Human Pancreatic Islet-Derived Progenitor Cell Engraftment in Immunocompetent Mice

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The potential for the use of stem/progenitor cells for the restoration of injured or diseased tissues has garnered much interest recently, establishing a new field of research called regenerative medicine. Attention has been focused on embryonic stem cells derived from human fetal tissues. However, the use of human fetal tissue for research and transplantation is controversial. An alternative is the isolation and utilization of multipotent stem/progenitor cells derived from adult donor tissues. We have previously reported on the isolation, propagation, and partial characterization of a population of stem/progenitor cells isolated from the pancreatic islets of Langerhans of adult human donor pancreata. Here we show that these human adult tissue-derived cells, nestin-positive islet-derived stem/progenitor cells, prepared from human adult pancreata survive engraftment and produce tissue chimerism when transplanted into immunocompetent mice either under the kidney capsule or by systemic injection. These xenografts seem to induce immune tolerance by establishing a mixed chimerism in the mice. We propose that a population of stem/progenitor cells isolated from the islets of the pancreas can cross xenogeneic transplantation immune barriers, induce tissue tolerance, and grow. (*Am J Pathol* 2004, 164:817–830)

A revelation in the field of tissue regeneration has been the finding that pluripotent stem cells, or multipotent progenitor cells, exist not only in embryo blastocysts and fetal gonadal ridges but also exist in probably all organs of the adult body.^{1–8} Stem cells manifest at least four important properties: they are highly mobile, have the ability for self renewal, differentiate into different cell lineages given exposure to appropriate local environmental stimuli known as growth factors or morphogens,¹ and may induce immune tolerance.⁹ Morphogens are typically provided by localized spatial regions of mesen-

chyme, so called mesenchymal niches. Many studies have been reported about how neural stem cells can be converted into blood (although controversial),¹⁰ and hematopoietic stem cells can be converted into brain,^{11,12} muscle,¹³ heart,¹⁴ and liver.^{15,16} It also has been shown that pancreas-derived stem cells can become liver¹⁷ and liver stem cells can differentiate into pancreas.¹⁸

Recently, stem/progenitor cells have been isolated from both the ducts of the exocrine pancreas,^{19,20} and the islets of Langerhans that make up the endocrine tissue of the pancreas.^{21–23} We have been characterizing a population of cells isolated from human pancreatic islets. These cells, nestin-positive islet-derived stem/progenitor cells (NIPs), initially express the protein nestin, a marker of neural stem cells, can be passaged extensively *in vitro*, and can be differentiated *in vitro* into islet-like clusters that produce islet hormones, eg, insulin and glucagon, by their exposure to known differentiation agents such as the insulinotropic, neogenic hormone, glucagon-like peptide-1.^{21–23} Analyses of NIPs by flow cytometry show that they contain a substantial subpopulation of side population cells, similar to pluripotent hematopoietic side population cells.²²

One aspect of human NIPs that we are currently investigating is their potential efficacy to produce insulin in amounts sufficient to achieve glycemic control when transplanted into diabetic mice. Although we initiated the studies by transplanting human NIPs under the kidney capsules of immunosuppressed nude mice, we discovered that human NIPs successfully engraft when transplanted into immunocompetent C57BL/6 mice without a requirement for immunosuppression. Here we show that nestin-positive cells in the pancreas do not express either class I or class II major histocompatibility (MHC) antigens, describe the transplantation studies and some morphological characteristics of the xenografts, and demonstrate the development of mixed chimerism in the

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mice by the detection of donor human Y-chromosome and human-specific antigens. Sixty days after the intravenous administration of human NIPs to immunocompetent mice, we find by flow cytometry that 1.5 to 9.0% of the hematopoietic cells in spleen, bone marrow, and peripheral blood leukocytes, express human HLA class I antigens. Further, these mice given NIPs by a single systemic intravenous injection develop focal regions of chimerism in multiple organs, including intestine, kidney, pancreas, heart, skeletal muscle, and brain, as detected by *in situ* hybridization using a human-specific probe to repetitive ALU sequences and by human ALU-specific polymerase chain reaction (PCR). We propose that human NIPs may induce a state of immune tolerance in immunocompetent mice such that the human tissue is recognized as self by the immune system of the mice. These findings suggest that when stem/progenitor cells derived from a human adult tissue (eg, pancreatic islets) are transplanted into immunocompetent mice they appear to induce tissue tolerance, resist graft *versus* host disease, and differentiate into specific cellular phenotypes, defined by the expression of markers of epithelial tissue (mixed keratins) and mesenchymal tissue (vimentin) as well as the human-specific marker antigen for hematopoietic tissue [leukocyte common antigen (LCA), CD45]. We suggest that our findings may hold promise for the use of these cells in future approaches to applications for the regeneration of degenerated tissues in human diseases.

