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Disproportionate Hyperproinsulinemia, β-Cell Restricted Prohormone Convertase 2 Deficiency, and Cell Cycle Inhibitors Expression by Human Islets Transplanted Into Athymic Nude Mice: Insights Into Nonimmune-Mediated Mechanisms of Delayed Islet Graft Failure

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To learn more about nonimmune-mediated islet graft failure, we transplanted different preparations (preps) of isolated human islets under the kidney capsule of streptozotocin (STZ)-diabetic nude mice. One month after the implantation of 1,000 or 2,000 islets, grafts were harvested for morphological, immunohistochemical, and ultrastructural analysis. Only a single islet prep cured the diabetes out of all the recipients, while the remaining preps showed only partial function after the implantation of 2,000 islets. Transplanted mice showed high circulating proinsulin levels but, with the exclusion of those bearing curative grafts, relatively low mature insulin levels. Engrafted β-cells showed positive carboxypeptidase E (CPE) and prohormone convertase 1 (PC1) staining, while prohormone convertase 2 (PC2) was undetectable. In contrast, PC2 was abundantly expressed by engrafted α-cells. Moreover, engrafted β-cells did not show evidence of replication, and preapoptotic β-cells, with intra- and extracellular amyloid deposition, were detected with electron microscopy. Cell cycle inhibitors $p16^{INK4}$, $p21^{WAF1}$, and $p27^{Kip1}$ were abundantly expressed in the islet grafts and showed a predominant nuclear localization. In conclusion, diabetic nude mice transplanted with human islets showed disproportionate hyperproinsulinemia and graft evidence of β-cell restricted PC2 depletion, amyloid deposition and β-cell death, and lack of β-cell replication with nuclear translocation of $p27^{Kip1}$ and $p21^{WAF1}$ that together may contribute to delayed graft failure.

Key words: Islet transplantation; Proinsulin processing; Prohormone convertase 2 (PC2); $p16^{INK4}$; $p21^{WAF1}$; $p27^{Kip1}$; Athymic nude mice

INTRODUCTION

Islet transplantation can successfully restore long-term exogenous insulin independence and improve glycemic control in patients with type 1 diabetes, but the number of patients who remain insulin independent declines progressively in 2–3 years (42). The numerous hurdles encountered by the islets during isolation, and after implantation, may account for these negative results (13,42,50). Recently, the "functional exhaustion" of a chronically overstimulated marginal β-cell mass has been implicated as a possible cause of graft failure (42). We previously reported that the delayed (5 years post-transplantation) functional decline of an initially successful islet graft is apparently unrelated to recurrence of autoimmunity, chronic rejection, and glucose toxicity

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(8). According to the "functional exhaustion" hypothesis, before the recurrence of hyperglycemia, this patient manifested a remarkable increase in circulating proinsulin levels, indicative of ongoing overstimulation of transplanted β-cells (8).

Islet transplantation into athymic nude mice allows us to study the function and the morphology of the engrafted β-cells in the absence of immune-mediated confounding factors (9,10). By using this model, it has been previously reported that transplanted human islets show rapid amyloid deposition and β-cell death, which are typical features of islet pathology in type 2 diabetes (5,16,33,51). It is generally believed that adult human islets do not replicate after transplantation, but little information is available on this issue. Cell proliferation relies on the activation of cyclins and cyclin-dependent kinases (CDKs), which act in G_1 to initiate S phase and in G_2 to initiate mitosis. To prevent uncontrolled proliferation, cyclin–CDK complexes are precisely regulated by two families of cell cycle inhibitors that block their catalytic activity (44). The first class of inhibitors includes INK4a proteins (such as $p16^{INK4}$) that bind only to CDK4-CDK6 kinases and not to cyclins and are therefore specific for early G_1 phase. The second family of inhibitors is composed of CIP/Kip proteins (such as $p21^{WAF1}$ and $p27^{Kip1}$) that inhibit all cyclins. Although only nuclear forms of CIP/Kip proteins should be probably considered as catalytic inhibitors of CDKs, $p21^{WAF1}$ and $p27^{Kip1}$ are also located in the cytoplasm where they might fulfill different functions, including the modulation of apoptotic pathways (7,12,14).

