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Rapid purification of human ductal cells from human pancreatic fractions with surface antibody CA19-9

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Abstract

Generating human insulin-secreting cells for cell therapy of diabetes represents a highly competitive world challenge. Human ductal cells can give rise to islets in vivo and in vitro. The goal of this study was to devise a rapid sorting method to highly purify human ductal cells from pancreatic tissue using a pan-ductal membrane antibody carbohydrate antigen 19-9 (CA19-9). Human pancreatic sections confirmed antibody specificity. The human exocrine fraction (30% ductal cells) was sorted with magnetic bead technology or by FACS. Immunocytochemistry post-sorting determined ductal cell content. The manual magnetic bead technique resulted in $74\% \pm 2$ ($n = 4$) CA19 positive cells. Whereas the automated AutoMACS technique ($n = 5$) yielded $92.6\% \pm 0.5$ CA19-9 positive cells with only a minor beta cell contamination ($0.2\% \pm 0.03$); cell yield post-sorting was $12.9\% \pm 2.5$ ($1.69 \pm 0.41 \times 10^6$ cells) with $51.7\% + 6.5$ ($n = 5$) viability post-sorting. The FACS ($n = 6$) resulted in $97.1\% \pm 0.82$ CA19-9 positive cells, a cell yield of $25.5\% \pm 5.6$ ($5.03 \pm 1.0 \times 10^6$), with $72.1\% \pm 6.1$ viability post-sorting.

Keywords: Human pancreatic ductal cell; Cell therapy of diabetes; Cell selection; Neogenesis; Surface marker; Alternative source of insulin-secreting cells

Radical improvements in the success of human islet transplantation have highlighted a foreseeable shortage of insulin-secreting cells in the near future [1]. Use of primary islets of Langerhans forbids all hope of developing this treatment on a large scale. Thus, generating human insulin-secreting cells for cell therapy of diabetes has become a major scientific and highly competitive world challenge. Insulin-secreting cells have been obtained in vitro from diverse sources including embryonic stem cells [2–5]. But problems include the lack of expression in these cells of key transcription factors like PDX-1 [6], the formation of teratomas post-transplantation [4], in addition to regulatory and ethical issues [7]. Several authors suggest that somatic stem cell sources can give rise to insulin positive cells including hepatic oval cells [8], splenic-derived cells [9], pancreatic nestin positive cells [10], and marrow-derived cells [11]; the latter two are highly disputed [12,13].

The description of the persistence of pancreatic precursors in the ductal epithelium capable of proliferating and differentiating in the adult pancreas offers an attractive, clinically relevant alternative for the production in vitro of homologous insulin-secreting cells. First suggested in vitro in man by our group in 1996 [14] ductal cells have been shown to give rise to endocrine islets in both mice [15] and men [16]. All of these reports, with the exception of one study involving a human pancreatic ductal cell line [17], have been unconvincing due to the contamination of starting preparations with mature endocrine islets. Thus, convincing evidence of human ductal cell differentiation depends on pure ductal starting preparations.

The goal of this study was to devise a rapid method to highly purify human ductal cells from pancreatic tissue using a ductal specific membrane antibody to carbohydrate antigen 19-9 (CA19-9). Carbohydrate antigen 19-9

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is a pan-ductal cell marker labeling the luminal surface of human ductal cells. CA19-9 reacts with sialosylfucosyllactotetraose, corresponding to sialylated blood group antigen Lewis A [18].

We previously described that the exocrine fraction which could be obtained after digestion of pancreata from healthy brain-dead donors [19] was a rapid and abundant source of viable human ductal cells (1.7×10^9/pancreas) [20]. Herein true human ductal cells have been purified from the exocrine fractions early on in culture prior to the acino-ductal switch using the surface antibody carbohydrate antigen 19-9 (CA19-9). Pure human ductal populations will subsequently allow us to perform a large scale analysis of gene and protein expression patterns of true ductal cells, in particular with reference to beta cells and dedifferentiated acinar-derived cells. This technique of human ductal cell purification allows a more precise study of ductal cell differentiation in vitro and in vivo.

