OBJECTIVE—Mesenchymal stem cells (MSCs) contribute to endothelial cell (EC) migration by producing proteases, thereby paving the way into the tissues for ECs. MSCs were added to our previously described composite EC islets as a potential means to improve their capacity for islet angiogenesis.

RESEARCH DESIGN AND METHODS—Human islets were coated with primary human bone marrow–derived MSCs and dermal microvascular ECs. The capacity of ECs, with or without MSCs, to adhere to and grow into human islets was analyzed. The survival and functionality of these composite islets were evaluated in a dynamic perfusion assay, and their capacity for angiogenesis in vitro was assessed in a three-dimensional fibrin gel assay.

RESULTS—ECs proliferated after culture in MSC-conditioned medium, and MSCs improved the EC coverage threefold compared with EC islets alone. Islet survival in vitro and the functionality of the composite islets after culture were equal to those of control islets. The EC-MSC islets showed a twofold increase in total sprout formation compared with EC islets, and vascular sprouts emanating from the EC-MSC–islet surface showed migration of ECs into the islets and also into the surrounding matrix, either alone or in concert with MSCs.

CONCLUSIONS—EC proliferation, sprout formation, and ingrowth of ECs into the islets were enhanced by MSCs. The use of composite EC-MSC islets may have beneficial effects on revascularization and immune regulation. The technique presented allows for pretreatment of donor islets with recipient-derived ECs and MSCs as a means of improving islet engraftment.

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The islets of Langerhans are micro-organs, with afferent and efferent blood vessels connecting the capillary network of the islets to the circulation system (1). Intra-islet endothelial cells (ECs) are fenestrated, and the density of the capillary network in the islets is ~10 times higher than that of the surrounding exocrine tissue (2,3). During the process of islet isolation before transplantation, the ECs in the islets lose their external vascular support; this situation contributes to their dedifferentiation, apoptosis, and necrosis during subsequent in vitro culture (4).

The formation of new capillaries during revascularization is a complex process that involves digestion of the vascular wall by proteases and the migration, proliferation, and differentiation of ECs (5). When blood vessels are assembled, ECs produce platelet-derived growth factor, which attracts supportive cells, including mesenchymal stem cells (MSCs) that can differentiate into pericytes (6).

We hypothesized that adding MSCs to our previously described composite EC islets (7) might improve the adherence of the ECs to the islets and subsequent vascularization because MSCs contribute to EC migration by producing proteases, thereby paving the way into the surrounding tissue for the immature EC sprouts (8). MSCs have also been shown to upregulate the expression of angiopoietin and vascular endothelial growth factor (VEGF) in ECs, contributing to an increase in angiogenesis and stabilization of the vasculature (9). Moreover, MSCs have been shown to possess important immune-modulating properties (10), and they do not trigger adaptive immune reactions, which could make them ideal in islet transplantation setting (11,12).

The present study describes a gentle and reproducible technique for forming EC-MSC islets that is designed to take into consideration the inherent characteristics of the various cell types involved and to take advantage of the anchorage-dependent growth of ECs and MSCs. Our data demonstrate that addition of MSCs to our composite islets enhanced the capacity of ECs to enclose the islets without compromising the functionality of the islets. Importantly, the MSCs stimulated EC sprout formation not only into the surrounding matrices, but also into the islets where intra-islet capillary-like structures were formed.

RESEARCH DESIGN AND METHODS

Isolation of islets of Langerhans. Human islets of Langerhans were isolated at the Division of Clinical Immunology at Uppsala University by using a modified semi-automated digestion-filtration method, then cultured in CMRL-1066 with supplements (islet medium) (13–15).

Islets were released for research after approval by the ethics committee at Uppsala University Hospital. Pancreata from 20 donors were used (4–7 donors per experiment). The purity of the islet preparations was 75–95%, with the exception of one preparation that was 49% pure.

