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ORIGINAL ARTICLE

Angiostatic factors normally restrict islet endothelial cell proliferation and migration: implications for islet transplantation

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Summary

New blood vessel formation in transplanted islets occurs within 7–14 days post-transplantation through both the expansion of donor islet endothelium and ingrowth of blood vessels from the implantation organ. However, several studies indicate that although the islets attract recipient blood vessels, the formed intra-islet vascular network is insufficient, which affects islet post-transplant function. This study aimed to develop an *in vitro* model to investigate the migration and proliferation properties of isolated liver and islet endothelium. Rat islet or liver endothelium was purified using Bandeiraea simplicifolia (BS-1)-coated Dynabeads. The liver endothelium displayed an increased migration and proliferation to islet-conditioned medium. These effects were fully prevented by adding a neutralizing vascular endothelial growth factor (VEGF)-antibody. In contrast, islet-produced VEGF failed to induce islet endothelial cell migration and only had marginal effects on islet endothelial cell proliferation. These properties could, however, be activated through blocking the effects of either endostatin, thrombospondin-1 or α_1-antitrypsin. In conclusion, VEGF may attract recipient blood vessels towards intrahepatically transplanted islets, but intra-islet vascular expansion is hampered by angiostatic factors present within the islets and the islet endothelium. Inhibition of angiostatic factors early after transplantation may provide a strategy to restore the islet vascular network and improve islet graft function.

Introduction

As whole pancreas transplantation is a major surgical procedure with associated risks [1], islet transplantation has been developed as a minimal invasive approach for beta-cell replacement in type 1 diabetes. However, the results have this far been rather poor. Generally, islets from at least two donor pancreata are needed to achieve similar 1-year insulin-independence rate as for whole pancreas transplantation, and in contrast to whole organ transplantation most islet grafts fail within a 5-year period [2]. One difference between the two modes of transplantation is that whole pancreas transplants have an intact endogenous vascular system, which is directly anastomosed to the recipient's circulatory system, whereas islets become disconnected from their vascular supply when they are isolated [3]. A new vascular system is formed within 7–14 days [4,5] through both the expansion of remnant donor islet endothelium and ingrowth of recipient blood vessels [6–8]. However, the resulting vascular density seems to be decreased compared to endogenous islets [9–11], and this is associated with an impaired oxygenation [10,12] and function of the islet grafts [13–15].

A large number of blood vessels is found in the connective tissue surrounding implanted islets, but few blood vessels are present within the transplanted endocrine tissue [9]. This implicates that the islets attract blood vessels, but fail to stimulate ingrowth, and/or the capability

for remnant intra-islet blood vessels to grow and connect to recipient blood vessels is insufficient. In this study, we developed an *in vitro* model with the aim to shed light on the mechanisms for islet revascularization. We investigated the capability of liver endothelium to migrate and proliferate towards stimuli secreted from islets, and islet endothelium to migrate and proliferate towards angiogenic stimuli within the islet. Notably, islet endothelial cells proved resistant to both migratory and proliferative stimuli, and as a second part of the study we tested means to activate these capacities through the inhibition of angiostatic factors present in the islets and the islet endothelium.

Materials and methods

Animals

Wistar–Furth rats were obtained from B&K Universal, Sollentuna, Sweden, and used in all experiments. Experimental procedures followed the 'Principles of laboratory animal care' NIH publication vol. 25, No 28, revised 1996, and were approved by the animal ethics committee for Uppsala University in accordance with Swedish law.

