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Treatment of diabetes mellitus with microencapsulated fetal human liver (FH-B-TPN) engineered cells

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ABSTRACT

Transplantation of whole human pancreases or isolated islets into patients with type 1 diabetes mellitus has been severely hampered by the scarcity of cadaveric human donor organs, which mandates search for insulin producing cells/tissue source alternatives. Recent progress in stem cell biology has started looking into functionally competent, insulin-secreting progenitor cells. It had been previously observed that induced expression of the β-cell transcriptional factor of the pancreatic and duodenal homeobox gene1 (PDX1), in human hepatocytes, may activate multiple features of the β-cell phenotype. These "FH-B-TPN" cells were shown to release insulin in response to physiological glucose stimulation both, in vitro and in vivo. However, because FH-B-TPNs lack the expression of a number of β-cell or non β-cell genes, and are associated with low insulin content, we aimed to determine whether these cells, upon physical manipulation and envelopment within "clinical grade" alginate-based microcapsules, would reverse hyperglycemia after graft into diabetic animal models.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disorder that leads to selective killing on pancreatic islet β-cells. The invariably abolished insulin secretion will induce severe hyperglycemia, associated with acute, as well as, over time, chronic secondary complications of the disease. To radically solve the problem posed by T1D different approaches have been attempted so far, including transplant of whole pancreatic or isolated, either free or microencapsulated islets of Langherans [1]. While validation of alternatives, for β-cell substitution is actually being in progress, special attention has been devoted to both pancreatic and extra-pancreatic, progenitor and embryonic or adult stem cells.

Staying with primary cell sources, the low mass of retrievable donor viable endocrine cells, suitable for transplantation, is a formidable obstacle. Such a limit could virtually be overcome, should nonendocrine cells, i.e., hepatocytes, that are known to express several molecules associated with the islet β-cell glucose-sensing apparatus, namely, glucose transporter 2 (Glut2) and glucokinase (GCK), be employed [2,3]. It has been shown [3] that cells derived from a subset of human fetal hepatocytes could be induced to express multiple β- or non β-cell related genes (i.e. glucagon) after the primary insertion of PDX1. These cells, named "FH-B-TPN cells" (FH-B-TPNs) showed an intracellular insulin storage, though it was very low (2% of the normal insulin content of human β-cells). Upon culture in serum-free, activin-supplemented culture media, FH-B-TPNs were able to alleviate hyperglycemia upon graft into immunoincompetent diabetic rodents.

However, cell therapy, whether be it based on primary adult, or progenitor, or adult/embryonic stem cells faces several problems. Aside of immune destruction upon graft into allo-/xenogenic hosts, function of the isolated cells could be facilitated by their embedding in a surrogate extra-cellular matrix. On this purpose, new developing strategies look into coupling need for readily available cell sources with artificial biomaterials, capable to prevent immune rejection and favor steady tissue differentiation and function. In effects, 3D, unlike conventional cell culture, closely mirrors the physiological tissue developmental conditions by enabling cell–cell and cell–matrix interactions. Additionally, 3D culture systems look necessary to induce spontaneous cell differentiation into pluripotent stem cell populations [4,5]. Moreover, 3D culture of multipotent adult stem cells seems to increase their adipogenic and osteogenic-oriented differentiation while enhancing secretion of trophic factors [6–8]. Fueled by need for "ad hoc" differentiation of stem cells into the intended cell phenotypes, the differentiation potential of multicellular stem cell aggregates in response to soluble factors has been evaluated showing that nutrient supply to the interior of the cell aggregates may limit the efficacy of such

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strategies [9,10]. Here comes the role of cell microencapsulation that, based on highly purified and nontoxic biopolymers, enables creation of both physical immunobarriers and soft and pliable microenvironment. Microencapsulation consists of entrapping cells within natural polymeric membranes, that also serve for immunoprotection of the embodied tissue. Thanks to microcapsules, only oxygen/nutrients, but not humoral and/or cellular mediators of the host's immune system, may cross the artificial membrane, thereby granting the microencapsulated cell/tissue survival and function. Furthermore, the capsular 3D microenvironment, consisting of ultrapure, endotoxin and protein-free alginates, has been shown to promote better growth, differentiation, and maturation of different cell types, including but not limited to, mesenchymal stem cells, mESCs, hESCs or hIPCs (human Insulin Producing Cells) [11].

