm. A calibration curve was constructed using medium containing recombinant hirudin (Refludan®) to obtain final concentrations between 0.019 and 0.2 µg/ml.

Measurement of Clotting Time Using a Free Oscillating Rheometer

Whole blood drawn into citrate tubes (Vacutainer™) was taken from healthy volunteers that had received no medication for at least 14 days. Clotting time in whole blood with addition of islet culture supernatants taken day 7 ($n = 5$) was measured in a four-channel free oscillating rheometer (ReoRox 4, Global Haemostasis Institute AB, Linköping, Sweden). As a control we used culture supernatants from untreated islets. The free oscillating rheometer used in this study has four measuring channels embedded in an aluminum block thermostat with temperature control. In our experiment the temperature was set at 37°C. Each measuring channel harbors a cylindrical, 12-mm-diameter plastic sample cup. For each measurement 100 µl sample, 1 ml whole blood, and 22 µl 1 M $CaCl_2$ was used for each cup, which was placed on a hollow shaft connected to the base of the instrument. Every 6 s, the cup was set into free horizontal oscillation around its vertical axis, and the damping and frequency of the oscillation was registered. Clotting times were identified by the software program of the instrument as the point of maximal damping.

Insulin, Hirudin, and DNA Contents of Islets

Islets in groups of 100 were homogenized by ultrasonic disruption in 300 µl phosphate-buffered saline containing 5 mM EDTA, 10 mM benzamide, 0.1 mg/ml of soybean trypsin inhibitor, and 1 mM PMSF ($n = 5$). A fraction of the homogenate was mixed with acid ethanol and insulin was extracted overnight at 4°C. The insulin concentration of the extract was measured with a commercial ELISA kit (Mercodia). Values are given as pmol/L. Another fraction of the homogenate was used for DNA measurement using commercial PicoGreen® dsDNA Quantitation Reagent and Kits. A third fraction of the homogenate was used for measurement of hirudin concentration by ELISA as described above.

*Transplantation of Transduced and Untreated Islets to C57BL/6J (*nu/nu*) Mice*

Adenoviral vector transduced and untreated islets were simultaneously transplanted as separate grafts to C57b1/6J nude (*nu/nu*) athymic mice under the left kidney capsule as previously described (9). In total five mice were transplanted. All mice were transplanted with two islet grafts, one composed of transduced islets and one of untreated islets from the same isolation. After 5 weeks, the animals were killed and the grafts were harvested with margin of 5 mm of adjacent kidney tissue and prepared for cryosectioning and immunohistological evaluation in light microscope.

Immunohistochemistry

Intact human islets and transplanted graft biopsies were snap frozen in precooled isopentane and subsequently stored at −70°C. Serial sections, 6–7 µm thick, were cut in a cryostat (−20°C), air dried, fixed in 100% cold acetone, and stored at −70°C. Islet sections were stained with polyclonal sheep anti-hirudin (Affinity Biologicals Inc.), and sections from the transplanted grafts were stained with guinea pig anti-insulin (DAKO, Carpinteria, CA, USA). After storage, the slides were washed in PBS and incubated in 0.6% H_2O_2 in PBS to inhibit endogenous peroxidase. Subsequently, 15-min incubations with normal rabbit serum to prevent nonspecific background staining were preformed on islet sections before the slides were incubated with the primary antibody. Transplanted graft sections were incubated with the primary antibody overnight at 37°C. After incubation with primary antibody islet sections were incubated with rabbit anti-goat Ig antibody (cross-reactive with sheep Ig; code No. Z0228; DAKO). Subsequent incubations of the sections with secondary antibodies were performed sequentially for 30 min, followed by three washes for 5 min in PBS between each step. After a final incubation of islet sections with goat PAP reagent (code No. B0157; DAKO) and graft sections with goat anti-rabbit Envision Ig antibody (code No. K4002; DAKO), the alkaline phosphatase reaction was developed using AEC Substrate Chromogen (code No. K3464; DAKO) for 15 min. The slides were thereafter counterstained with Mayer's hematoxylin and mounted in glycerin gelatin. The islets and transplanted graft biopsies were thereafter analyzed under a light microscope.

Statistics

All results are presented as mean ± SEM. The Student's paired *t*-test was used to compare paired data (hir-

udin-expressing islets and controls) in different islet isolations.