Cell culture. MSCs were isolated from human adult bone marrow as previously described (10). In brief, bone marrow was harvested from the iliac crest of adult volunteers (n = 8) after approval from the ethics committee at Huddinge University Hospital. The cells were classified as MSCs on the basis of their ability to differentiate into bone, fat, and cartilage and by flow cytometric analysis (positive for CD29, CD44, CD73, CD166, and CD105, but...
negative for CD14, CD34, and CD45 and were used from passage 3-9 (FACScalibur; Becton Dickinson, Franklin Lakes, NJ). Potential EC contamination of the MSC population was assessed by flow cytometry (Becton Dickinson) (n = 3). The cells were stained for CD90 (1:100; BD Bioscience Pharmingen, San Diego, CA), the EC pan marker CD31 (1:100; Becton Dickinson), and the lectin Ulex europaeus agglutinin-I (UEA-1; 1:100; Vector Laboratories, Peterborough, U.K.). Human dermal microvascular ECs of HDMECs (PromoCell, Heidelberg, Germany) derived from adult dermis were cultured on 1% gelatin-coated flasks in EC Growth Medium Microvascular with supplements (EC medium; PromoCell). The ECs were used from passage 3-12.

Cell labeling. MSCs and ECs were labeled using CellTracker (CT) green and CT orange CMRA, respectively, according to the manufacturer’s protocol (Molecular Probes, Eugene, OR).

Thymidine assay and coculture of cells. Thymidine assays were performed according to a standard proliferation protocol measuring [3H]thymidine ([3H]thymidine (1μCi/ml) incorporation (16). ECs were plated in 24-well plates, with 25 × 10^4 cells/well in EC medium with supplements for 24 h and then cultured in EC medium without supplements but containing 0.5% fetal bovine serum (starvation medium) for an additional 24 h (basal control) or stimulated with either VEGF-A (20 ng/ml; Peprotech, London, U.K.) in starvation medium or MSC-conditioned medium (supernatant from 24-h MSC cultures in starvation medium). [3H]thymidine incorporation (at 20 – 24 h) was determined by liquid scintillation counting. Triplicate samples were assayed in three individual experiments. A mixture of 25 × 10^4 CT green-labeled MSCs and 25 × 10^4 CT orange-labeled ECs were cocultured in eight-well glass culture slides (Becton Dickinson) with EC medium and analyzed by fluorescence microscopy after 3 days.

Coating of islets of Langerhans with ECs and MSCs. Islets of Langerhans (10,000–30,000 islet equivalents) were incubated within 2 – 5 days after isolation with 0.5 × 10^5 CT orange-labeled ECs (EC islets) or with 0.25 × 10^5 CT green-labeled MSCs in combination with 0.25 × 10^5 CT orange-labeled ECs (EC-MSC islets) in a total volume of 500 μl. The cell suspensions were incubated at 37°C for 2–3 h in suspension culture tubes in EC medium and mixed gently twice per hour during the incubation. Thereafter, the EC islets, EC-MSC islets, and untreated control islets were cultured for up to 6 days at 37°C in EC medium or islet medium (CMRL, Invitrogen) until analyzed. The medium was changed every second day. Quantification of EC adherence was performed on EC islets and EC-MSC islets after 1 and 4 days of culture.

In vitro islet survival assay and cytokine and insulin content determination. A total of 100 islets per donor (n = 6) were hand picked: 50 islets were coated with 5 × 10^6 MSCs and 5 × 10^6 ECs, and 50 islets were untreated. All islets were cultured for 6 days in EC medium, with a medium change every second day. The number of remaining islets in the respective groups were counted after 1, 3, and 6 days of culture, and the islet number was confirmed by the use of a stereomicroscope (Leica Microsystems, Diagnostica, Stamford, CT), and insulin (Mercodia) contents were measured using a Gyrolab workstation (Gyros, Uppsala, Sweden), and the values were compared using a two-tailed Wilcoxon test for paired data, with significance set at P ≤ 0.05.