Methods

All reagents were obtained from Sigma–Aldrich, France, unless otherwise specified.

Specificity of antibody. Control pancreatic sections of normal pancreata were used to determine the specificity of the CA19-9 antibody (NCL-CA19-9, Novocastra, UK).

Human pancreatic cultures. Human pancreata were harvested from adult brain-dead donors in agreement with French Regulations and with the Ethical Committee of our Institution. Pancreatic tissue was digested according to the Automated Ricordi technique with modifications [21] followed by density gradient purification. The exocrine fraction was recovered in the pellet after islet purification, washed, and cultured in Dulbecco's minimum essential medium (DMEM, Invitrogen, Scotland) with 3 g/L glucose containing 10% fetal calf serum (FCS, Euro Bio Laboratories, Les Ulis, France), 1% insulin, transferrin, selenium (ITS), and 50 μg/ml Geneticin (G418) to limit fibroblast overgrowth [22], as described [19]. After 12–36 h culture (37 °C, 5% CO_2) exocrine cells were detached (trypsin–EDTA 1× diluted 1:5), washed, and clusters were further dissociated to single cell suspensions with Splittix solution (1.8 mg/ml; Biomedia, France) or Splitase (Autogenclear, Bioclear, France) at 37 °C. Ethylenediaminetetraacetic acid (EDTA), 5 mM alone provided a more gentle dissociation over 2.5 h and was subsequently adopted. Cells were washed, filtered (70 μm), resuspended in culture medium with 10% FCS, and placed in the incubator for a 90 min recovery period.

Flow cytometry analysis and FACS. A kinetic study of CA19-9 labeling was performed in flow cytometry on 1–7 day cultured, fixed cells. Briefly, trypsinized cells were fixed in Stabilcyte Cell stabilization Buffer (BioE, France) and used within 2 months according to the manufacturer's instructions. Incubation of fixed cells or fresh cells (FACS) with CA19-9 antibody (1 μg/ml/10^6 cells) or the isotypic antibody (Negative Control IgG1, Dako) was followed by FITC-conjugated anti-mouse IgG antibody (Dako France). Cells were systematically refiltered on a 40 μm filter prior to sorting. Analysis and cell sorting were performed using a FACS Vantage (Becton–Dickinson, France). Dead cells labeled with propidium iodide (2 μg/ml) were excluded.

Magnetic bead sorting. The cells were incubated (1 h, 4 °C) with the CA19-9 antibody (1 μg/ml) followed by incubation with magnetic beads coated with a mouse IgG (kit Dynal CELLection Pan Mouse IgG, Dynal, France, SA). Following filtration (70 μm screen) labeled cells were selected with a magnet as described by the manufacturer; beads were subsequently dissociated from cells following DNAse treatment.

To sort in the automated bench-top magnetic cell sorter (AutoMacs Miltenyl Biotec, France), dissociated cells ($n = 6$) were incubated with CA19-9 antibody (0.25 mg/ml/10^7 cells) for 5 min at 4 °C. After several washes in buffer (PBS, 0.5%, culture grade bovine serum albumin, and 0.75 mg/ml EDTA) goat anti-mouse IgG micro beads (Miltenyl Biotec, Paris, France) were added (15 min, 4 °C). Cells were suspended in buffer (10^7 cells/ml) and sorted with the positive selection program ('Possel') according to the manufacturer's instructions.

Controls post-sorting. Viability was determined throughout the procedure with trypan blue. Cytospins (Cytospin 3, Shandon Scientific, Cheshire, UK) were prepared on cell suspensions (700 rpm, 3 min) before and after sorting on positive and negative fractions. Phenotypic controls included evaluating the percentage of ductal (CA19-9), endocrine (chromogranin A (Dako), proinsulin/insulin (Biogenesis: clone 5E4/3), c-peptide (TEBU Bio SA, France: clone MFB-HB)), and acinar (amylase (Sigma)) positive cells. As described [20], antibodies were revealed with EnVision system (Dako, France) using either D-3,3-diaminobenzidine (DAB), Pthaloblue or Pthalored chromogen kits (Kirkegaard & Perry Lab, Gaitherburg, MD). Nuclei were stained with Carazzi's hematoxylin. Controls included replacing the primary antibody with PBS containing BSA (1%). Nonspecific binding was blocked with goat serum (30 min).