RESULTS

Hirudin Content in the Culture Supernatant (ELISA)

In all experiments, transduction resulted in hirudin expression ($n = 5$). After 3 days of culture hirudin expression was first detected, gradually increased, and reached plateau levels 6–7 days after transduction. A decrease was observed after day 7 as a consequence of a total medium change. The results are presented as mean level of hirudin (ng/ml) ± SEM ($n = 5$) (Fig. 1A).

Chromogenic Substrate Assay

In all experiments, transduction resulted in expression of hirudin ($n = 5$). Transduction efficiency of Av3-HHV1-treated human islets was assessed at 72 h after gene transfer. To obtain a reference curve recombinant hirudin was added to culture medium to obtain final concentrations between 0.02 and 0.2 μg/ml, respectively. The curve was linear over the entire concentration range. Hirudin expression showed a similar pattern as seen after the ELISA analysis. A plateau level of hirudin was reached after day 5, although the levels were 30–40% lower compared to the ELISA analysis. The results are presented as mean level of hirudin (ng/ml) ± SEM ($n = 5$) (Fig. 1B).

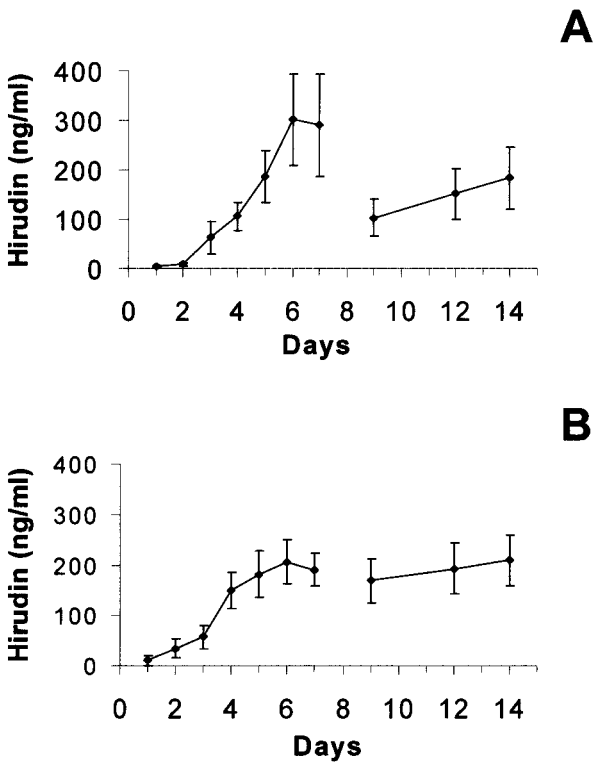

Figure 1. Hirudin levels over time in the culture supernatants of human islets transduced with adenoviral vector Av3HHV1, as measured by hirudin ELISA. Data are presented as mean level of hirudin (ng/ml) ± SEM ($n = 5$) (A). Hirudin levels over time in the culture supernatants of human islets transduced with adenoviral vector Av3HHV1, as measured by a chromogenic substrate assay based on the antithrombin binding properties of hirudin. Parallel experiments with untreated control islets were all negative (not shown). Data are expressed as mean level of hirudin (ng/ml) ± SEM ($n = 5$) (B).

Insulin, Hirudin, and DNA Content

In the five different isolations the level of hirudin/DNA content was found to be comparable or higher than the insulin/DNA content levels. At the same time the insulin/DNA levels in the transduced islets were found to be lower ($p < 0.05$) compared to untreated control islets (2.86 ± 0.9 and 4.56 ± 1.6, respectively). The results are presented as mean level of insulin or hirudin (pmol/μg DNA) ± SEM ($n = 5$) (Fig. 2).

Immunohistochemical Staining of Islets After Transduction

Immunohistochemical staining of frozen and sectioned islets from five different isolations on day 7 after transduction showed hirudin expression in a majority of the islet cells, both in the core and in the periphery (Fig. 3A). Parallel experiments with untreated control islets were all negative (not shown).

Graft Survival of Islets Transduced With Adenoviral Vector and Untreated Controls in nu/nu *Mice*

After 5 weeks the grafts were harvested and processed for immunohistochemistry (transduced islets, $n = 5$; control islets, $n = 5$). The morphology indicated successful engraftment and survival of both transduced (Fig. 3B) and untreated islets (not shown).