To learn more about nonimmune-mediated causes of islet graft failure, we measured circulating insulin and proinsulin levels and the residual graft β-cell mass in STZ-diabetic nude mice transplanted with human islets. Our prediction that circulating proinsulin to insulin ratios would correlate inversely with the outcome of the graft and the residual β-cell mass was confirmed only in part. Notably, transplanted β-cells did not show prohormone convertase 2 (PC2) immunoreactivity, which, conversely, was abundant in the α-cells. Ultrastructurally, the grafts showed degranulated and preapoptotic β-cells with intra- and extracellular deposition of islet amyloid pancreatic polypeptide (IAPP) fibrils. Moreover, transplanted islets showed negative Ki67 staining and abundant expression of $p16^{INK4}$, thereby confirming the lack of replication of engrafted β- and α-cells. Cell cycle inhibitors $p21^{WAF1}$ and $p27^{Kip1}$ were expressed in the islets grafts, which showed selective nuclear localization.

MATERIALS AND METHODS

Human Islet Isolation

Islets were isolated using the Ricordi method (39). Liberase (Roche Pharmaceuticals, Indianapolis, USA) was utilized for pancreatic digestion at a concentration of 1.4 mg/ml. Experiments reported in this study were performed with the islets obtained from 11 consecutive preparations (preps) (donors age ranging from 44 to 60 years) considered unsuitable for clinical transplantation due to low islet yield.

Pretransplantation Characterization

Transplantations were performed after an overnight culture under standard conditions in Medium 199 (Euroclone, Celbio, Milan, Italy), 10% FCS (Euroclone), and penn/strep. Before transplantation, islet preps were characterized for insulin content and islet cell death as previously described (31).

Characterization of 5-Day Cultured Islets

Aliquots of 2,000 unpicked islets from four isolations were cultured for 5 days, under the conditions described above for measurements of β-cell mass, morphological and ultrastructural analysis, and RNA extraction. The β-cell mass was measured on freshly isolated, overnight, and 5-day cultured pelleted islet aliquots as previously described (9,10).

Graft Recipients

Transplantations were performed in male Swiss nude mice aged 5–7 weeks. These mice were made diabetic with a single IP injection of streptozotocin (180 mg/kg of b.w.; Sigma, St. Louis, MO, USA) freshly dissolved in citrate buffer at pH 4.5. Only mice with blood glucose levels ≥ 19.5 mmol/L and failed native β-cell regeneration (unpublished observations) were used as graft recipients.

Graft Composition, Transplantation, and Outcome

Grafts were composed of unpicked islet aliquots (purity 60–80% by dithizone staining) differing in islet number (1,000 vs. 2,000) and transplanted under the left kidney capsule as previously described in detail (10). After transplantation, random (nonfasted) blood glucose levels were measured twice weekly (10). Fully Effective Prep (FEP) was the only one in which 1,000 islets were sufficient to restore recipients' normoglycemia; Partly Effective Preps (PEPs) were those in which 2,000 islets reduced recipients' hyperglycemia only in part. One month after transplantation, after 6 h of fasting, mice were bled and euthanized and grafts quickly removed. Recipients' sera were stored at −20°C until assayed for human insulin by MEIA (IMX System, Abbott Laboratories, IL, USA) and for proinsulin by ELISA (Dako, Cambridgeshire, UK). For comparison purposes, insulin and proinsulin were also measured in the sera of 10 nondiabetic subjects. Insulin and proinsulin were expressed in pmol/L and circulating proinsulin to insulin ratios

were calculated as percent proinsulin over insulin concentrations.

β-*Cell Mass in the Harvested Grafts*

Grafts were peeled off of the kidney, weighed, embedded in OCT, and snap frozen. Grafts were then sectioned with a cryostat and the β-cell mass of insulin-stained sections was measured using a morphometric approach as previously described (17) and expressed in milligrams.