Materials and Methods

Reagents

Basic fibroblast growth factor and epidermal growth factor were obtained from Sigma (St. Louis, MO). Leukemia inhibitory factor was obtained from Chemicon (Temecula, CA). The Y-chromosome DNA hybridization probe labeled with Spectrum Red was purchased from Vysis Inc. (Downers Grove, IL) and antibodies were from BD Pharmingen (Lexington, KY).

Mice

C57BL/6 mice, 8 to 10 weeks old, were obtained from Charles River Laboratories (Wilmington, MA) for use in the transplantation of human NIPs either under the kidney capsule or for systemic administration, by injection into the tail vein.

Isolation and Culture of NIPs and Karyotype Analysis

Human islet tissue was obtained from the Juvenile Diabetes Research Foundation Center for Islet Transplantation, Harvard Medical School (Boston, MA), and the Diabetes Research Institute, University of Miami School of Medicine (Miami, FL). NIPs were isolated and propa-

gated as described previously.²³ Briefly, islets were washed and cultured in RPMI 1640 medium containing 10% serum, 11.1 mmol/L glucose, antibiotics, sodium pyruvate, β -mercaptoethanol, and growth factors. Within several days, nestin-positive cells grew out from islets. These cells were cloned and expanded in medium containing 20 ng/ml each of basic fibroblast growth factor and epidermal growth factor or, 10 ng/ml of human leukemia inhibitory factor (Chemicon) in the presence or absence of serum. In certain instances the cells were maintained in the absence of serum. Chromosomal analysis and karyotyping were performed at the Dana Farber/HCC cytogenetics core laboratory, Brigham and Women's Hospital, Harvard Medical School.

Administration of Human NIPs to Immunocompetent Mice

Between 10^5 and 10^6 NIP cells prepared from human male or female donor islets were transplanted under the kidney capsules of 26 female C57BL/6 mice without immunosuppression. The transplantation procedure has been described previously.²⁴ The mice were sacrificed 15 to 60 days after the transplantations and the kidneys containing the NIP grafts were removed for histomorphological and biochemical analyses including fluorescence *in situ* hybridization (FISH) for the detection of human Y-chromosome, immunocytochemistry for detection of human-specific antigens, and detection of enhanced green fluorescent protein (EGFP). Four mice were each given a total of 10^5 to 10^6 NIPs intravenously via the tail vein, administered twice 1 month apart. Sixty days after the first intravenous injection of cells, the mice were sacrificed. Spleen, bone marrow, and peripheral blood were analyzed for the presence of chimerism by fluorescence-activated sorting of cells using the human marker HLA-A, -B, -C. The pancreata, kidneys, livers, hearts, skeletal muscle, intestines, brains, and lungs were collected from the mice for *in situ* hybridization using a DNA probe specific for the detection of human Y-chromosome, and human repetitive ALU DNA sequences as measures of tissue microchimerism.

Antibodies

The rabbit anti-human nestin was a gift from Dr. C. Messam (National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD). The mouse monoclonal antibody against human CD-45; HLA-A, -B, -C (class 1 HLA marker); mixed keratins; and vimentin were purchased from BD Pharmingen. MHC I and II antibodies were from Serotec Inc. (Raleigh, NC).

Immunocytochemistry

Frozen kidneys with grafts were embedded in OCT compound and 5- μ m tissue sections were prepared. Tissue sections were fixed in acetone, blocked with normal goat

serum, followed by avidin D and biotin-blocking solution, and then incubated overnight in mouse anti-human antibodies to CD45 (LCA) vimentin, and mixed keratins or in anti-rat antibodies to MHC I, MHC II, as described previously.²¹ Sections were rinsed with phosphate-buffered saline (PBS) and incubated with biotinylated goat anti-mouse serum. Sections were then immersed in diaminobenzidine substrate solution and a red-brown precipitate was visualized. Immunostaining for insulin, glucagon, and PDX-1 of the subrenal capsular grafts was performed on 5- μ m serial sections of frozen tissue. Antisera used were: guinea pig anti-insulin IgG and guinea pig anti-glucagon serum (Linco Research Inc., St. Charles, MO) and polyclonal antisera to human PDX-1 (IPF-1) (anti-serum R253²⁵). Normal guinea pig IgG and normal rabbit serum served as controls. Immunoreactivity was developed using both the glucose oxidase method, with nitro blue tetrazolium as the substrate, and the peroxidase method, with diaminobenzidine as the substrate (Vector Laboratories, Burlingame, CA).