Measurement of Clotting Time Using Free Oscillating Rheometer

As shown in the diagram, islets transduced with Av3-HHV1 secreted hirudin in quantities large enough to be detected in the ReoRox assay. A 2.2 ± 0.4-min (29.0 ± 6.2%; $p < 0.05$) delay in clotting time in human whole blood was observed after addition of supernatants taken from Av3HHV1 transduced islets. Corresponding untreated islet supernatants were used to obtain reference clotting time values. All samples tested were collected on day 7 after transduction. The results are presented as mean clotting time (min) ± SEM ($n = 5$) (Fig. 4).

Quality/Viability Test

Specific function was evaluated using a dynamic perfusion system. Both transduced (hirudin and null vectors) and untreated islets responded to glucose stimula-

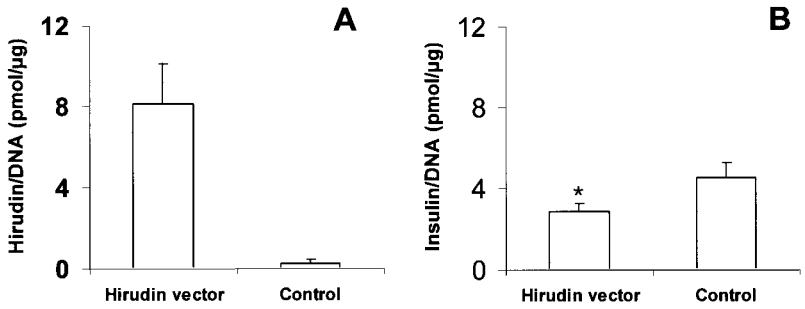

Figure 2. Hirudin/DNA ratio (A) and insulin/DNA ratio (B) of human islets transduced with adenoviral vector Av3HHV1 and the corresponding untreated controls. The level of hirudin/DNA content was found to be comparable or higher than the insulin/DNA content levels. At the same time the insulin/DNA levels in the transduced islets were found to be lower ($p < 0.05$) compared to untreated control islets. Data are expressed as mean level of insulin or hirudin (pmol/µg DNA) ± SEM ($n = 5$).

tion. However, relative to the two types of control islets, the hirudin-secreting islets had an impaired insulin secretion capacity. The results are presented as mean level of insulin (pmol/L) ± SEM ($n = 5$ and $n = 3$) (Figs. 5 and 6).

DISCUSSION

As a conceivable strategy to reduce IBMIR, we demonstrated that expression of biologically active hirudin can be induced in intact human islets of Langerhans using a replication defective adenoviral vector. The ability of the hirudin vector to induce synthesis of hirudin in the islets was tested using two independent techniques: a chromogenic substrate assay and an ELISA. The chromogenic substrate assay measures the level of functional thrombin-binding hirudin present in the sample while the ELISA shows the total amount of hirudin present. This could explain why the chromogenic substrate assay indicated slightly lower levels of hirudin in comparison to the ELISA method. After 3 days of culture, following