Results

The specificity of the surface antibody CA19-9 was confirmed by immunohistochemistry on sections of human pancreata (Fig. 1A). In normal human pancreatic tissue ($n = 3$) $24.2 \pm 1.9\%$ of cells express CA19-9, including main duct, interlobular, intralobular, intercalated, and centroacinar ductal cells.

Following the purification phase of the pancreatic islet isolation technique, the light density gradient fractions including the islet rich fraction (Fig. 1B) contained large ductal fragments (inset). The pellet fraction, containing the heavier exocrine fraction which is often discarded after islet purification, can be recovered from the COBE bag (Figs. 1C and D). Figs. 1E and F show that 12 h cultures of human exocrine fractions contain approximately 30% of ductal cells (cytokeratin 19 positive), 60% acinar (amylase positive), and 1–5% endocrine cells (chromogranin A positive). Initial experiments demonstrated that the trypsinization of human exocrine fractions cultured overnight in suspension resulted in poor viability (<20%). Adherent cultures survived trypsinization more readily with $77.9\% \pm 4.9$ viability ($n = 4$, range 72–98%). Thereafter, exocrine fractions were systematically cultured overnight in monolayer prior to sorting.

A preliminary study using flow cytometry analysis quantified the increase in CA19-9 positive cells during the acino-ductal switch of exocrine fractions (Fig. 2). Ungated light scatter profile of the human exocrine fraction population demonstrating 2 populations of large cells with high granulosity (SS) and small cells with

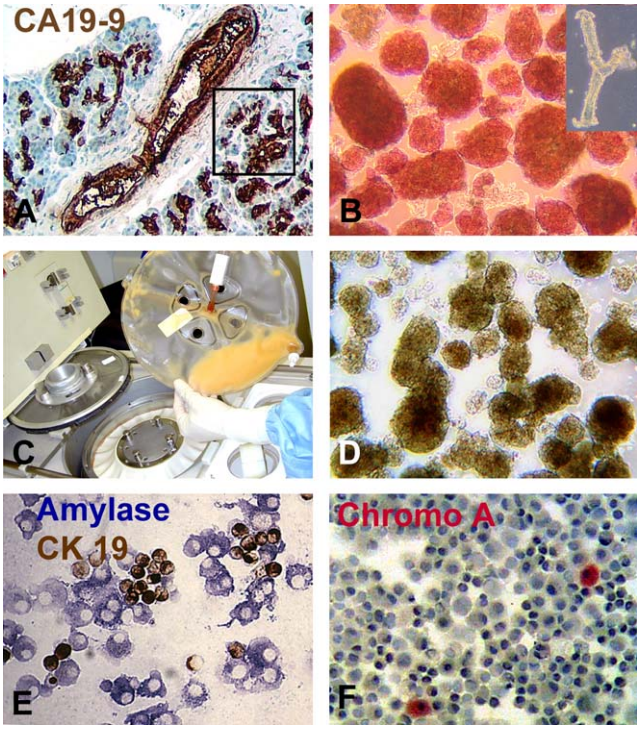

Fig. 1. (A) Immunohistochemical characterization in human pancreatic section with surface CA19-9 antibody revealed with DAB (10×) showing specificity for all ductal cells, including centroacinar cells. (B) Inverted microscopical aspect of the light islet rich fraction colored with dithizone following density gradient purification which contains ductal fragments (inset). (C) After islet purification, the pellet which contains the heavier exocrine fraction which is routinely discarded can be recovered from the COBE bag. (D) Inverted microscopical aspect of the human exocrine fraction. Immunohistochemistry of human exocrine fraction after 12 h of culture. (E) Cells (30–35%) are cytokeratin 19 positive ductal cells (DAB, brown), and 60% amylase positive acinar cells (Pthaloblue), and (F) whereas only 1–5% fractions are chromogranin A positive endocrine cells (PhaloRed).