Islet isolation and culture

Islets were isolated by using collagenase digestion followed by separation on a density gradient. Briefly, under deep anaesthesia with sodium pentobarbital (200 mg/kg i.p.; Apoteket, Gothenburg, Sweden), a laparatomy was performed and the pancreas exposed. After ligation at the ampulla of Vater, 5 ml of collagenase solution (from *clostridium histolyticum*; F. Hoffman-La Roche, Basel, Switzerland) was injected into the pancreas via the common bile duct. The animal was killed and the pancreas dissected free from surrounding tissues, removed and incubated in a water bath for approximately 16 min at 37 °C. The islets were separated from exocrine tissue by a density gradient (Histopaque-1077) centrifugation at 900 *g* for 22 min. After washing, islets were handpicked and groups of 150 islets were maintained free-floating at 37 °C (air:CO_2; 95:5) in culture medium RPMI 1640 with 11.1 mmol/l glucose (Sigma-Aldrich, Irvine, UK) supplemented with L-glutamine (200 mmol/l; Sigma-Aldrich), benzylpenicillin (100 U/ml; Roche Diagnostics Scandinavia, Bromma, Sweden), streptomycin (0.1 mg/ml; Sigma-Aldrich) and 10% (vol/vol) fetal calf serum (FCS; Sigma-Aldrich).

Islet-conditioned culture medium

After 4 days of culture to minimize contamination with exocrine tissue and passenger leucocytes, islet-conditioned culture medium was obtained by culturing islets in fresh culture medium for another 48 h. The medium was thereafter centrifuged for 2 min at 800 *g* and the supernatant was collected and stored at −70 °C until used.

Isolation and culture of islet endothelial cells

Outgrowth of islet mesenchymal cells on a collagen matrix was stimulated using a modification [16] of a previously described protocol [17]. Vascular sprouts growing out from freshly isolated islets were removed before expanding cells reached confluence. Cells were detached with 0.25% (wt/vol) trypsin (Invitrogen Life Technologies, Gaithersburg, MD, USA). By the use of Bandeiraea (Griffonia) simplicifolia (BS-1)-coated Dynabeads (Dynal Biotech, Oslo, Norway), endothelial cells are separated from contaminating cells, and a purity of more than 90% is achieved [16,18]. This was also confirmed in this study by immunostainings for CD31 and VE-cadherin.

Isolation and culture of endothelial cells from the liver

Wistar–Furth rats were anaesthetized with sodium pentobarbital (60 mg/kg i.p.) and the liver was perfused retrogradely with 25 ml perfusion buffer (142 mmol/l sodium chloride, 6.7 mmol/l potassium choloride, 10 mmol/l HEPES) [19]. The liver was then perfused with a collagenase solution (25 mg collagenase from Clostridium Histolyticum; Roche Diagnostics, Mannheim, Germany) dissolved in 8 ml Hanks' balanced salt solution (The National Bacteriological Laboratory, Stockholm, Sweden), dissected out and cut into pieces. The tissue was transferred to vials containing collagenase solution, and shaken vigorously. The emulsion was washed and the liver endothelial cells were extracted using BS-1-coated Dynabeads [16]. The beads with the bound endothelial cells were resuspended in endothelial cell culture medium [16] in a collagen-coated 24-well culture dish.

Endothelial cell migration assay

Migratory properties of islet and liver endothelium towards islet-produced stimuli (islet-conditioned culture medium) were assessed by using a Boyden chamber with collagen-coated polycarbonate membrane filter of 8.0 μm pore size (Whatman, Springfield Mill, UK). Migration attractants were added to the lower chambers, whereas the cell suspensions were added to the upper chambers. In some cases, vascular endothelial growth factor (VEGF, 20 ng/ml; Sigma-Aldrich), neutralizing antibodies to VEGF (1 μg/ml; Lab Vision, Fremont, CA, USA), endostatin (10 μg/ml; Chemicon, Temecula, CA, USA), thrombospondin-1 (tsp-1, 1 μg/ml; Lab Vision), α_1-antitrypsin (α_1-AT, 10 μg/ml; Sigma-Aldrich) or a nonsense antibody

(10 μg/ml) (IgG control; R&D Systems, Abingdon, UK) were added to the wells with migration attractants. After incubation at 37 °C (air:CO_2, 95:5) for 4 h (liver endothelium) or 5 h (islet endothelium), the cells were fixed and stained with DAPI (Vector Laboratories, Burlingame, CA, USA). The number of migrating cells was counted under a fluorescence microscope.