RESULTS

Interaction between ECs and MSCs. Before coculture, the purity of each cell population (EC and MSC) was assessed by fluorescence-activated cell sorter analysis. This analysis showed that the MSCs stained positive for the stem cell marker CD90 but were negative for the EC marker CD31 and did not bind the human EC lectin UEA-1 (Fig. 1A), indicating an absence of contaminating ECs. The ECs were negative for CD90 but were positive for CD31 and bound UEA-1 (Fig. 1B). MSC-conditioned medium induced an increase in EC proliferation, with the magnitude of the increase being similar to that produced by the addition of 20 ng/ml VEGF-A to the culture medium (Fig. 1C).

To analyze the potential cell growth effects by MSCs on ECs, we cocultured MSCs (CT green) and ECs (CT orange) in EC medium at defined cell densities for 3 days. Morphological analysis of these cultures showed that the cells grew either on top of each other or aligned with one another, indicating interactions between the cells (Fig. 1D).
Growth of ECs in double layers is an atypical phenomenon, indicating unusual growth interactions between the ECs and MSCs.

**Improved islet coating by combining ECs and MSCs.**
ECs are adherent cells that interact with the extracellular matrix. We therefore evaluated the adherence of ECs (CT orange) and MSCs (CT green) to isolated islets. After 3-h incubation of islets and ECs (EC islets), a few ECs were found to adhere to the islet surface (Fig. 2A, left panel). Combining ECs and islets with MSCs (EC-MSC islets) had no apparent effect on the coating of the islets by ECs at this early time point (Fig. 2A, right panel). However, after only 1 day of incubation, a threefold increase in the islet surface covered by ECs was observed in the presence of MSCs, as compared with EC islets (Fig. 2B, quantification in Fig. 2C). Morphological characterization of the ECs adhering to the islet surface repeatedly showed that the ECs flattened out and aligned themselves with the surface of the islets in the presence of MSCs (Fig. 2D). Analyses of sectioned EC islets and EC-MSC islets stained for insulin showed ECs adhering to both insulin-positive and insulin-negative cells in the islet preparations after 24 h of incubation (Fig. 2E–G). After 4 days in suspension culture, there was a tendency toward a decrease in the islet surface area covered by ECs in both EC islets and EC-MSC islets; however, this difference did not reach statistical significance (n = 6; P = 0.16 and P = 0.06, respectively). A few cell clusters remained uncoated in all groups (Fig. 2B, right panel, arrowheads).

**EC islet and EC-MSC islet survival and function.** Islet loss during the process of coating was evaluated by incubation of 50 hand-picked islets with ECs and MSCs according to the coating protocol. No loss of islets was observed during the formation of EC-MSC islets; however, a gradual decrease of ~20% in islet number was observed in both groups during the 6-day culture period (Fig. 3A). Cytokine analysis showed insignificant differences in the levels of IL-6 (0.005 ± 0.005, 0.07 ± 0.06 pmol/μg DNA), IL-8 (0.3 ± 0.1, 0.9 ± 0.04 pmol/μg DNA), TF (0.1 ± 0.01, 0.3 ± 0.07 pmol/μg DNA) and MCP-1 (0.2 ± 0.2, 0.6 ± 0.1 pmol/μg DNA).
pmol/µg DNA) in control islets and EC-MSC islets, respectively. The insulin content/DNA was 1.5 ± 0.4 ng insulin/ng DNA for the control islets and 0.9 ± 0.3 ng insulin/ng DNA for the EC-MSC islets on day 6.

**FIG. 2.** Islet coating by ECs and MSCs. Islets were incubated with ECs (red; A and B, left panel) or ECs and MSCs (red and green, respectively; A and B, right panel) for 3 h (A) or 24 h (B). Some islets remained uncovered in all groups (B, right panel, arrowheads). EC adherence was quantified by calculating the surface area of digital micrographs of EC islets (black) and EC-MSC islets (white) (% of total islet area) after 1 day of culture (C) (means ± SEM; n = 7; *P = 0.03). ECs in the presence of MSCs were seen to flatten and coat the surface of the islets (D, enlarged islet from B, right panel). Insulin expression was determined by immunofluorescent staining of sectioned control islets (E), EC islets (F), and EC-MSC islets (G) after 24 h of culture. The images E-G show insulin (green), EC (red), and nuclear staining with DAPI (blue). Scale bars = 100 µm. (Please see http://dx.doi.org/10.2337/db07-0981 for a high-quality digital representation of this figure.)