Morphology, Immunohistochemistry, and Electron Microscopy

Two surgical and three autopsic normal pancreases, four cases of 5-day cultured islets, and six cases of 2,000 islet grafts harvested from mice either cured or not cured by transplantation were fixed in buffered formalin for 24 h and routinely processed for paraffin embedding. After microwave antigen retrieval, 5-μm-thick sections were incubated with primary antibodies at 4°C for 18–20 h before undergoing the avidin-biotin complex (ABC) procedure (22). Sections were then immersed in 0.03% 3,3-diaminobenzidine tetrahydrochloride and counterstained with Harris' hematoxylin. The following antibodies and antisera were used: anti-synaptophysin (BioGenex Laboratories, San Ramon, CA, USA; monoclonal at 1:100 dilution), anti-glucagon (Milab, Malmo, Sweden; polyclonal at 1:1250 dilution), anti-insulin (BioGenex Laboratories, San Ramon, CA, USA; monoclonal at 1:200), anti-proinsulin (Novo Nordisk, Hiller?-dgarde, Copenhagen, Denmark; monoclonal at 1:200), anti-prohormone convertase 1 (ABC Affinity Bioreagent, Golden, CO, USA; polyclonal at 1:50), anti-prohormone convertase 2 (NOVUS Biologicals, Littletown, CO, USA; polyclonal at 1:50), anti-carboxypeptidase E (BD Transduction, Franklin Lakes, NJ, USA; monoclonal at 1:50), anti-$p27^{Kip1}$ (BD Transduction, Franklin Lakes, NJ, USA; monoclonal at 1:200), anti-$p16^{INK4}$ (BD Pharmingen, San Diego, CA, USA; monoclonal at 1:200), anti-$p21^{WAF1}$ (Oncogene Research, Nottingham, UK; monoclonal at 1:100), anti-bcl2 (Dako, Carpintera, CA, USA; monoclonal at 1:40), anti-caspase cleavage product of cytokeratin 18 clone M30 (Hoffmann-LaRoche, Basel, SW; monoclonal at 1:100), and anti-Ki67 (Dako, Carpintera, CA, USA; monoclonal at 1:100). Apoptag staining was performed as suggested by the manufacturer (Chemicon International, Tamecula, CA, USA). The percentage of positive cells for various antibodies employed was evaluated by counting at least 200 cells for each case (five of normal pancreas, four of cultured islets, and six of transplanted islets under the kidney capsule).

Colocalization studies were performed with double-label immunostaining according to Mason and Sammons (32) and to Lan et al. (26) and/or using serial consecutive sections. Confocal microscopy was performed as previously described (15) as well as ultrastructural examination, electron microscopy, and immunocytochemistry (16). For the immunogold technique, samples were incubated with anti-IAPP antibody (Peninsula Laboratories, Belmont, CA; at 1:500) and then with 1:50 gold-tagged goat anti-rabbit (EY Laboratories, San Mateo, CA, USA) (16).

Immunofluorescence Stainings

After microwave antigen retrieval, 5-μm-thick sections from normal pancreases, 5-day cultured islets, and islet grafts harvested from transplanted mice were incubated with primary antibodies (PC1, PC2, CPE, $p27^{Kip2}$) overnight at 4°C followed by 2-h incubation with a biotin-conjugated secondary antibody and fluorescein isothiocyanate (FITC)-conjugated streptavidin. To identify α- and β-cells, sections were subsequently double stained with anti-hormone antibodies (glucagon, insulin, and proinsulin) for 2 h at room temperature, followed by the appropriate secondary antibodies. Secondary antibodies were from Jackson Laboratories. Confocal microscopy was performed as previously described (15); to minimize the bleed through, sequential sections were taken with low iris diameter.

To evaluate the percentage of insulin- or glucagon-positive cells with nuclear $p27^{Kip1}$ staining, sections derived from three different cases of transplanted mice were triple stained with $p27^{Kip1}$, insulin, and glucagon. Transplanted islets were imaged, and the number of β- or α-cells with nuclear $p27^{Kip1}$ were manually counted. At least 150 insulin-positive and glucagon-positive cells for each case were counted. Data are expressed as a mean ± SE. Comparisons between the two groups were performed by Student's dependent *t*-test, and $p < 0.05$ was considered statistically significant.

$p16^{INK4}$ and $p27^{Kip1}$ Semiquantitative RT-PCR

Total RNA was extracted from overnight- and 5-day cultured islets using the RNAfast method (Molecular Systems, San Diego, CA, USA). Semiquantitative RT-PCR was performed as previously described (15). Sequences of the oligonucleotides used in the RT-PCR analysis for $p16^{INK4}$ were as follows: forward 5′-cccgcttt cgtagttttcat-3′, reverse 5′-ttatttgagctttggttctg;-3′ and for $p27^{Kip1}$: forward 5′-agatgtcaaacgtgcgagtg-3′; reverse 5′-tctctgcagtgcttctccaa-3′.

Statistical Analysis

In the figures and tables, *n* indicates the number of experiments or animals; data are expressed as mean ± SE. Comparisons between groups were performed by

factorial two-way analysis of variance (ANOVA), and $p < 0.05$ was considered statistically significant.

RESULTS

Metabolic Outcome of the Grafts

Transplantation of 1,000 islets did not induce any reduction of blood glucose levels in five of the seven preps; a small significant decrease and the reestablishment of normoglycemia were observed only with islets of preps F and G, respectively (Table 1). Transplantation of 2,000 islets reduced glycemic levels in all the recipients, but normoglycemia was achieved only with islets of prep G. Therefore, six out of the seven preps were considered Partly Effective Preps (PEPs, preps A–F), while there was only one Fully Effective Prep (FEP, prep G).