Endothelial cell proliferation assay

The proliferatory properties of liver and islet endothelium were investigated using a Bürker chamber. Islet endothelium was seeded to a collagen 24-well culture dish and cultured at 37 °C (air:CO_2, 95:5) in endothelial cell culture medium [16]. After 24 h, the cells in some of the wells were directly counted. Culture medium or islet-conditioned culture medium with VEGF (20 ng/ml), neutralizing antibodies to VEGF (1 μg/ml), endostatin (10 μg/ml), tsp-1 (1 μg/ml), α_1-AT (10 μg/ml) or a nonsense IgG antibody (10 μg/ml) (R&D Systems) were added to the endothelial cells in the remaining wells. After 48-h incubation at 37 °C, also the number of cells in these wells was counted in a Bürker chamber.

Statistical analysis

Values are expressed as the mean ± SEM. When only two groups were compared, Student's two-tailed *t*-test was used. Multiple comparisons between data were performed using analysis of variance (ANOVA) and Bonferronni's *post hoc* test. Statistical evaluations were calculated by comparisons of total number of proliferating or migrating cells to each stimulus. For all comparisons, *P*-values <0.05 were considered statistically significant.

Results

Endothelial cell migration

Rat liver endothelium had a higher tendency to migration than rat islet endothelial cells towards islet-conditioned culture medium (Fig. 1). Liver endothelial cells also displayed an increased migration towards islet-conditioned culture medium when compared to migration towards control medium (RPMI 1640 + 10% FCS). This chemo-attractant effect of the islet-conditioned culture medium was fully prevented by addition of a neutralizing VEGF-antibody (Fig. 2a). Moreover, addition of VEGF but not a nonsense antibody to culture medium caused migration of liver endothelial cells (Fig. 2a). The supplementation of neutralizing antibodies directed towards endostatin, tsp-1 or α_1-AT to the islet-conditioned culture medium had no additive effect on the liver endothelial cell migration.

Although we extended the time for migration from 4 to 5 h, we could not find a positive chemo-attractant effect of either RPMI 1640 + 10% FCS or islet-conditioned culture medium on rat islet endothelial cells. In all cases, only ca. 0.5% of the islet endothelial cells migrated (Figs 1 and 2b). However, when neutralizing antibodies directed towards either endostatin, tsp-1 or α_1-AT was added, the migratory capacities of islet endothelial cells towards islet-conditioned culture medium were improved (Fig. 2b). No additive effects were observed when all three antibodies were applied. Neither a VEGF-antibody, nor a nonsense IgG antibody, had any effects on islet endothelial cell migration.

Endothelial cell proliferation

Islet-conditioned culture medium had a stimulatory effect on rat liver endothelium proliferation compared to control medium (RPMI 1640 + 10% FCS) (Fig. 3a). As for the liver endothelial cell migration, this effect of the islet-conditioned culture medium was fully preventable by addition of a neutralizing VEGF-antibody (Fig. 3a). Moreover, addition of VEGF, but not a nonsense antibody, to culture medium caused increased proliferation of the liver endothelial cells. The supplementation of neutralizing antibodies directed towards endostatin, tsp-1 or α_1-AT to the islet-conditioned

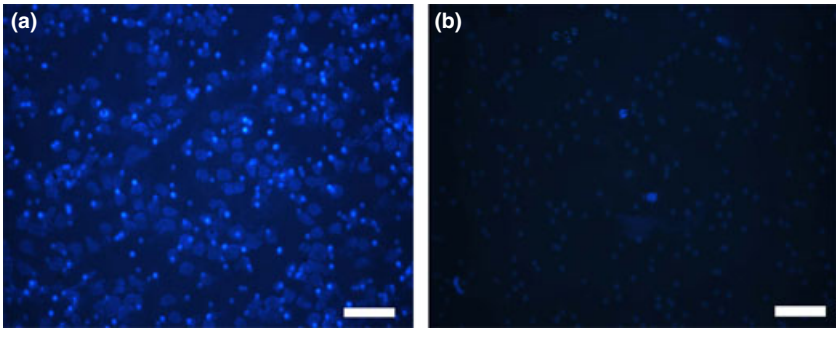

Figure 1 Migrating endothelial cells stained with DAPI. About 2.3% of the liver endothelium (a) migrated towards islet-conditioned culture medium, in contrast to only 0.5% of the islet endothelial cells (b). Scale bars represent 100 μm.