low granulosity. Ungated fluorescence intensity profile of CA19-9 staining on logarithmic scale vs number at day 2 of culture showing a highly (32.3%) and weakly (67.7%) fluorescent CA19-9 population. The mean percentage of CA19-9 positive cells in the cultured exocrine fraction of $n = 5$ pancreata was $23 \pm 2.7\%$ (day 1), 37.4 ± 3.8 (day 2), $40.3 \pm 3.7\%$ (day 3), $47.9 \pm 5.3\%$ (day 5), and $50.5 \pm 5.2\%$ (day 7). Compared to initial day 1 levels, the expression of CA19-9 became statistically significantly ($p < 0.05$) different after 2, 3, 5, and 7 days culture. Therefore, sorting was routinely performed prior to 2 days culture (bold box) to avoid sorting de-differentiated acinar cells that take on ductal markers, including CA19-9 expression.

Results of the three techniques are summarized in Table 1. The manual magnetic bead technique ($n = 4$) resulted in $74 \pm 2\%$ CA19-9 positive cells after 12 h culture. Immunohistochemistry revealed that the source of the poor purity was linked to the recovery of clusters of cells composed of CA19-9 positive and negative cells. Systematic filtration through a 40 μm porosity reduced cell yields but improved purity and was thus implemented immediately prior to subsequent sorting protocols.

The AutoMACS technique was performed on 28.3 ± 4 h cultured exocrine fractions with a post-trypsin viability of $77.9\% \pm 4.9$ ($n = 5$, range 64–98%), and a viability before sorting of $59.5\% + 4.2$ (range 49–73%). A total of $22.6 \times 10^6 \pm 7.5$ viable cells were sorted resulting in a cell yield of $1.69 \pm 0.41 \times 10^6$ (range $1.1–2.9 \times 10^6$), a $12.9\% \pm 2.5$ yield (range 3.9–21.3%). Post-sorting immunohistochemistry demonstrated that $92.6 \pm 0.5\%$ of cells were CA19-9 positive (range 78–99%) with only a minor endocrine contamination $0.2 \pm 0.03\%$. Post-sorting viability was $51.7\% + 6.5$ ($n = 5$). We noted that CA19-9 positive cells expressed CA19-9 with different intensities. We tested different conditions attempting to optimize the immunolabeling to select only for cells highly expressing the antigen, however we were unable to achieve this in the Automacs (results not shown).

Fluorescent activated cell sorting was tested as a third approach (Fig. 3) in six preparations following 41.1 ± 4.4 h of culture. The fluorescence intensity profile plot (log scale) with reference to size (forward scatter, FSC) demonstrates the CA19-9 positive and negative populations. The positive fraction (R5 + R4) could be further gated based on small (R4) and large (R5) CA19-9 positive cells. The isotypic antibody negative control (dotted line) is superposed on the CA19-9 plot. After gating for dead cells and debris, CA19-9 positive cells represented $31.1\% \pm 3.2$ ($n = 4$) of the exocrine cell fraction. Immunohistochemistry confirmed that the cells were $97.1\% \pm 0.82$ CA19-9 positive (range 97–99%). Cell viability with trypan blue was $76.7\% + 5.3$ after cell dissociation and $72.1\% \pm 6.1$ post-sorting. Habitually $23.7 \times 10^6 \pm 6.3$ cells were sorted with a yield of $5.03 \times 10^6 \pm 1.03$ CA19-9 positive cells, equivalent to a sorting yield of $25.5\% \pm 5.6$ ($n = 4$). Cell morphology post-sorting demonstrated the presence of some damaged cells following FACS purification. FACS allowed both the separation of highly fluorescent cells from less intensely labeled cells, and the highly CA19-9 positive cells could be separated by size. The latter approach selects for the smaller true centroacinar ductal cells. The small highly fluorescent cells represented $16.1\% \pm 2.4$ of cells. Of the $23.7 \times 10^6 \pm 6.3$ cells sorted, $2.59 \times 10^6 \pm 0.6$ were small highly fluorescent cells. Viability was $51\% \pm 4.9$ following sorting.