Before the implantation, islet preps were similar in terms of viability (data not shown), while insulin content was about fivefold higher in the FEP than in the PEPs (2,400 μU vs. 455 ± 60 μU of insulin/μg of total protein, $n = 6$). One month after the implantation, pooled glycemic levels of the recipients of 1,000 and 2,000 islets from PEPs were 25.0 ± 0.6 and 19.4 ± 0.8 mmol/L, respectively, $p < 0.05$ (Table 2). Conversely, mice transplanted with the FEP were all normoglycemic and the recipients of 1,000 and 2,000 islets showed similar blood glucose levels (5.8 ± 0.2 and 5.5 ± 0.2, respectively) (Table 2).

Graft β-Cell Mass and Secretory Parameters in Graft Recipients

β-Cell mass of 1,000 and 2,000 islet grafts performed with PEPs was 0.6 ± 0.01 and 1.18 ± 0.1 mg, respectively ($p < 0.05$), while that with the FEP was 1.6 ± 0.2 and 2.6 ± 0.5 mg, respectively ($p < 0.05$) (Table 2). For comparison in vitro, the β-cell mass of 2,000 islets aliquots decreased from 5.1 ± 0.9 at day 0 to 3.4 ± 0.8 at day 1 ($p < 0.05$ vs. day 0, $n = 4$) to 2.8 ± 0.4 mg at day 5 ($p < 0.02$ vs. day 0, $n = 4$). Mature insulin levels were significantly higher in the FEP than in PEP recipients while proinsulin levels were similar and remarkably high in both the FEP and PEP recipients (Table 2). Due to the higher insulin levels, circulating proinsulin to insulin ratios were significantly lower in the FEP than in the PEP recipients. Mice transplanted with 1,000 FEP islets showed a proinsulin to insulin ratio of 146%, which was significantly higher than the 45% detected in the recipients of 2,000 islets ($p < 0.02$). For comparison purposes, the mean proinsulin to insulin ratio detected with our assays in the sera of 10 nondiabetic subjects was of 3.7 ± 1.2%.

Expression of CPE, PC1, and PC2 in Native, Cultured, and Transplanted Islets

Native islets, islets cultured for 5 days, and islet grafts showed positive CPE staining in both insulin-positive and insulin-negative cells (Fig. 1A–C). In contrast, PC1 and PC2 were undetectable in native (Fig. 1D, G) and cultured islets (Fig. 1E, H). In the islet grafts, PC1 staining was positive in insulin-positive and insulin-negative cells (Fig. 1F). Conversely, strong PC2 positivity was exclusively localized in insulin negative cells (Fig. 1I). Because overstimulated β-cells may have few mature insulin granules, sections of islet grafts were also double stained with antibodies against proinsulin or glucagon and PC1 or PC2 (Fig. 2). While PC1 was detected in both proinsulin- and glucagon-positive cells (Fig. 2C, F), PC2 was exclusively localized in glucagon-positive β-cells (Fig. 2I, L).

Morphology and Ultrastructure of Cultured and Transplanted Islets

Morphology of cultured islets showed β-cells with degenerative features such as loss of cell aggregation and reduction of cell size with hyperchromic nuclei suggestive of initial apoptosis. In the islets grafts, we de-

Table 1. Metabolic Outcome of the Grafts: Glycemic Levels Measured Before and 1 Month After Transplantation of 1,000 or 2,000 Islets Under the Kidney Capsule of STZ-Diabetic Nude Mice

	1,000-Islet Grafts				2,000-Islet Grafts			
Prep.	Pre-Tx	Post-Tx	*n*	*p*	Pre-Tx	Post-Tx	*n*	*p*
A	27.5 ± 1	22.2 ± 2	8	NS	30.1 ± 1	25.6 ± 1	6	<0.05
B	33.0 ± 0.5	33.1 ± 0.5	9	NS	32.5 ± 1	16.9 ± 2	9	<0.02
C	27.4 ± 1	18.3 ± 7	4	NS	26.1 ± 3	14.5 ± 3	4	<0.05
D	28.2 ± 2	22.8 ± 3	5	NS	30.6 ± 1	20.4 ± 3	5	<0.05
E	22.2 ± 1	17.1 ± 2	3	NS	30.1 ± 2	20.2 ± 1	3	<0.05
F	32.4 ± 0.5	22.4 ± 0.5	3	<0.02	33.3 ± 1	14.9 ± 3	3	<0.02
G	27.1 ± 1	5.8 ± 0.2	8	<0.001	25.2 ± 0.5	5.5 ± 0.2	6	<0.001