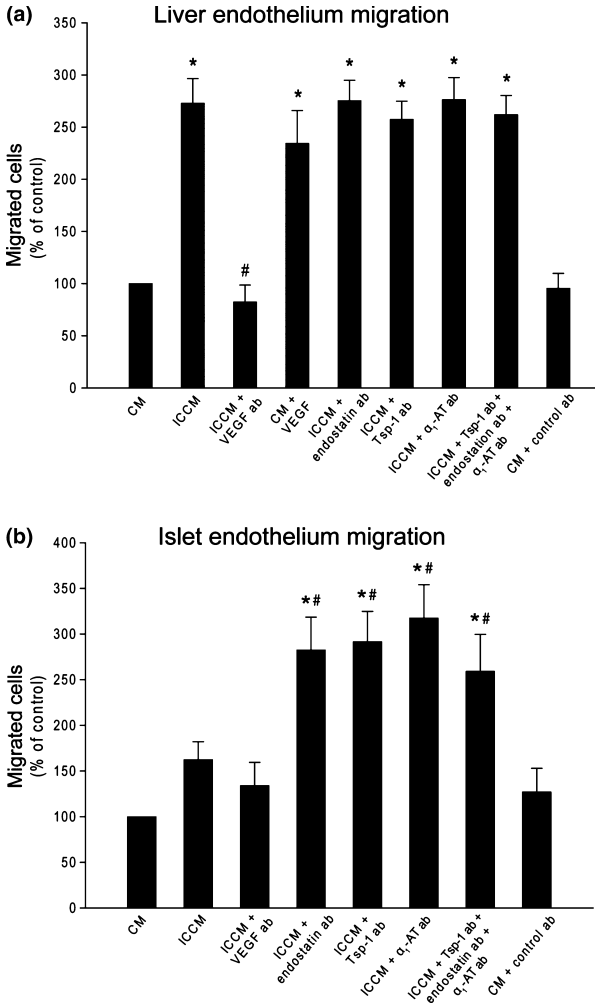

Figure 2 Rat liver endothelial cell (a) and islet endothelial cell (b) migration towards culture medium (CM) with or without a nonsense IgG antibody (10 μg/ml), islet-conditioned culture medium (ICCM), or ICCM with neutralizing antibodies to vascular endothelial growth factor (VEGF; 1 μg/ml), endostatin (10 μg/ml), thrombospondin-1 (tsp-1; 1 μg/ml) or α_1-antitrypsin (α_1-AT; 10 μg/ml). For liver endothelial cells, also the migration towards CM with addition of VEGF (20 ng/ml) was investigated. The number of migrating cells towards RPMI 1640 without serum was set to 0%, and the number of migrating cells towards CM without added VEGF or nonsense antibody to 100%. All values are expressed as mean ± SEM for seven experiments. *$P < 0.05$ when compared to CM, and #$P < 0.05$ when compared to ICCM.