Human ALU Sequence Histochemistry

Tissue sections were hybridized with a human ALU DNA probe kit following the recommendations of InnoGenex (San Ramon, CA). ALU sequences are repetitive DNA elements that are unique to primates. The human ALU DNA probe does not hybridize to mouse DNA at the specified hybridization conditions. Briefly, proteinase K reagent was added to cover the sections and was incubated at room temperature for 10 to 15 minutes. Sections were then washed, refixed with 1% formalin, and hybridized with the human ALU probe at 37°C overnight. Then, tissue sections were washed repeatedly with PBS-Tween 20 buffer and incubated with primary antiserum to the ALU probe followed by incubation with secondary antibody. Subsequently, sections were immersed in streptavidin-peroxidase substrate solution and the color was developed with an amino ethyl carbazole chromogen (Sigma). Sections were counterstained with hematoxylin and mounted in SuperMount for visual microscopic examination.

PCR

PCR amplification of human Alu-sx and mouse c-mos sequences. DNA was extracted using the QIAamp DNA extraction kit (Qiagen, Valencia, CA). Alu-sx primers were ALU-forward: 5'-GGCGCGGTGGCTCACG-3' and ALU-reverse: 5'-TTTTTGTAGACGGAGTCTCGCTC-3'. Primers for c-mos were MOS-forward: 5'-GAATTCAGATTGTG-CATACACAGTGACT-3', and MOS-reverse: 5'-AACATTTTTCGGGAATAAAAGTTGAGT-3'. DNA amplification and primer selection were as previously described.²⁶ PCR conditions were 94°C for 5 minutes followed by 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 60 seconds, and final extension 72°C for 10 minutes. PCR products were resolved by electrophoresis on a 1.5% agarose gel, and transferred overnight to a cellulose filter. Southern blot hybridization was performed by auto-

radiographic detection with an ALU internal probe: 5'-CCACTTTGGGAGGCCGAGGCGGGTGGATCATGAGG-TACAAGGTGAAACCC-3'. Further confirmation of the presence of human sequences was achieved by cloning of the PCR fragments into the pCRII plasmid by TA cloning (Invitrogen Corp., Carlsbad, CA) and direct sequencing.²⁶

Transfection of NIPs

Human NIPs, maintained in long-term cultures containing serum and human leukemia inhibitory factor, were transfected with a plasmid expressing the EGFP according to published protocols (GenePorter; Gene Therapy Systems, Inc., San Diego, CA). Cells were transfected sequentially twice within a period of 4 days to enhance the number of transfected cells. By this method ~20 to 30% of the cells expressed the green fluorescent protein.

FISH

FISH was performed with a commercially available kit according to the manufacturer's recommendations (Vysis). Briefly, 5- μ m frozen sections on silanized slides were fixed with methanol and acetic acid, washed with PBS and 2 $\times$ standard saline citrate at 73°C for 2 minutes. Then, sections were immersed in pepsin solution at 37°C for 5 minutes, washed in 2 $\times$ standard saline citrate at room temperature for 1 minute, and dehydrated for 1 minute in 85% and 100% ethanol successively. The CEP Y-chromosome probe (orange fluorescence) mixture was denatured at 73°C, and applied onto the sections. Hybridization was performed at 37°C. Sixteen hours later, the slides were washed in 2 $\times$ standard saline citrate for 1 to 4 minutes and mounted in a 4,6-diamidino-2-phenylindole (blue fluorescence)-containing solution to allow visualization of the nuclei.

Flow Cytometry Analysis

Peripheral blood leukocytes and bone marrow cells as well as splenocytes were isolated for flow cytometry analysis.²⁷ Cells were washed in Hanks' buffer containing 10% bovine serum albumin, incubated with an Fc-receptor blocking solution, and then incubated with phycoerythrin-labeled mouse anti-human HLA-A, B, C antigen. Cells obtained from animals that received tail vein injections of human NIPs, as well as control mice that did not receive NIPs, were analyzed by flow cytometry.