Table 2. Graft β-Cell Mass, Blood Glucose, Insulin and Proinsulin Levels, and Proinsulin/Insulin Ratios 1 Month After Transplantation of 1,000 and 2,000 Islets from PEPs and FEP Into STZ-Diabetic Mice

	PEPs 1,000 Islets	PEPs 2,000 Islets	*p*	FEP 1,000 Islets	FEP 2,000 Islets	*p*
Graft β-cell mass (mg)	0.06 ± 0.01 ($n = 32$)	1.18 ± 0.1 ($n = 30$)	$p < 0.05$	1.6 ± 0.2* ($n = 8$)	2.6 ± 0.5* ($n = 6$)	NS
Blood glucose (mmol/L)	25.0 ± 0.6 ($n = 32$)	19.4 ± 0.8 ($n = 30$)	$p < 0.05$	5.8 ± 0.2† ($n = 8$)	5.5 ± 0.2† ($n = 6$)	NS
Insulin (pmol/L)	20 ± 3 ($n = 23$)	28 ± 6 ($n = 25$)	NS	64 ± 12 ($n = 8$)	118 ± 32 ($n = 6$)	NS
Proinsulin (pmol/L)	43 ± 5 ($n = 23$)	72 ± 7 ($n = 25$)	NS	93 ± 12* ($n = 8$)	53 ± 5* ($n = 6$)	NS
Proinsulin/insulin ratio (%)	217 ± 36 ($n = 23$)	251 ± 72 ($n = 25$)	NS	146 ± 36‡ ($n = 8$)	45 ± 11† ($n = 6$)	$p < 0.02$

*$p < 0.01$ versus PEPs.
†$p < 0.001$ versus PEPs.
‡$p < 0.05$ versus PEPs.

tected degenerated and preapoptotic β-cells in both euglycemic and diabetic mice [blood glucose 7.3 ± 1.5 ($n = 3$) vs. 21.6 ± 3.4 mmol/L ($n = 3$); $p < 0.02$]. Quantification of insulin- and glucagon-positive cells showed a reduction in β-cell percent ratio from normal native (74.8% of total islet cells) to cultured (35%) to transplanted islets (7.1%). By contrast, the α-cell percent ratio increased from native (35%) to cultured (47%) to transplanted islets (95.5%) (Table 3). Electron microscopy of the islet grafts confirmed the presence of sparsely granulated β-cells with degenerative features such as pyknotic nuclei and dilated cysternae of the endoplasmic reticulum. Densely packed IAPP-positive fibrillar deposits were observed in the reticulum cysternae and in the extracellular spaces (Fig. 3A, B). Ultrastructurally, engrafted α-cells appeared wealthy and well granulated (data not shown).

Expression of Protein Involved in Apoptosis and Cell Cycle Control

Staining with M30 and apoptag showed massive apoptosis in cultured islets (Fig. 4A) that was limited to the β-cells (Fig. 4C–F). The mean percentage of apoptotic cells, evaluated by counting at least 200 cells, was 22%, ranging from 10% to 51%. Conversely, transplanted islets did not show positive M30 and apoptag staining in all six cases examined (Fig. 4B). Antiapoptotic bcl2 protein was negative in endocrine cells of both cultured and transplanted islets, while positive in contaminant transplanted ducts (data not shown). Ki67, a marker of cell proliferation (24,43), was negative in both cultured and transplanted islets, indicating the absence of active cell proliferation (Fig. 4G–I). Rare Ki67-positive cells were detected in contaminant ducts but not in the endocrine component of the grafts (Fig. 4H, I). Cell cycle inhibitor $p16^{INK4}$ was expressed by native, cultured, and transplanted islets (Fig. 5D, I, N) and localized in the nuclei and cytoplasm (Fig. 5N, inset). $p27^{Kip1}$ (Fig. 5A, F, K) showed a different pattern of expression: in native islets the majority of the cells showed cytoplasmic and nuclear staining (Fig. 5A), while $p27^{Kip1}$ was undetectable in cultured islets (Fig. 5F). Strong $p27^{Kip1}$ immunoreactivity resumed in transplanted islets but was exclusively localized to the nucleus (Fig. 5K, inset). Percentage of $p27^{Kip1}$-positive cells decreased from native (72%) to cultured islets (9.6%) but increased after transplantation (55%) (Table 3). Culture-induced $p27^{Kip1}$ reduction was confirmed by RT-PCR, where 5-day cultured islets showed a 40% reduction of $p27^{Kip1}$ mRNA as compared to the overnight cultured. Conversely, $p16^{INK4}$ mRNA remained stable in overnight and 5-day cultured islets (data not shown). Similar to $p27^{Kip1}$, native islets showed positive $p21^{WAF1}$ staining of both nuclei and cytoplasm; $p21^{WAF1}$ was undetectable in cultured islets and resumed in transplanted islets where it was localized exclusively in the nuclei (not shown). Percentage of $p21^{WAF1}$-positive cells was 16 in native islets and 23 in transplanted islets (Table 3). Triple label immunostaining with antibodies against $p27^{Kip1}$, insulin, and glucagon showed that $p27^{Kip1}$ was preferentially localized in the nuclei of the β-cells and, at a lower extent, also in the α-cells (Fig. 6). The mean percentage of insulin-positive and glucagon-positive cells with nuclear $p27^{Kip1}$ staining was 63.24 ± 4.91% and 21.35 ± 7.98%, respectively ($p < 0.05$).