Discussion

Different strategies exist to obtain purified ductal cells including tertiary 3D cultures of ducts in collagen [23], selective attachment [16], and density purification coupled with serum free culture [24]. Ductal cultures can

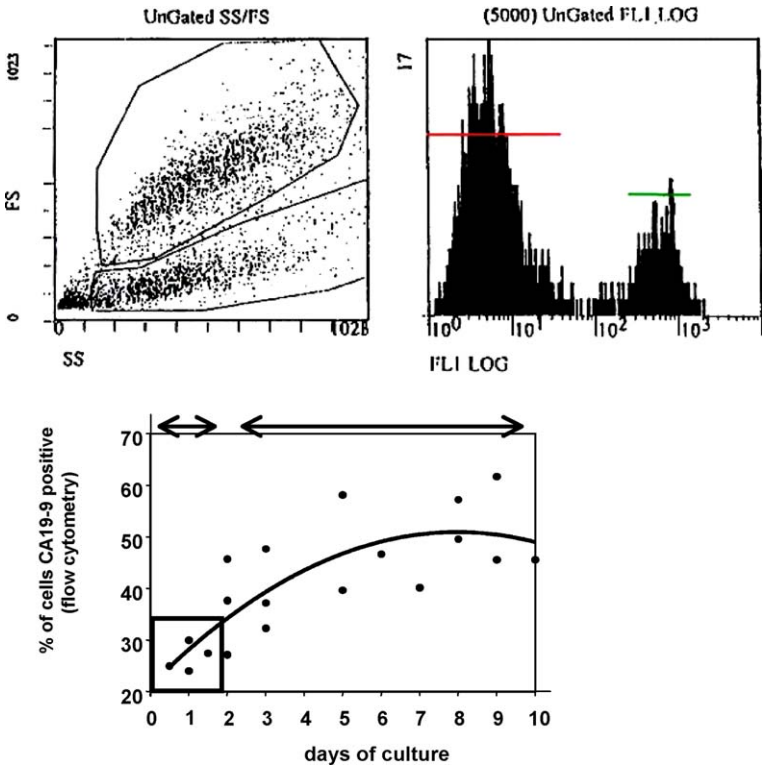

Fig. 2. Dissociated exocrine fraction stained with CA19-9 followed by secondary fluorescein isothiocyanate labeled antibody. (Left) Ungated light scatter profile of the human exocrine fraction population demonstrating 2 populations: cells with high granulosity (SS) and small cells with low granulosity. Ungated fluorescence intensity profile of CA19-9 staining on logrithmic scale vs number at day 2 of culture showing a highly (32.3%) and weakly (67.7%) fluorescent CA19-9 population. A kinetic graft of flow cytometry ($n = 6$) results (means ± SEM) of the percentage of highly fluorescent CA19-9 positive cells in culture. Sorting was routinely performed prior to 2 days culture (bold box) to avoid sorting dedifferentiated acinar cells that take on ductal markers.

Table 1
Quantitative comparison of the three sorting techniques

Technique	*n*	Time	CA19-9 (% + SEM)	Insulin (% + SEM)	Viability (% + SEM)	Yield (%)
Manual bead	4	12 h	74 ± 2	ND	ND	ND
AutoMacs	5	28.3 ± 4	92.6 ± 0.5	0.2 ± 0.03	51.7 ± 6.5	12.9 ± 2.5
FACS	6	41.1 ± 4	97.1 ± 0.8	—	72.1 ± 6.1	25.5 ± 5.6

also be obtained via dedifferentiation of exocrine fractions [19,22,25] or islets [26,27]. Microdissection of ductal cells is another option for RNA, DNA or protein expression studies, with inherent quantitative problems. Herein, we opted for the macrodissection of ductal structures with the ductal specific antibody Sialyl Lewis A carbohydrate antigen 19-9. Other antibodies were tested including cystic fibrosis transmembrane receptor and carbonic anhydrase 2 yielding poor results (results not shown).