Our previous studies showed that β-cell precursors, derived from in vitro human islet cell monolayers, may be induced not only to express higher levels of β-cell markers, such as GK and GLUT2, but also to gain back glucose-stimulated responsiveness ability [12]. Upon envelopment in alginate/poly-L-ornithine (NAG/PLO) microcapsules, these cells survive long-term and enable blood glucose lowering in diabetic rodents. Because cell aggregation, induced by our ultrapure alginates seems favor differentiation, we have promoted this process mechanically [13–15]. FH-B-TPNs, since their fetal origin, potentially are prone to differentiation towards other cell lineages, such as adipocytes, osteocytes and neurons. Trans-conversion between liver and pancreas is conceivable, given the common developmental pathways of the two organs: in fact, both derive from appendages of the upper primitive foregut endoderm. It is plausible that the late separation of liver and pancreas, during organogenesis, might have left pluripotent cells that are capable of originating both tissue cell lineages [16,17]. Liver cells in culture expressed lower levels of the epithelial marker E-Cad and higher levels of N-Cad as compared to whole liver tissue. Such a switch in cadherins expression usually hallmarks an epithelial to mesenchymal transition (EMT) process, which also occurs in pancreatic islet cells in culture. Both tissues show many common features, including responsiveness to glucose and a large group of specific transcriptional factors [18].

The transdifferentiation capacity towards the β-cell phenotype may be enhanced by special culture conditions in low serum media with no induction additives [19].

The aim of our work was to determine whether FH-B-TPNs, genetically engineered to produce insulin by PDX1 gene insertion, would retain stem cell markers oriented towards β-cell phenotype differentiation. We thought that this potential would benefit from either cell culture in serum-free medium and subsequent cell aggregation, or microencapsulation within alginate-based membranes, that serve for ECM-like biomatrices. Ultimately, starting from FH-B-TPNs, we aimed at developing an insulin-producing, encapsulated cell product, throughout its in vivo graft testing into nonimmunosuppressed diabetic animal models.

2. Materials and methods

2.1. Cell culture

FH-B-TPNs (a kind gift from Prof. Shimon Efrat, University of Tel Aviv, Sackler Medical School, Tel Aviv, IL), were cultured in Dulbecco's Modified Eagle's Medium (DMEM 25 mM glucose; Celbio), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and G418 (400 μg/ml) (Normal Medium; NM) at 37 °C and 5% CO_2. The cells were cultured by dilution in fresh medium to a density of about 1×10^6 cells/flask every three days, after treatment with 0.05% trypsin/EDTA (Gibco, Milan, Italy). Cell viability was assessed by Trypan Blue exclusion test (0.1% in 0.9% NaCl; Sigma, Milan, Italy).

FH-B-TPNs, had been obtained by Prof. Shimon Efrat [20] by immortalization and transduction with a viral vector encoding the catalytic subunit of human telomerase (hTERT), while the PDX1 gene was inserted using a second viral vector into a single-cell clone of hTERT-expressing fetal liver cells.

2.2. Immunocytochemistry

Immunocytochemistry was performed on cultured single cells, or cell aggregates, or encapsulated cell aggregates. Both, free and encapsulated cell aggregates were cryosectioned (9 μm thick sections) upon fixation in 3.7% PFA o/n, while unencapsulated cells were allowed to adhere onto glass slides (12 mm in diameter), and subsequently treated with 3.7% PFA for 10′. In both instances, permeabilization (when necessary) with 0.01% triton x-100 in D-PBS for 15′ and block with 1%BSA in D-PBS for 1 h were performed. The primary antibody was added overnight, while a secondary antibody was added for 1 h (Supplementary Tables 5,6). Cell nuclei were counterstained with Hoechst 33342 (1:200, Sigma), mounted on Mowiol®4-88 (Calbiochem), and prepared according to the manufacturer's instructions, while 0.6% 1,4-diazabicyclo(2.2.2)octane (DABCO, Sigma), as an anti-fading agent. Images were collected on Axio Observer.A1 fluorescence microscope (Zeiss) equipped with AxioVision software driven camera. Eosin/Haematoxylin staining to assess morphology of both free and encapsulated cell aggregates was applied.