Results

Cytogenetic Analysis of NIPs

We have described previously the procedures for the isolation and culture of the NIPs.²¹⁻²³ We have now examined the karyotype of the NIPs. Analysis of chromosome preparations of a NIP culture (clone Hu-6a, passage 06) revealed a 46, XY diploid cell line in two cells

Figure 1. Karyotype of human NIPs. The NIPs were passaged in continuous culture for 5 months. The NIP culture was derived from pancreatic islets obtained from a male donor pancreas. The karyotype is that of a diploid (euploid) 46, XY male.

analyzed (Figure 1). Analyses of three additional cells revealed 46 chromosomes.

Transplantation of NIPs Under the Kidney Capsules of Immunocompetent Mice

NIP cultures were prepared from islets obtained from several different human male and female donors provided by islet procurement and transplantation centers. Human NIPs (10^5 to 10^6 cells) were transplanted under the kidney capsules of 26 immunocompetent female C57BL/6 mice throughout the course of 2.5 years. The mice were sacrificed from 15 to 60 days after the transplantations. Eight of the mice died within 24 hours of causes related to the anesthesia and/or surgery. In 10 of 18 mice the NIPs successfully engrafted and grew into readily visible masses of tissue (Figure 2A). The NIPs transplanted into 8 of 18 mice failed to engraft. We then set out to determine whether the grafts contained human

Figure 2. Engraftment of human NIPs in immunocompetent mice. **A:** Mouse kidney removed 35 days after transplantation of human NIPs under the kidney capsule. The mouse was a female C57BL/6 without immunosuppression. The whitish mass of tissue at the top of the kidney (arrow) is the expanded NIP graft, contrasting with the kidney tissue. The engraftment of NIPs has been achieved in 10 of 26 of nonimmunosuppressed mice so transplanted and examined from 15 to 60 days after the xenograft. **B:** A human NIP transfected with a plasmid expressing EGFP viewed under UV light. The cells expressing EGFP were transplanted under the kidney capsule of an immunocompetent mouse without immunosuppression. **C:** Gross morphology of the human NIP graft expressing EGFP in a kidney removed 15 days after transplantation of the NIPs. The graft (G) and kidney (K) were viewed under UV light. **D:** EGFP-positive cells present in explant cultures prepared from the graft shown in **C** (5 days of culture).

Figure 3. Histomorphology of a human NIP graft in an immunocompetent mouse. **A:** Tissue sections (H&E stained) of a C57BL/6 nonimmunosuppressed mouse kidney and human NIP graft 35 days after transplantation of human NIPs under the kidney capsule. K, Mouse kidney; G, human NIP graft. Note that the transplanted human NIP cells grow and are not rejected by the mouse recipient of the graft. **B:** The graft contains localized regions of glandular-like tissue (liver). **C:** Magnification of glandular-like liver tissue in graft. **D:** Mesenchymal-stromal-like tissue in the graft. **E:** Magnification of the stromal-like tissue in the graft. **F:** Nestin-positive cells in the pancreas do not express either class I or class II MHC antigens. Dual immunostaining (green) of a rat pancreas with antisera to MHC class I antigen (**left**) and MHC class II antigen (**right**). Nestin-positive cells are immunostained in red. The absence of yellow cells, which would indicate that a cell co-expresses nestin and MHC antigens, shows that NIPs do not express either MHC class I or class II antigens. **Dashed lines** denote boundaries of pancreatic islets. Original magnifications: $\times 100$ (**A**); $\times 1000$ (**C**); $\times 400$ (**E**).

tissue (xenografts). First, we transfected human NIPs with a plasmid expressing EGFP as shown in Figure 2B. These transfected NIP cultures were then transplanted under the kidney capsule of a mouse. Fifteen days later, the kidney was removed and under UV light the graft showed intense EGFP fluorescence (Figure 2C). Later, the graft was excised, enzymatically dissociated, and cultured for an additional 5 days. Transfected NIPs

present in explant cultures showed expression of EGFP (Figure 2D).