DISCUSSION

Islet transplantation can successfully restore exogenous insulin independence in patients with type 1 diabetes; however, the function of the graft declines over time and insulin therapy must be eventually resumed (42).

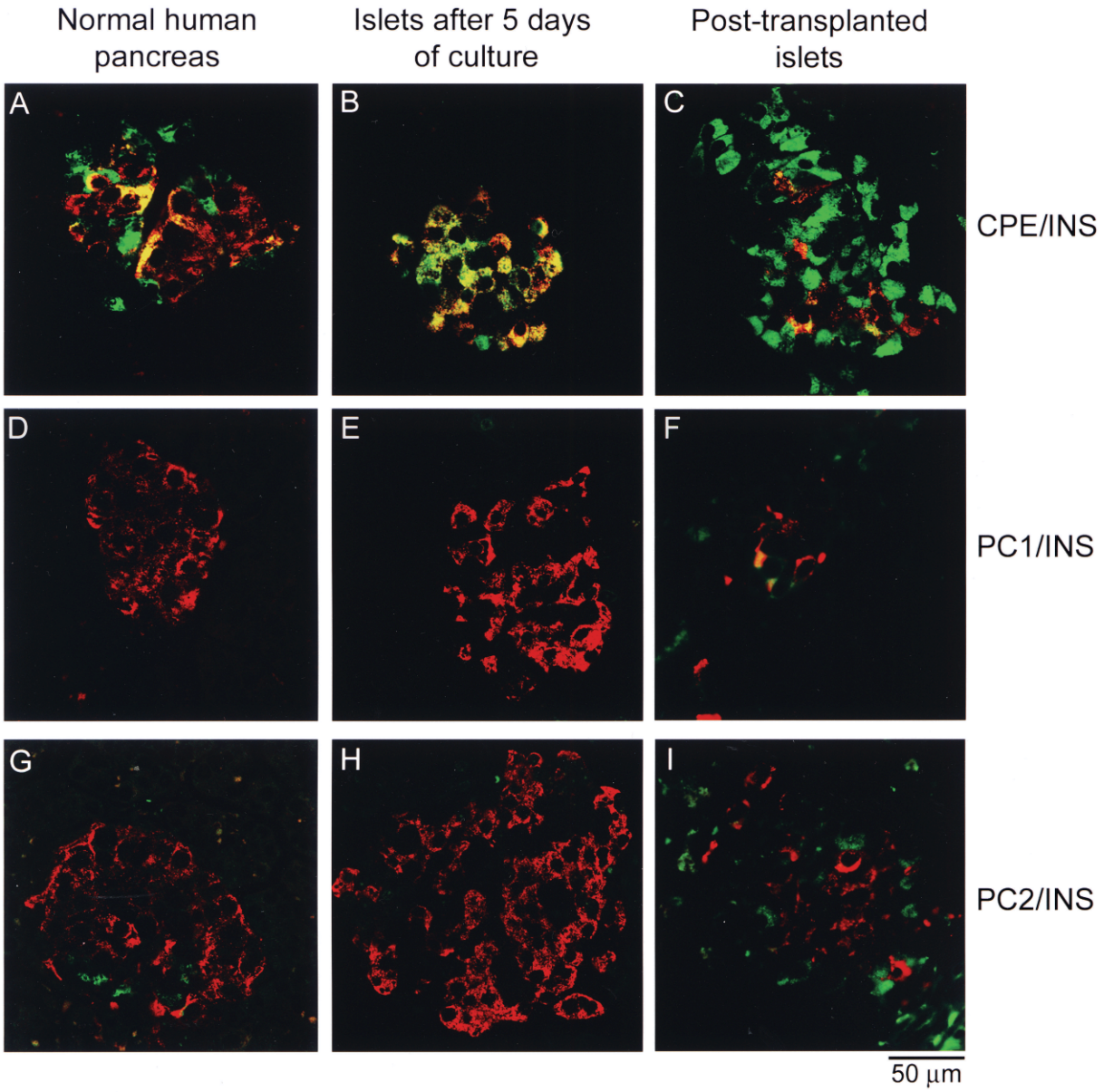

Figure 1. PC1, PC2, and CPE expression in autopsic pancreas and in cultured and transplanted islets. Normal native (A, D, G), 5-day cultured (B, E, H), and transplanted islets (C, F, I) were double stained for insulin (in red), CPE (A–C), PC1 (D–F), and PC2 (G–I) (in green). The yellow staining in the merge indicates colocalization. Images were obtained using the confocal microscope. CPE was highly expressed by insulin-positive cells in either native, cultured, or transplanted islets. Note that both PC1 and PC2 were negative in cultured islets but positive in islet grafts. In the latter, PC1 was present in both insulin-positive (yellow/orange staining in F) and insulin-negative cells (red staining in F), while PC2 was detected exclusively in insulin-negative cells. The absence of PC1/2 staining in the native islets may be ascribed to the long period of tissue ischemia (postmortem sample) and the different fixation protocol.

The "functional exhaustion" of a marginal, chronically overstimulated, β-cell mass has been indicated as a likely explanation, and the increase in circulating proinsulin levels detected before the return of the hyperglycemia supports this hypothesis (8).

To better understand the mechanisms responsible for nonimmune-mediated islet graft failure, we transplanted human islets into STZ-diabetic nude mice. The outcome of our grafts was considerably poor because 1,000 islets restored normoglycemia only in one out of the seven series of transplantations shown in Table 1. Concerns about the quality of the islet preps is confirmed by a