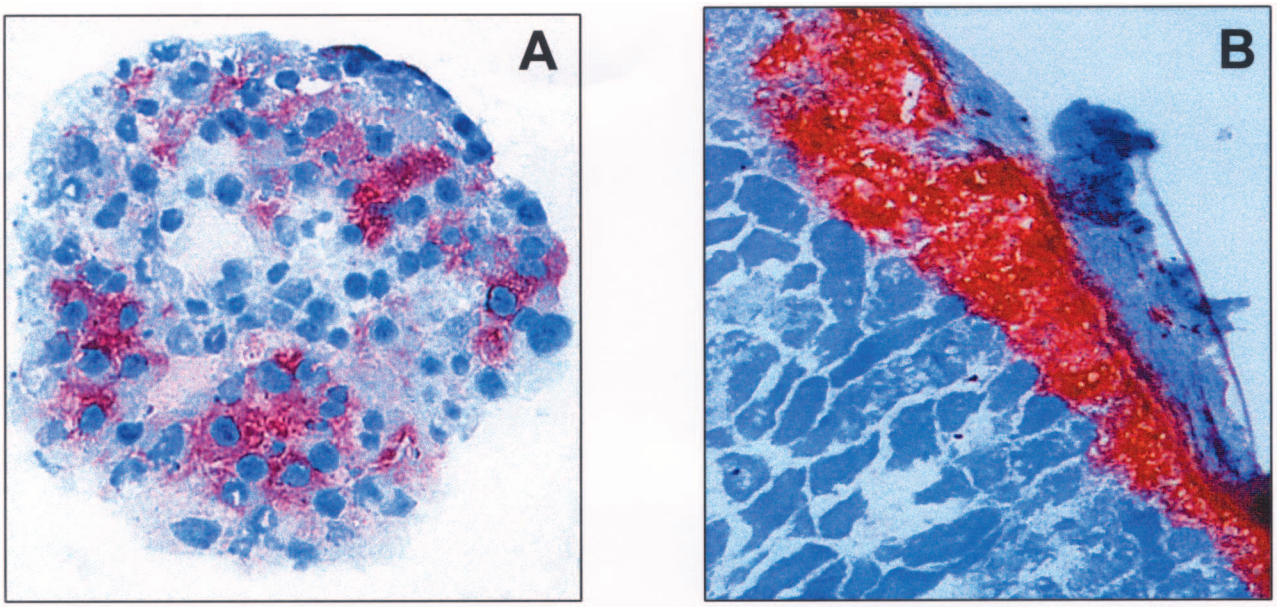

Figure 3. Immunohistochemical staining of cryosectioned human islets, transduced to express hirudin. The islets were prepared for immunohistochemistry on day 7 after transduction with adenoviral vector Av3HHV1. Parallel experiments with untreated control islets were all negative (not shown) (A). Immunohistochemical staining of serial sections from grafted human islets after transplantation under the kidney capsule in athymic (*nu/nu*) nude mice. The figure shows the expression of insulin in the graft 5 weeks after transplantation (B). Magnification ×200.

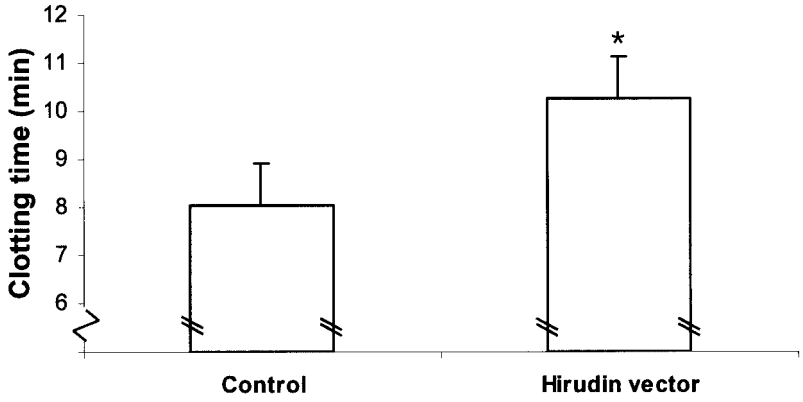

Figure 4. Clotting time in human whole blood after addition of culture supernatants taken from Av3HHV1-treated islets or corresponding untreated control islets. There was a delay in clotting time of 2.2 ± 0.4 min (29.0 ± 6.2%; $p < 0.05$). Data are presented as mean clotting time (min) ± SEM ($n = 5$).

the transduction, hirudin was first detected and the levels then gradually increased and reached plateau levels after 6–7 days. A delay in clotting time of approximately 30% could be observed in 1 ml of human whole blood with addition of 100 µl of islet culture supernatants taken from Av3HHV1 transduced islets in comparison to their corresponding controls. An increase in clotting time together with a hirudin content comparable to the insulin content in transduced islets indicates that systemic effects may be observed in transplanted patients.