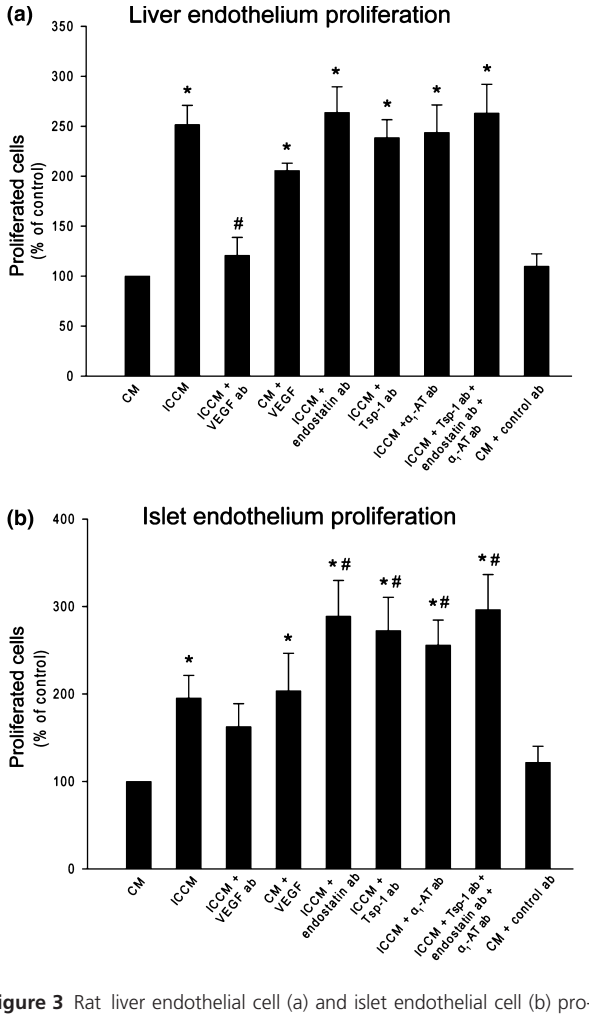

Figure 3 Rat liver endothelial cell (a) and islet endothelial cell (b) proliferation when stimulated with culture medium (CM) with or without added vascular endothelial growth factor (VEGF; 20 ng/ml) or nonsense IgG antibody (10 μg/ml), islet-conditioned culture medium (ICCM), or ICCM with addition of neutralizing antibodies to VEGF (1 μg/ml), endostatin (10 μg/ml), thrombospondin-1 (tsp-1; 1 μg/ml) or α_1-antitrypsin (α_1-AT; 10 μg/ml). The number of proliferating cells to RPMI 1640 without serum was set to 0%, and the number of proliferating cells towards CM without added VEGF or nonsense antibody was set to 100%. All values are expressed as mean ± SEM for seven experiments. *$P < 0.05$ when compared to CM, and #$P < 0.05$ when compared to ICCM.

culture medium had no additive effect on the liver endothelial cell proliferation.

Islet endothelial cell number increased very slowly during culture. A slight stimulatory effect of islet-conditioned culture medium on islet endothelial cell numbers was observed when compared to control (RPMI 1640 + 10% FCS). This effect could be abolished by addition of a VEGF-antibody to the islet-conditioned culture medium, and was mimicked by addition of VEGF but not a nonsense antibody to control culture medium (Fig. 3b). However, a further increase in islet endothelial cell proliferation was obtained after addition of neutralizing antibodies directed towards either endostatin, tsp-1 or α_1-AT (Fig. 3b) to the islet-conditioned culture medium. As for the effects on cellular migration, no additive effects were observed when more than one of the antibodies was applied.

Discussion

Pancreatic islets normally have a dense vasculature, which is of importance to provide oxygen and nutrients, allow for an accurate glucose sensing and disperse hormones to the systemic circulation. Recent evidence also indicates an important role for the islet endothelium in sustaining beta-cell functions and growth [18,20]. Insufficient revascularization of the transplanted islets may therefore be one of the contributing factors to the unsatisfying results currently seen in clinical islet transplantation [17,21]. The molecular events within the islet tissue, which are permissive for a better revascularization, are largely unknown. Despite the constitutive secretion of a number of proangiogenic factors from pancreatic islets, including VEGF and hepatocyte growth factor, adult pancreatic islet endothelial cells are normally in a quiescent state with a very low proliferation rate [18]. Increased expression of several of these factors occurs early after transplantation [22–24], but even inhibition of VEGF signalling during these circumstances does not seem to affect the revascularization process [25]. To develop strategies to stimulate the formation of a new appropriate islet capillary network, we need to explore which factors in the islets that normally attract recipient liver endothelium, as well as to stimulate the proliferation and intra-islet migration of islet endothelium. In the present work, we developed an *in vitro* model for this benefiting from our recently established technique for isolation and purification of microvascular endothelial cells using BS-1-coated Dynabeads [16].