2.3. Transcriptional expression analysis by RT-PCR and qPCR

Total cellular RNA was extracted from the cultured cells or aggregates using a commercial kit (SV Total RNA Isolation System; Promega, Milano, Italy) according to the manufacturer's instructions. Total RNA extraction from the encapsulated cell aggregates required that the capsules were dissolved using 55 mM Na citrate for 5′ followed by centrifugation at 1200 rpm to retrieve the cell aggregates. After assaying RNA by agarose gel electrophoresis, cDNA was synthesized from 0.5 to 1 μg total RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Milan, Italy). The obtained cDNA was used as a template for PCR reaction containing 300 nM of the primer pairs for amplification of the indicated gene product. RT-PCR primers were designed using sequences from GenBank (htpp://www.ncbi.nlm.nih.gov/Genbank) (Supplementary Table 7). qPCR amplifications were performed using the SsoFast EvaGreen Supermix as directed by the manufacturer (Bio-Rad Laboratories). The amplification conditions were optimized for the MxPro 3000 Stratagene and assays were run in triplicate in two step PCR reaction under the following conditions: Taq polymerase activation, 95 °C for 30″ followed by 40–45 cycles at 95 °C for 10″, at the indicated annealing temperature for 10″. PCR products were demonstrated to be a single PCR product by melting curve and electrophoresis analysis.

2.4. Differentiation protocols

2.4.1. Osteogenic

Osteogenic differentiation was induced by incubating the cells monolayers with 10^{-8} M dexamethasone, 0.2 mM ascorbate phosphate, 10 mM β-glycerolphosphate in α-MEM (Invitrogen) and 10% v/v FBS [21] After 3 weeks, the cells cultured and treated in 24-well plates were fixed with 10% formaldehyde, and stained with 2% freshly prepared Alizarine Red, pH 4.2, for 3 min; calcium deposition was visualized and photographed. Total RNA was extracted from FH-B-TPN's, cultured and treated in 75 cm^2 flasks, retro transcribed, and assayed by qPCR for osteopontin gene expression (see supplementary data).

2.4.2. Adipogenic

Adipogenic differentiation was induced in the confluent FH-B-TPN cell cultures by treatment with 0.5 mM 1-methyl-3-isobutylxanthine, 10^{-6} M dexamethasone, 10 μg/ml insulin, and 200 μM Indomethacin in Dulbecco's (DMEM) high glucose (Invitrogen) with 10% FBS [21]. At 3 weeks of culture, the cells incubated in 24-well plates were fixed with 10% formaldehyde, and treated with fresh Oil Red O for 10 min, at room temperature. Lipid droplets were detected and photographed. At the same time, total RNA was extracted from the cells, cultured and treated in 75 cm^2 flasks as above reported. cDNA was assayed by qPCR for PPARγ2 and FABP4 genes expression (see Supplementary data).

2.4.3. Neural

Neural differentiation was pre-induced by overnight treatment with b-fibroblast growth factor (bFGF; Peprotech, LiStarFish, Milano, Italy) (10 ng/ml) in DMEM (Biospa) and 20% FBS (phase 1), followed by 5 h induction with 2% DMSO and 200 μM butylated hydroxyanisole in DMEM plus 2% FBS (phase 2) [22]. FH-B-TPN differentiation capacity into neural cells was assayed in comparison with untreated controls. We performed morphological evaluation and qPCR for Tubβ3 (see Supplementary data) gene expression after transdifferentiation.

2.5. Differentiation of FH-B-TPN into insulin-secreting cells

Endocrine differentiation of FH-B-TPN into insulin-secreting cells followed three steps:

1) Culture maintenance in serum free medium (SFM)

The cells were placed in Dulbecco's modified Eagle's medium (DMEM-HG; 25 mM glucose) containing antibiotics in the presence of ITS 1× (10 mg/ml insulin, 5.5 mg/ml transferrin, and 5 mg/ml selenium, Gibco, Milan, Italy) throughout 6 days.