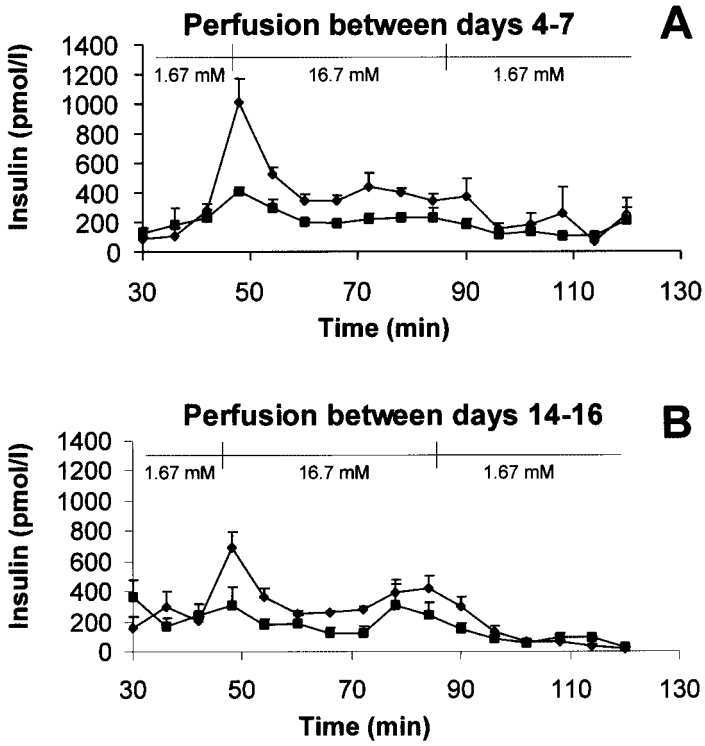

Figure 5. Islet function tested in a dynamic perfusion system. Islets from five different pancreata transduced with Av3HHV1 (black squares) or corresponding untreated controls (black diamonds). The figure shows insulin release from the islets after stimulation with 1.67 or 16.7 mmol/L glucose at two different time points. Data are expressed as mean level of insulin (pmol/L) ± SEM ($n = 5$).

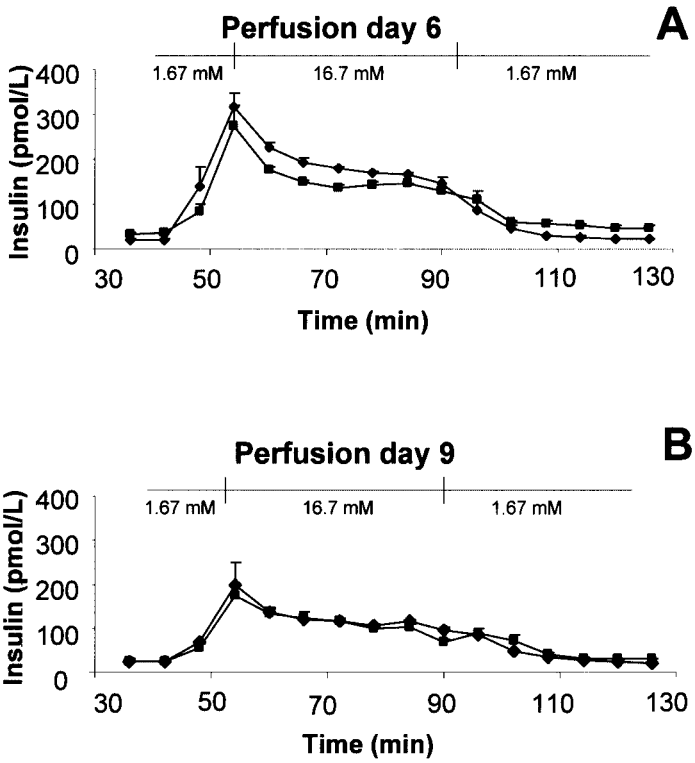

Figure 6. Islet function tested in a dynamic perfusion system. Islets from three different pancreata transduced with Av3null (black squares) or corresponding untreated controls (black diamonds). The figure shows insulin release from the islets after stimulation with 1.67 or 16.7 mmol/L glucose at two different time points. Data are presented as mean level of insulin (pmol/L) ± SEM ($n = 3$).

However, while severe systemic effects could be minimized, the microenvironment around the islets will still create a substantial concentration gradient that will inhibit coagulation and clot formation in the immediate vicinity of the islets.