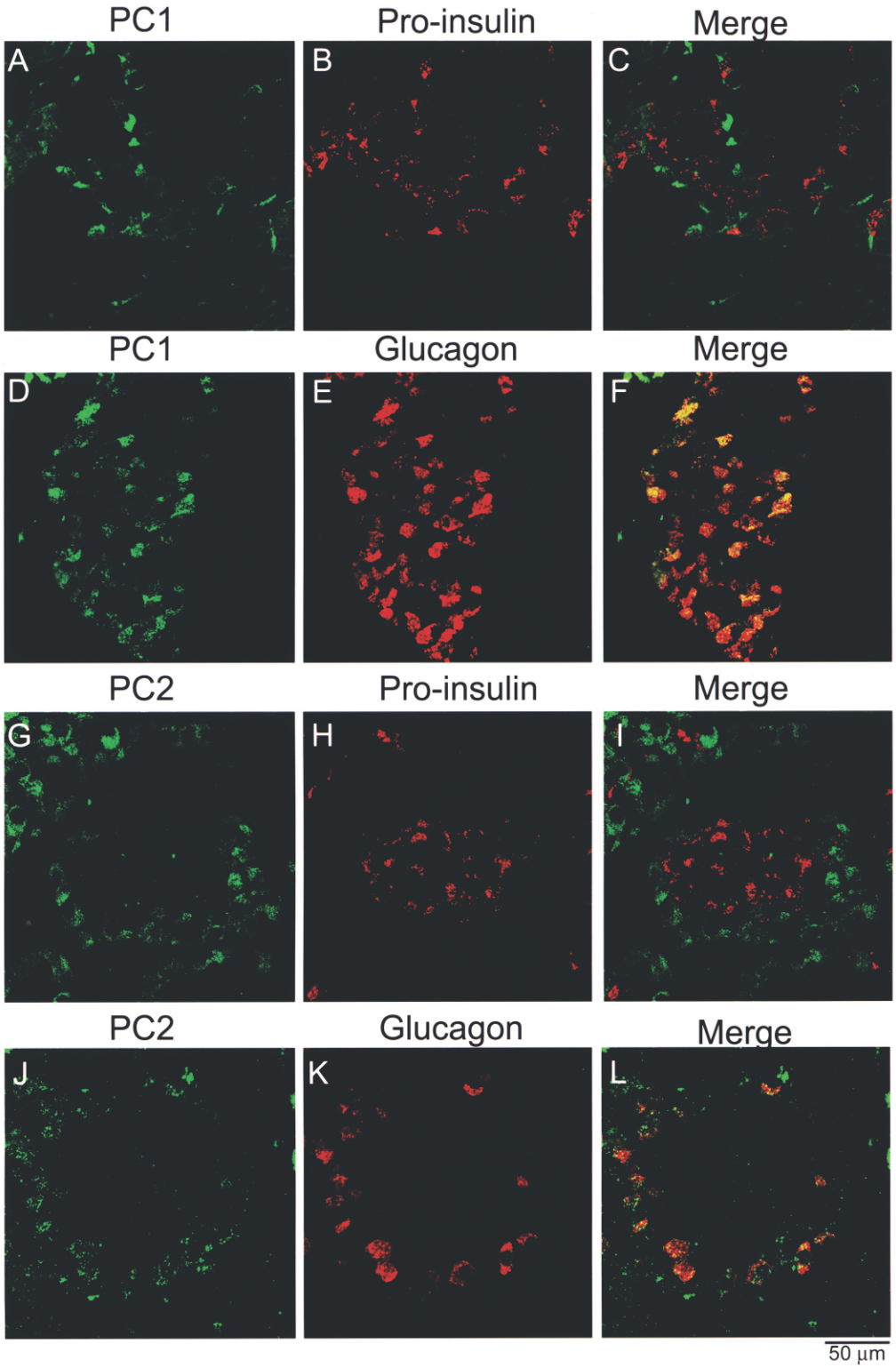

Figure 2. PC1 and PC2 staining in transplanted islets. Islet grafts were double stained for proinsulin (B, H) or glucagon (E, K) (in red) and for PC1 (A, D) or PC2 (G, J) (in green). Note that PC1 was expressed by both proinsulin-positive cells (C) and by the glucagon-positive cells (F). In contrast, PC2 was absent in proinsulin-positive cells (I) while abundantly expressed in glucagon-positive cells as shown by the yellow staining in the merge (L). These results were representative of the staining performed in three different grafts.

Table 3. Percentage of Insulin-, Glucagon-, $p27^{Kip1}$-, and $p21^{WAF1}$-Positive Cells in Native Pancreases, in 5-Day Cultured Islets, and in Transplanted Islets 1 Month After Transplantation

	Insulin	Glucagon	$p27^{Kip1}$	$p21^{WAF1}$
Native islets	75%	35%	72%	16%
5-Day cultured islets	35%	47%	10%	not detected
Transplanted islets	7%	95%	55%	23%

The percentage of positive cells was evaluated by counting at least 200 cells for each case (five of normal native pancreas, four of cultured islets, and six of transplanted islets under the kidney capsule).

previous paper where 1,000 human islets restored normoglycemia in only 23% of the recipient mice (1). In another study, 33% of all islet preps failed to restore normoglycemia with the implantation of 2,000 islets (36). Better outcomes have also been reported, but pretransplantation glycemic levels were not reported in that study (46). The relationship between the