**FIG. 3.** Islet numbers, functionality, and dynamic insulin release of EC-MSC islets. Control islets (black) and EC-MSC islets (white) were counted after 1, 3, and 6 days (A) (mean of islet number ± SEM; n = 6; *P = 0.03; when the numbers of control islets on day 6 were compared with those of control islets on day 1). Insulin release was measured from control islets (black) and EC-MSC islets (white) cultured for 1 day (B) or 3–4 days (C). B and C show means ± SEM, n = 4.
Sprouting and invasion of fibrin gels. To evaluate the effect of angiogenesis, uncoated and coated islets were cultured in three-dimensional fibrin gels (Fig. 4). The number of sprouts per islet after 1–4 days in culture for control islets (gray), EC islets (black), and EC-MSC islets (white) (B) (mean of total sprout count ± SEM; n = 6; *P = 0.03; when EC-MSC islets were compared with control islets and EC islets at 48 h and 3–4 days). C and D: MSC-induced EC (red) with elongated sprouts in EC-MSC islets (C), as compared with EC-islets (D). E: EC sprout length measured in EC-MSC islets (white) and EC islets (black) (mean of total EC sprout length/islet ± SEM; n = 6; *P = 0.03). Scale bars = 100 μm. (Please see http://dx.doi.org/10.2337/db07-0981 for a high-quality digital representation of this figure.)

Insulin release by EC-MSC islets showed preserved dynamics during high-glucose stimulation after 1 and 3–4 days compared with control islets (Fig. 3B–C). No significant differences were seen in the stimulation indexes between control islets and EC-MSC islets at 1 day (5,090 ± 2,190 and 4,540 ± 1,300 pmol · min⁻¹ · 1⁻¹, respectively) or 3–4 days of culture (4,560 ± 1,330 and 5,090 ± 800 pmol · min⁻¹ · 1⁻¹, respectively).

Sprouting and invasion of fibrin gels. To evaluate the effect of angiogenesis, uncoated and coated islets were cultured in three-dimensional fibrin gels (Fig. 4A and C–D), in which ECs formed defined sprouts of patched ECs, and MSCs formed thin cords of aligned cells (21,22). After only 48 h, the total number of sprouts in the composite EC-MSC islets was higher than that in the control or EC islets. This difference remained after 3–4 days of culture (Fig. 4B).

When the islets came into close contact with each other, sprouts of ECs were formed between the EC-MSC islets, indicating paracrine stimulation (Fig. 4C, arrowhead). EC-MSC islets retained sprout formation over time, whereas in EC islets, the sprouts were disrupted and the ECs were separated from the islets (Fig. 4C–D). The median (± 1 quartile) EC sprout length in the EC-MSC islets was 900 μm (700–1,380 μm) and only 680 μm (300–840 μm) in the EC islets. Also, the total EC sprout length per islet was longer in the EC-MSC islets than in the EC islets (Fig. 4E).

Figure 5 shows the typical morphology of sprouts emanating from a composite EC-MSC islet after 2 days in culture. At this early stage, the sprouting was characterized by a complex course of events involving EC sprouts interacting with MSCs at the EC sprout base (Fig. 5A), ECs in close contact with MSCs (Fig. 5B–C), ECs at the leading tip of an MSC-initiated sprout (Fig. 5D), and MSCs at the leading tip of an EC sprout (Fig. 5E). ECs also migrated in groups to form a completely naked sprout with no apparent connection to the islet (Fig. 5F). Sprouts of ECs in close interaction with MSCs occurred twice as frequently as EC sprouts with nonaligned MSC cords. In the sprouts in which close cell interactions occurred between ECs and MSCs, there was a preference for ECs as the leading tip cells.