Examination by hematoxylin and eosin staining of the histology of the grafts showed circumscribed tissue with considerable pleomorphism without evidence of invasion into the adjacent kidney parenchyma (Figure 3A). In various grafts, and tissue sections of individual grafts, focal regions of tissue were seen that had the appearance of

Figure 4. Immunohistochemical analyses of human NIP grafts in immunocompetent mice. **A:** Immunostaining for human-specific LCA, CD45, in a section of mouse kidney transplanted (kidney capsule) with human NIPs examined 35 days after the transplant. The brown-colored cells (shown in the figure as grayish-black) are the cells in the xenograft that express human LCA in the recipient mouse. K, Kidney; G, graft. **B:** LCA immunostaining of the graft shown at higher magnification. **C:** Keratin immunostaining. **D:** Vimentin immunostaining. **E:** Detection of pancreatic endocrine hormones insulin and glucagon, and pancreas- and duodenal-specific homeobox transcription factor, PDX-1 in serial sections of expanded grafts of human NIPs 15 days after transplantation under the kidney capsules of immunocompetent C57BL/6 mice. Islands of pancreatic endocrine-like tissue are found scattered about in the graft, which grew to ~50% the volume of the kidney without evidence of invasion or oncogenicity. The **top row** shows two representative fields of the grafts. GP-IgG and normal rabbit serum are the control antisera for insulin and glucagon/PDX-1, respectively (**bottom row**). Immunostaining done by glucose oxidase method (shown in the figure as grayish-black). Immunocytochemistry was also done by diaminobenzidine (Vectastain ABC) with similar results. **Asterisk** denotes a burnout defect artifact in the CCD camera. Original magnifications: $\times 100$ (**A, E**); $\times 400$ (**B-D**).

glandular epithelial tissue (Figure 3, B and C). The predominant tissue type appeared to consist of mesenchymal stromal-like tissue (Figure 3, D and E). These observations indicate that human NIPs engraft and survive for at least 60 days after transplantation under the kidney capsules of immunocompetent mice and appear to represent different tissue types. It should be noted that heretofore transplantation of human tissue xenografts into immunocompetent mice results in a complete rejection of the graft after 5 to 10 days.^{24,27,28} Therefore human NIPs may have a special property by which to induce the host (mouse) to recognize them as self and not to reject the xenograft. In this regard we show that the nestin-positive cells within rat pancreatic islets do not express either

MHC I or MHC II antigens (Figure 3F). Thus NIPs, as they reside in the islets, may be immunologically naive because they appear not to express either MHC I or MHC II antigens.

Examination of the xenograft tissues by immunohistochemistry using antibodies specific for human-specific LCA (CD45) a marker of hematopoietic tissues, keratin, an epithelial cell marker and vimentin, a mesenchymal marker, revealed clusters of immunopositive cells within the grafts concentrated along the border of the graft with the kidney (Figure 4). The more peripherally located tissue in the graft is probably of mouse origin, indicating a host *versus* graft cellular proliferative response, but not typical of a vigorous graft rejection response. The reac-

Figure 4. *Continued*

tion is more typical of a delayed sensitivity granulomatous response with fibrosis and scarring, rather than an acute rejection with lymphocytic infiltration.

We also examined the grafts for the expression of pancreatic endocrine tissue. In one mouse, a large graft examined 15 days after transplantation of human NIPs showed scattered islands of tissue that immunostained positive with antisera to the hormones insulin, glucagon, and the transcription factor PDX-1 (Figure 4E). The expression of endocrine tissue was particularly prominent in this mouse, but was also observed to a lesser extent in another transplanted mouse.

Evidence for Tissue Microchimerism Originating from Kidney NIP Grafts

Because it seemed reasonable that the acceptance of the human xenografts by the mice was because of tissue tolerization by the establishment of chimerism we examined the various organs of the graft-bearing mice for

evidence of microchimerism. Therefore immunohistochemical staining with antibodies to human LCA (CD45) was performed and revealed apparent microchimerism in the kidney parenchyma (Figure 5). The establishment of microchimerism of donor cells in graft recipients is known to induce tolerance to donor tissue.^{29–35} In particular, the dendritic cells, special antigen-presenting T cells, are known to induce tolerization.^{36,37} To test for the presence of chimerism in organs of the female recipient mice other than the kidney, the FISH assay specific for the human male Y-chromosome (donor tissue), was performed. By semiquantitative assessment of human Y-chromosome-positive nuclei present in 20 to 30 × 400 fields of tissue sections the prevalence of human cells was estimated to be 0.02 to 0.2%. This analysis indicates that microchimerism is widespread throughout many organs of the mouse including, kidney, pancreas, skeletal muscle, liver, and heart (Figure 6). These findings in mice transplanted with human NIPs show that a preparation of human tissue islet-derived cells engraft when transplanted under the kidney capsules of

Figure 5. Evidence for the establishment of microchimerism of human tissue in the kidney parenchyma of an immunocompetent mouse given a subrenal capsular graft of NIPs 35 days before. **Left:** Renal tubules within a section of kidney immunostained with an antibody specific for human LCA (CD45). **Right:** Low-power micrograph of the NIP engraftment kidney as it appears 35 days after the NIP transplant under the kidney capsule. Immunostained with antibody to LCA/CD45. G, Graft; K, kidney. Original magnifications: $\times 400$ (left); $\times 100$ (right).

fully immunocompetent mice and suggest that the mechanism for the acceptance of the xenografts is the establishment of tissue microchimerism.