By the used method, endothelial cells are identified by their cell surface expression of α-D-galactosyl residues, which in islets and liver are selectively expressed by the endothelium [9,26,27]. Islet-conditioned culture medium caused liver endothelial cells to migrate and proliferate in our assay, suggesting that some islet secretory product has the capacity to stimulate attraction of recipient endothelium following islet transplantation. This is also consistent with our earlier observations *in vivo* that transplanted islets induce a dense vascular network in their immediate vicinity, which fails to develop if plastic microbeads of similar size as islets instead are implanted [9]. The potent proangiogenic factor VEGF-A is produced by the beta cells [28,29] and has recently been shown to be pivotal for the development and maintenance of the dense microvascular network in endogenous islets [30–32]. We tested the hypothesis that the main islet secretory product that stimulated liver endothelial cells to migrate and proliferate was VEGF, by administering VEGF-neutralizing antibodies to the islet-conditioned culture medium. This also blocked the induced liver endothelial cell proliferation and migration. Moreover, addition of VEGF to the culture medium mimicked the boosting proliferatory and migratory effects obtained with islet-conditioned culture medium. The addition of neutralizing antibodies directed towards endostatin, tsp-1 or α_1-AT to the islet-conditioned medium had no additive effect on the liver endothelial cell proliferation and migration.

The formation of a dense microvascular network surrounding transplanted islets, but with few intra-islet blood vessels, suggests incapability to proliferation and migration of endothelial cells within islets. Migration and proliferation of endothelial cells within islets may be important to connect remnant intra-islet blood vessels to recipient blood vessels, and to support ingrowth of capillaries towards the beta-cell rich islet core. In our *in vitro* model, we observed that islet endothelial cells normally have a very low capacity for both migration and proliferation towards islet secretory products including VEGF [14,15]. This probably reflects a normal close balance between proangiogenic and angiostatic factors in pancreatic islet and islet endothelial cells. Marked overexpression of VEGF in islets [14,15], or a shift in VEGF postreceptor signalling in favour of angiogenic effects [18], has been shown feasible strategies to stimulate angiogenesis and improve intra-islet revascularization following experimental transplantation. However, un-opposing the actions of the normally occurring proangiogenic factors, through silencing the angiostatic factors, could be even more effective, especially since some of these latter factors exert their effects through inducing apoptosis in proliferating endothelium [33]. Moreover, strategies employing short-term treatment with pharmacological inhibitors for involved angiostatic factors are likely to be much more feasible in the clinical setting, than trying to increase the expression of proangiogenic factors in the transplanted tissue.

Earlier the importance of the angiostatic factor α_1-AT, produced by the endothelial cells themselves, for the low basal proliferation rate in islet endothelium was disclosed [34]. However, presence of this factor in the endothelium also seems to be of importance to prevent the development of type 1 diabetes, at least in nonobese diabetic mice [35]. We therefore decided to test not only to inhibit α_1-AT, but also two other potent angiostatic factors, i.e. endostatin and tsp-1, which has been described to be produced by islets including the islet endothelial cells themselves [27,36–38]. Notably, addition of neutralizing antibodies to endostatin or tsp-1 showed to be as potent as inhibition of α_1-AT to improve islet endothelial migration and proliferation in our assays. At least tsp-1 may be important to prevent islet vascular expansion *in vivo*, as tsp-1 deficient mice have been described to not only have hyperplastic islets but also have markedly hypervascular islets [37]. In our recent experiments, depletion of tsp-1 in islets for transplantation also seems to improve islet

graft revascularization and function [39]. Most interestingly, there seemed to be no additive effects to even further increase islet endothelial cell migration and proliferation by blocking all three investigated angiostatic factors. This may suggest that intervention towards only one of these factors is needed to fully activate the angiogenic properties of islet endothelium.

Based on our results in the herein developed *in vitro* model for islet graft revascularization, we conclude that inactivation of either endostatin, tsp-1 or α_1-AT in pancreatic islets for transplantation may be possible strategies to restore the islet vascular network and improve islet graft function.

Authorship

ÅJ, MJ and POC: designed study. ÅJ, JO and MJ: performed research, collected data and analyzed data. ÅJ and POC: wrote paper.

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