Vessel formation in islets coated with ECs and MSCs. After 24 h of culture in EC medium, the EC islets were stained for CD31 to reveal endogenous islet capillaries (Fig. 6A, green). An ingrowth of added ECs (CT orange) connecting to the native CD31-positive islet capillary network was frequently observed (Fig. 6A, arrowheads). To achieve a stable expression of DsRed, we infected the ECs with DsRed-labeled lentiviral particles. DsRed-infected ECs showed almost complete transduction after 2 weeks of culture, as visualized by merged phase-contrast and fluorescence microscopy images (Fig. 6B). The proliferative capacity of the DsRed EC in response to VEGF was unaffected (data not shown).
Confocal microscopy analyses of DsRed EC islets and DsRed EC-MSC islets after 6 days of suspension culture showed that the DsRed ECs had penetrated into the islets (Fig. 6C). Especially in the EC-MSC islets, the DsRed ECs had formed a dense network of capillary-like structures penetrating into the islets (Fig. 6D). The presence of DsRed ECs within the islets was scored at day 3 and 6. Whereas the EC islets had a mean score of 0.7 ± 0.2, the EC-MSC islets showed a higher level of penetration, with a mean score of 1.6 ± 0.4 (Fig. 6E). A comparison of the score distribution for the EC islets and EC-MSC islets showed that those with a score of 0 or 1 were predominantly EC islets, whereas the islets reaching a score of 2 or 3 were predominantly EC-MSC islets (Fig. 6F).

DISCUSSION

In the present study, modern tissue engineering was applied as a strategy to enhance the process of islet angiogenesis by combining ECs and supportive MSCs with isolated islets in culture. The capacity of the ECs to cover the islet surface was markedly enhanced by the presence of MSCs. This effect is most likely not only a result of enhanced adherence, but also a reflection of a change in the phenotype of the ECs that allowed them to stretch toward and interact with the islet surface, together with an increase in their proliferation. Importantly, in an established in vitro model of angiogenesis, the MSCs also stimulated EC sprout formation, not only into the surrounding matrices, but also into the islets where intra-islet capillary-like structures were formed.

Microvascular ECs were used to obtain an active EC phenotype with a capacity for angiogenesis that resembles a tentative future clinical situation involving harvesting autologous ECs from fat tissue (23). As compared with the aortic ECs that were used earlier (7), microvascular ECs are less covered by pericytes and, therefore, relatively more susceptible to remodeling (24). The relative capacity of EC populations to coat microcarrier surfaces is affected by the source from which they were obtained (25) and their expression of integrins, such as α5β1, α1β1, and αvβ5. The same relationship, possibly reflecting variations in integrin expression (25), seems to hold true for their ability to coat islet surfaces (cf. [7]).

Established in vitro models of angiogenesis involve culturing ECs in three-dimensional polymers, such as fibrin or collagen gels (26,27). By using this model system, we were able to demonstrate the induction of sprout formation of ECs in the presence of MSCs, a response that could be monitored over time by confocal microscopy. Similarly, Ghajar and colleagues (8) have demonstrated stimulatory effects of MSCs on angiogenesis in three-dimensional fibrin gels. The induced vessel formation was due in part to degradation of the fibrin matrix by proteases produced by the MSCs, and sprout formation was enhanced even in matrices in which the matrix density was too high for the ECs to invade when cultured alone (8). This capacity of MSCs may be of particular importance in facilitating EC migration into dense micro-organs such as islets. In addition, MSCs most likely contributed growth factors and extracellular matrix production that serve to encourage the stabilization and maturation of the EC sprouts (9). This effect of MSCs was evidenced by the increased sprout formation by EC-MSC islets in fibrin gels, where close interactions between EC sprouts and MSCs occurred to a high degree. The MSCs express platelet-derived growth factor (PDGF) receptors and may respond to PDGF production by the ECs, leading to enhanced cell interactions (6,28). A majority of the EC sprouts that interacted closely with MSCs in the fibrin gel also showed a thicker sprout phenotype, suggesting that a maturation process occurred. In contrast, several of the sprouts emanating from the EC islets eventually collapsed and detached from their base at the islet surface.

Examination of sections of composite islets showed that the added ECs formed connections to the native islet capillaries. This growth of ECs into the islets was analyzed by scoring confocal microscopy z-stacks. A marked increase in the number of ECs growing into the islets was observed in the presence of MSCs. In these samples, the ECs formed capillary-like structures deep within the EC-MSC islets, indicating a prompt and enhanced angiogenic capacity.