Human ductal cells represent approximately 25–30% of total pancreatic cells. Digestion and purification of the human pancreas with the combined Ricordi/Edmonton technique yields large ductal cells (main duct, interlobular, and intralobular) copurifying with the islets and other light density fractions. Small ductal cells including intercalated and in particular centroacinar cells purify with the exocrine pellet fraction. This study concentrated on purifying ductal cells from the exocrine pellet fraction. However, since CA19-9 is a pan-ductal marker the technique equally could be used to purify the larger ductal structures in the lighter density gradient fractions.

The exocrine pellet fraction is an abundant source of human ductal cells. In certain culture conditions without serum, the acinar cells have been reported to die of apoptosis [28–30]. As we and others have shown [25], the ductal phenotype arises as the result of the proliferation of the 30% preexisting ductal cells as well as the de/transdifferentiation of the 60% acinar cells [19,22]. The

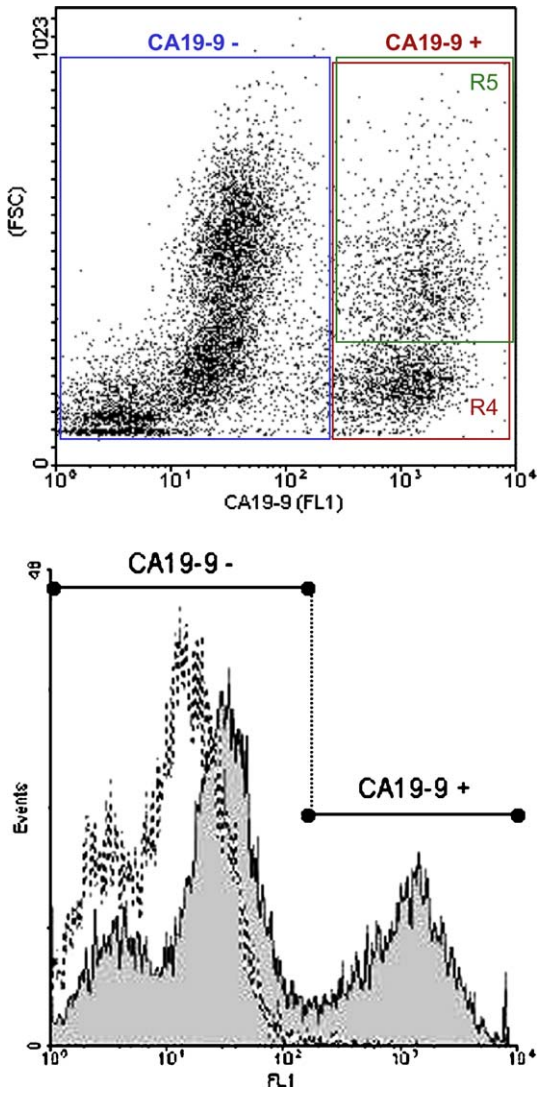

Fig. 3. FACS analysis of CA19-9 stained exocrine fraction fluorescence intensity profile (log scale) plot vs forward scatter (size) demonstrates the CA19-9 negative population and the positive fraction (R5 + R4) which could further be gated according to R4 small cells and R5 large cells. Labeling with the isotype-matched antibody control (dotted line) superimposed over the CA19-9 staining profile.

kinetic study of the exocrine pellet fractions more precisely characterized this phenotypic acinar-ductal switch to determine the point at which the expression of CA19-9 increased statistically over initial levels. In subsequent studies, sorting was always performed prior to 2 days culture to obtain true ductal cells and avoid sorting dedifferentiated acinar cells that take on ductal marker expression, notably CA19-9 expression, with increasing culture duration.