Establishment of Microchimerism by Administration of Human NIPs Systemically to Immunocompetent Mice

To further investigate the potential capabilities of human islet-derived NIPs to successfully engraft in immunocompetent, nonimmunosuppressed mice we injected human NIPs (10^5 to 10^6 cells) into the tail veins of four immunocompetent C57BL/6 mice. The mice were sacrificed 60 days after the systemic injection of the NIPs. The establishment of micro/mixed chimerism was examined by flow cytometry of cells extracted from mouse bone marrow, spleen, and peripheral blood and by histohybridization of several organs of the mouse using a hybridization probe specific for the detection of human genome-specific ALU-repetitive DNA sequence elements, an assay for human tissue that became available during the course of these studies. ALU-repetitive DNA sequences are specific to the human genome and at the proper hybridiza-

tion conditions the ALU hybridization probe does not cross-hybridize to mouse DNA.

The results of the flow cytometry analyses using human-specific HLA-A, -B, -C monoclonal antibody showed substantial chimerism in bone marrow, spleen, and peripheral blood leukocytes of 0.71 to 9.2% (Table 1 and Figure 7). The nonspecific background of fluorescent cells was determined to be 0.6% for spleen and peripheral blood leukocytes and 1.7% for bone marrow (Figure 7). By human ALU histohybridization chimerism was found in all tissues examined: small intestine, kidney, heart, skeletal muscle, liver, pancreas, and brain (Figure 8). The distribution of ALU-positive cells in the tissues was very heterogeneous with limited focal regions of positive cells. Most of the tissue was negative, therefore, to obtain a semiquantitative overall assessment of the extent of human tissue microchimerism in the various organs of the mice, PCR products (semiquantitative) derived from the mouse organs were compared to that of a human DNA standard. It is estimated that the percentage of human cells in the organs (microchimerism) ranged overall from 0.15% (brain) to 0.005% (kidney, lung) although the distribution of human cells was heterogeneous in the mouse tissues (Figure 9). These findings further dem-

Figure 6. FISH of human Y-chromosome in tissues of a female mouse 45 days after the transplantation of 10^6 human male NIPs under the kidney capsule of an immunocompetent C57BL/6 mouse. **A**, Kidney; **B**, liver; **C**, skeletal muscle; **D**, exocrine pancreas; **E**, endocrine pancreas (islet). One to three human Y-chromosome-positive cells were observed in representative fields. Original magnifications, $\times 400$.

Table 1. Mixed Chimerism in Immunocompetent Mice 60 Days After Intravenous Delivery of Human NIPs

Mouse No.*	% Chimerism (HLA-A, -B, -C (PE) [†]		
	Marrow	Spleen	PBLs
1	2.28	ND [‡]	ND
2	1.57	4.91	3.56
3	1.73	9.2	0.71
4	1.33	5.06	ND

*NIP cells (100,000 to 1,000,000) were given intravenously via tail vein injection.

[†]Determined by flow cytometry using human HLA-A, -B, -C antigen as marker.

[‡]Not determined.

onstrate that human NIPs can take up residence in and thrive in various organs of immunocompetent mice for at least 60 days after their systemic administration into the mice.

Discussion

The results of these studies provide evidence that stem/progenitor cells, specifically stem/progenitor cells isolated from human pancreatic islets (NIPs), can cross a xenogenic barrier, as they engraft and grow when transplanted into immunocompetent mice. The purpose of this

initial study was not to extensively characterize the phenotypes of the NIPs in the various organs of the mice after their transplantation, but rather to demonstrate that a selected population of cells of human origin (NIPs) can engraft in immunocompetent mice without the requirement for immunosuppression. The findings presented herein demonstrate engraftment of a human tissue in immunocompetent mice. At this time it is not known whether such nestin-expressing cells derived from adult tissues can cross xenogenic barriers without host *versus* graft rejection, graft *versus* host disease, and oncogenicity, or whether our findings are unique to pancreatic islet-derived stem/progenitor cells.