Expression of biologically active hirudin from human islets may contribute to a marked graft protective effect. Hirudin not only prevents the terminal step of coagulation, the fibrin formation, but also inhibits other thrombin-catalyzed reactions, such as the activation of factors V, VIII, and XIII and platelet activation. The direct inhibitory action of hirudin on thrombin has valuable therapeutic potential as an anticoagulant. Although heparin is widely used as an anticoagulant, it cannot inactivate fibrin-bound thrombin, a major stimulus for thrombin growth. It also requires endogenous cofactors, such as antithrombin III. Furthermore, systemic administration of heparin may induce serious bleedings, and in rare occasions heparin-induced thrombocytopenia. In contrast, hirudin inhibits thrombin directly, both in the free and in the thrombus-bound state, and does not require any endogenous cofactors. Moreover, it does not induce a high degree of bleeding when administrated to animals or humans (10). The main disadvantage with hirudin when used in the clinic is that, in rare cases, patients who have been preexposed to the drug, especially within recent months, have an increased risk of developing severe anaphylactic reactions. Forthcoming experiments in large-animal models will demonstrate if hirudin alone is able to control IBMIR in vivo or whether the thrombin inhibitor should be combined with some additional drug. Further, the islet protective effect after transduction should be compared with that achieved applying systemic blockade of coagulation.

Islets were exposed to low and high glucose levels in order to examine whether the insulin secretion from the islets was affected after transduction with adenoviral vectors. Both transduced and nonexposed islets responded to glucose stimulation, but a difference in capacity could be detected in favor of islets not expressing hirudin.

The level of hirudin content was found to be comparable or even higher than the insulin content levels, on both a total amount and a molar comparison. The insulin levels in the hirudin-secreting islets were found to be lower compared to untreated control islets. Taken together this indicates that the additional protein synthesis induced may have affected the capacity of producing insulin and may also provide an explanation for the ob-

served impaired glucose response in hirudin-secreting islets. This explanation is supported by the fact that islets exposed to empty vector had a normal function, thus not suggesting that the transduction procedure itself had an influence on the insulin-secreting capacity. In this context it should however be noted that while only the β-cells express insulin, hirudin is likely to be expressed also in other cell types within the islets and also in contaminating exocrine cells.

Several steps in the process of IBMIR serve as targets for pharmacological intervention as a mean of preventing the reaction and promoting the engraftment of the islets. Different strategies to pretreat the islets before transplantation, such as adenoviral gene transfer, would be advantageous compared to systemic inhibition, because it would have little effect on the hemostasis in the recipient. Compared to other types of viral vectors (e.g., retroviruses), which induce permanent gene expression, adenoviruses are particularly suitable to overcome IBMIR during the immediate posttransplant period. Because adenoviral vectors only induce a transient expression of the inserted gene, it will leave the islets unaffected after a few weeks (i.e., the time needed for completion of engraftment). Another advantageous safety aspect, compared to many other gene therapies, is that for the purpose of islet transplantation only the grafted tissue will be exposed to the infective adenovirus in vitro prior to transplantation. Consequently, any potential side effects related to the direct patient exposure to the virus are avoided. Alternative strategies to adenoviral gene transfer would be to physically cover or modify the islet surface, thereby reducing the exposure of factors that might trigger the inflammatory response. For instance, heparinization is already in clinical use to reduce activation of the coagulation cascade on a variety of biomaterials exposed to blood. Ultimately, hirudin-transduced islets will have to be evaluated in animal models. Unfortunately, rodent models are not suitable for this purpose. First of all, it will be difficult to avoid the confounding xenoreactive processes that will take place when human islets are transplanted intraportally into rodents. Moreover, human tissue factor derived from the islets, which has been shown to be the initiator of IBMIR in human blood, has a less pronounced effect in rodents due to intraspecies molecular incompatibilities. Human islets may therefore not trigger IBMIR in the same way as in the allograft situation. Also, there exist anatomical differences: the transplanted islets, due to their size, will be entrapped in the larger portal branches in a rodent whereas in the human situation they will end up in the distal part of the portal vessels. Hence, it is likely that the most relevant model will be in nonhuman primates.

The primary aim of this work was to demonstrate the possibility to use adenoviral vectors to induce expression of protective proteins in human islets as a mean to prevent IBMIR. This study demonstrates that the adenoviral transduction may interfere with the insulin production in the islets and that a balance between transgene expression and islet function after transduction will have to be determined in order to obtain optimal islet graft function after transplantation.

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