The reexpression of ductal cell markers like CA19-9 has equally been reported for islets [23]. Prior to sorting exocrine fractions were less than 5% endocrine. The Automacs approach yielded high purity ductal cell fractions with extremely low endocrine contamination post-sorting ($0.2\% \pm 0.03$). The FACS procedure is longer, results in higher cell damage, and requires a trained technician or engineer to run the apparatus. Although the purity was superior with FACS sorting, for the reasons listed above we routinely adopted the Automacs procedure over the FACS.

Convincing evidence using a human ductal cell line suggests that ductal cells can differentiate in the presence of GLP-1 if they express the transcription factor PDX-1 [17]. Neurogenin 3, a proendocrine transcription factors can also drive ductal cells into an endocrine pathway leading to an increased expression of beta specific markers including insulin. Besides these two mechanistic studies, the endocrine differentiation from ductal precursors in the majority of the duct to islet studies has been somewhat unconvincing due to the systematic contamination of starting preparations with mature endocrine islets, including both our original article [14] and Bonner–Weir's [16] spectacular article considered as the 'proof of concept.' Moreover, Otonkoski's group suggests that NCAM endocrine depleted human islet/duct rich fractions no longer undergo differentiation in experimental conditions described by Bonner–Weir [16]. Doubts on the endocrine precursor nature of ductal cells have recently been shed following tracer studies [32]. Thus, convincing evidence of human ductal cell differentiation depends on pure ductal starting preparations which can be obtained with the methods described herein, following purification with the surface marker carbohydrate antigen 19-9.

The Edmonton group [30] assessed the long-term function of clinical human islet grafts in 11 patients and reported surprisingly a correlation between the number of ductal cells transplanted and an increased acute insulin response to glucose (AIRg) at 2 years post-transplant ($r = 795$; $p < 0.005$). These results would suggest that grafts composed of a higher number of ductal cells exhibit enhanced long-term function which could possibly result from a greater frequency of islet cell neogenesis from ductal cells post-transplant. This technique applied to the light density fractions post-islet purification yielding large ductal cells in parallel with the denser fractions, yielding the smaller centroacinar and intercalated ductal cells, would allow us to establish which ductal cells give rise to endocrine islets in vivo.

Islet neogenesis in pathological pancreata is observed in both small and large ductal structures. Although the neogeneic capacity of the large ductal cells was demonstrated by Bonner–Weir's and Peck's groups, the former was unable to achieve islet neogenesis from the smaller centroacinar ductal cells associated with the pellet layer [16]. The literature suggests that the centroacinar cells can differentiate into endocrine islets. We demonstrated that ductal cells from the exocrine fraction reexpressed PDX-1 and early neuroendocrine markers (unpublished observation, J.K.C.), suggesting that these cells have indeed endocrine precursor

potential. One study now suggests that the small ductal cells derived from the human exocrine fraction can become insulin positive cells in vitro [29]. Pipeleers' group recently has confirmed that post-natal duct-cell preparations derived from the human exocrine fraction of young donors under 10 years old can generate new beta cells in vivo [31]. Cell sorting techniques could determine if the veritable human ductal cells differentiate post-transplantation and more precisely which type of ductal cells, and if indeed the ductal cells derived via dedifferentiation from the abundant acinar cells in the discarded pellet contribute to this phenomenon.

Conclusion

The surface marker CA19-9 can be used for the purification of human ductal cells. The automated magnetic cell sorting technique (Automacs) is a simple rapid (90 min) technique requiring little operator expertise, consistently yielding large numbers of pure human ductal cells from pancreatic tissue. The automated system exists for the clinical grade preparation of cells (Clinimax) and may provide a simple means to rapidly isolate human ductal precursor cells to be transplanted together with human islets.

Pure human ductal populations will subsequently allow us to perform a large scale analysis of gene and protein expression patterns of true ductal cells, in particular with reference to beta cells and dedifferentiated acinar-derived cells. This technique of human ductal cell purification will subsequently allow more precise studies of ductal cell differentiation in vitro and in vivo.

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