However, Hori and colleagues³⁸ have recently reported the successful engraftment of nestin-positive, neurospheres derived from neural stem cells under the kidney capsules of immunocompetent allogeneically mismatched mice. These stem cells did not express MHC I or MHC II antigens and differentiate into glial and neurons concomitant with their extinction of nestin expression. A conclusion of their findings was that neural stem cells are immunoprotected. Perhaps, pancreatic islet-derived stem cells are similarly immunoprotected, as we report herein.

Although it was not the explicit intention of these initial studies to demonstrate differentiation of the NIPs into pancreatic endocrine tissue,²³ we did find focal areas of tissue that co-stained with antisera to the endocrine markers insulin, glucagon, and PDX-1. Endocrine tissue staining was particularly pronounced in one graft (Figure 4E) but was also seen to a lesser extent in a few of other grafts. We suggest that these findings represent an additional demonstration of principle that a subpopulation of cells exists within the NIPs that is capable of differentiating into pancreatic endocrine tissue.²³

The possibility has been raised that certain of the observations of the transdifferentiation of stem cells delivered into mice may be an artifact because of the fusion of marked stem cells with somatic cells, thus marking the somatic cell by introducing the marker into a pre-existing differentiated somatic cell.^{39,40} Although we cannot categorically exclude the possibility that extensive cell fusion has occurred in our studies, cell fusion appears to be a relatively rare event.⁴¹ The establishment of human Y-chromosome-positive cells originating from the human NIPs transplanted under the kidney capsules was widespread in all of the organs examined showing one to three Y-chromosome-positive cells per $\times 400$ magnified field of view. It should be noted that the efficiency of detecting the presence of the human Y-chromosome in a nucleus in a 5- μ m tissue section is ~ 20 to 50% because of the residence of the Y-chromosome in a single locus of the nuclear chromatin. Thus, the extent of human Y-chromosome chimerism is estimated to be 0.20 to 0.02% of all cells in the various organs examined. Furthermore when the human NIPs were delivered to the mice by systemic intravenous injection, the extent of tissue chimerism was substantial when analyzed by histohybridization using a probe to human-specific ALU repeat sequences. As such, every human chimeric cell will be detected in the nuclei of a 5- μ m tissue section. Because

Figure 7. Flow cytometry using fluorescence-activated cell sorting of cells obtained from immunocompetent mice 60 days after systemic administration of human NIPs; the tissues are: **A**, bone marrow (BM); **B**, spleen (side population (Sp)); and **C**, peripheral blood leukocytes (PBL). The fluorescent marking probe was a monoclonal antibody to HLA antigens A, B, and C. The flow cytometry data shown correspond to mouse 2 in Table 1.

Figure 8. Histohybridization to detect human-specific ALU-repetitive sequences in tissues of female immunocompetent mice 60 days after a single intravenous injection of 10^6 human NIPs. **A**, The NIPs in culture used for the intravenous systemic injections in mice; **B**, small intestine; **C**, kidney; **D**, heart; **E**, skeletal muscle; **F**, liver; **G**, pancreas; **H**, brain. The brown-stained nuclei are positive for the presence of human-specific ALU sequences. The blue nuclei counterstained with hematoxylin are negative for human ALU sequences. **B** is from mouse 1 and **C** and **D** are from mouse 2 in Table 1. Original magnifications, $\times 400$.

Figure 9. Detection of human ALU-repetitive sequences and mouse c-mos in different organs of transplanted mice by PCR. **A:** Ethidium bromide staining of a SDS-PAGE gel. **Lane 1**, 100-bp molecular weight marker; **lane 2**, positive control (60 pg of human DNA); **lane 3**, negative control (240 pg of mouse DNA); **lanes 5 to 11**, 20 ng of DNA from different organs of a transplanted animal (**lane 5**, liver; **lane 6**, brain; **lane 7**, muscle; **lane 8**, kidney; **lane 9**, intestine; **lane 10**, lung; and **lane 11**, heart). **B:** Southern blot of gel shown in **A** using a labeled ALU sequence probe. **C:** Semiquantitative densitometric analysis of products detected in the Southern blot shown in **B**.

of the large DNA target presented by the 3 to 6×10^5 copies of the human ALU sequence the histohybridization conditions used to detect these sequences was stringent. The density of human ALU-positive cells in the tissue sections of the various organs is difficult to assess because ALU-positive cells were not evenly distributed throughout the sections, but rather were present at relatively high density in scattered focal regions and at low

density, or not detectable at all, in other regions of the tissue sections. Some focal regions of chimerism in the brain, heart, and kidney were in the range of 20 to 5% by counting ALU-positive and ALU-negative cell nuclei per selected field in sections of the various organs. By semi-quantitative analysis of the tissues, which includes the entire tissue, the PCR prevalence of chimerism appears to be between 0.16 to 0.005%, with the brain being the highest.