The technique described here for forming EC-MSC islets has been designed to take into consideration the specific needs for in vitro support of the various cell types involved and to take advantage of the anchorage-dependent growth
FIG. 6. Analysis of intra-islet vessel formation by accompanying ECs. Sectioned islets, coated with EC (red) and stained for CD31 (green), showed connections between accompanying EC and intra-islet capillaries (A, arrowheads). Yellow-colored vessels indicate CD31-positivity on added ECs. The last figure in panel A shows an enlargement of the connecting ECs. The merged phase-contrast and DsRed fluorescence microscope images/micrographs of infected human dermal microvascular ECs show an almost complete transduction efficiency with the DsRed lentiviral vector (B). C and D: DsRed ECs visualized in composite islets after 6 days of culture, EC islets alone (C), and EC islets in the presence of unlabeled MSCs (D). The insert in the last panel shows islets in bright-field with a DsRed EC overlay. Scale bars in A = 50 μm. Scale bars in B-D = 100 μm. Scoring of DsRed ECs in the islets showed an increased EC ingrowth in EC-MSC islets (white) compared with EC islets (black) (E) (mean score ± SEM; n = 7; *P = 0.03). F: Score distribution for DsRed EC ingrowth into EC-MSC islets (white), as compared with EC islets (black). (Please see http://dx.doi.org/10.2337/db07-0981 for a high-quality digital representation of this figure.)
of ECs and MSCs. By utilizing the inherent characteristics of the different cell types, we were able to establish a gentle and reproducible technique. Most importantly, no additional loss of islets occurred during the formation of EC-MSC islets and their subsequent culture (6 days), when compared with untreated islets. A culture medium designed to support microvascular EC survival was applied to promote EC adhesion and proliferation on the islet surface unafecting the islet function. Equally important is that the culture conditions applied did not promote the pro-inflammatory and procoagulant capacity of ECs (29,30), i.e., there was no difference in the level of expression of TF, IL-6, IL-8, or MCP-1 between the EC-MSC and control islets.

In the present communication, human ECs, MSCs, and islets were used. One limitation of this approach in the experimental setting is that evaluation of composite EC-MSC islet grafts in small animal models are not easily performed because of several significant species-specific differences in important areas such as angiogenesis, tissue repair, and innate immunity (31). Also, severely immunodeficient mice (SCID, nu/nu, and Rag) trigger strong innate immune responses that mainly target the vasculature of a transplanted organ due to the phylogenetic disparity between the two species, i.e., human-to-mouse is a discordant xenotransplantation model triggering activation of neutrophils, natural killer cells, macrophages, the complement and coagulation systems, and naturally occurring xenoreactive antibodies (32–39).

MSCs are currently being evaluated in clinical trials for their capacity to control severe graft-versus-host disease (40). Composite EC-MSC islet grafts could benefit from the immune regulatory effects of MSCs and from improved revascularization, thereby promoting transplantation at an extrahapatic site. This strategy requires a reliable source of recipient-derived ECs or endothelial precursor cells; however, human endothelial precursor cells can be obtained in relatively large numbers by sorting for circulating angiopoietin receptor Tie-2-positive cells (41) or from adipose-derived stem cells in human fat tissue (42,43). Our promising results with EC-MSC islets strongly suggest that they can contribute to the success of future clinical islet transplantation.

ACKNOWLEDGMENTS

This study was supported by grants from the Swedish Medical Research Council (K2005-72X-12219-09A and K2008-55X-12219-12-3), the Swedish Medical Research Council/Vinnova/Swedish Foundation for Strategic Research (60761701), the National Institutes of Health, the Novo Nordisk Foundation, Barndiabetesfonden, the Swedish Diabetes Association, and the Juvenile Diabetes Research Foundation.

The authors thank Daniel Brandhorst for assistance in statistical analysis, Deborah McClellan for editorial assistance, and the Nordic Network for Clinical Islet Transplantation for providing islet preparations.

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