In addition to our demonstration of successful xenograftment without immunosuppression, we provide evidence that the lack of host *versus* graft rejection seems to result from the establishment of tolerizing mixed chimerism of the transplanted human tissues in mouse organs. More than 30 years ago it was recognized that transplanted organs (allografts) in human recipients become genetic chimeras.^{34,42} In 1992, Starzl and colleagues³¹ showed that not only is the donor organ populated by host recipient cells, but that the host recipient organs became populated by donor cells. This mutual cross-population of transplanted donor cells and recipient organs was termed microchimerism. This microchimerism was proposed as a mechanism to explain the induction of immune tolerance (host *versus* graft) and the avoidance of graft *versus* host disease; owing to the capacity of passenger hematopoietic cells within the donor organ to convert to dendritic T cells. Dendritic cells are specialized highly efficient antigen-presenting cells that function via the thymus to re-educate host tissue-reactive T cells to recognize the donor allograft as self. It was postulated that the mixed chimeric state results from a two-way interaction between donor and recipient leukocytes vying for dominance, similar to a two-way mixed lymphocyte reaction.^{31,34} The balance between the recipient immune system and the donor leukocytes that determines whether the outcome is graft rejection (recipient leukocytes dominate), graft *versus* host disease (donor leukocytes dominate), or a mutually acceptable tolerant state develops between the transplant and host acceptance. It has been suggested that the donor-tolerizing dendritic cells must originate from a population of stem cells present in the donor organ to explain the existence of foreign tissue tolerance by the host for 30 years or more.³³ Some organ transplant recipients have self-discontinued immunosuppression therapy and the grafts are maintained functionally intact, because of the induction of immune tissue tolerance via the establishment of microchimerism, macrochimerism, or mixed chimerism.³⁰

Much effort has been made in the co-administration of donor blood transfusions or bone marrow transplants concomitant with organ transplants, but their relevance for graft acceptance is still a matter of debate.^{29,32,35} The potentially immunomodulating and tolerizing mechanisms of donor-specific transfusions and donor bone marrow transplants appear to be similar and include induction of anergy, stimulation of anti-HLA antibodies, provision of soluble antigen, suppressor cell and/or veto cell activities, clonal deletion, regulation of all surface molecules, regulation of cytokines, promotion of microchimerism, or any combination of these circumstances.⁴³ A conjecture is that stem cells, resident in and released

from the donor transplants, are responsible for tolerization, perhaps in addition to passenger immunotolerant-prone dendritic T antigen-presenting T cells.^{33,36,37,44} We suggest that our findings of xenoengraftment of the human NIPs transplanted under the kidney capsules, or by intravenous injection into the systemic circulation, of non-immunosuppressed mice (the cells grow and are not rejected by the usual criteria of a rapid, vigorous rejection of a xenograft) manifest a property of stem-like progenitor cells as they appear to induce immune tolerance and thereby resist host *versus* graft rejection. Somehow the NIPs must re-educate the host immune system to recognize the donor stem/progenitor cells as self. This may occur by mechanisms of either microchimerism (peripheral), macrochimerism (central), or both (mixed chimerism).

The possible efficacy of NIPs to restore injured tissue when used as regenerative medicine remains unknown. These proof-of-principle studies as yet need to be done. Because of the finding of tolerization and the establishment of tissue chimerism after the administration of human tissue-derived cells to immunocompetent mice it is tempting to speculate that NIPs or NIP-like cells may conceivably be useful for the therapeutic induction of tissue tolerance. One might conjecture that such induction of tolerance could be a component of the protocol in certain organ transplantation procedures, and thereby eliminate the need for immunosuppression. For example, when a voluntary organ (eg, kidney) donor is identified, tissue could be obtained from the donor, NIP or NIP-like cells could be isolated from the tissue and expanded *in vitro*. The cells could then be administered to the potential recipient to tolerize the recipient to the donor organ. The induction of a state of tolerance to the donor tissue in the recipient could be determined by taking a skin biopsy from the donor and determining successful test engraftment in the potential recipient.

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