severity of pretransplantation glycemic levels and the outcome of human islet implants has not been addressed in mice. Blood glucose >300 mg/dl may be sufficient to consider a mouse diabetic (23), but reestablishment of normoglycemia in mice with mild hyperglycemia is presumably easier than in mice with severe hyperglycemia (500–600 mg/dl), such as those utilized in this study.

We here confirmed previous reports showing that the β-cell mass of human islet grafts decreases over time, while endocrine non-β-cells remain quantitatively stable (8,9,16) (Fig. 5L, M). Islet transplantation is followed by a dramatic remodeling process that is accompanied by massive β-cell death (11). Treatment with exogenous insulin partially prevents β-cell graft death (11), suggesting that hyperglycemia-induced β-cell overstimulation may be implicated in delayed graft failure. The abnormality most clearly linked to β-cell overstimulation is disproportionate hyperproinsulinemia (6,28,34,40,41), which is absent in patients with a combined pancreatic–kidney graft (2). In contrast, we observed severe disproportionate hyperproinsulinemia in mice bearing human islet grafts, regardless of their function (Table 2). This was not unexpected because isolated human islets may also secrete abnormally high proinsulin amounts in vitro (4,15). Circulating proinsulin to insulin ratios of mice bearing curative grafts (FEP recipients) were lower than those of PEPs recipients (Table 2). The higher residual β-cell mass found in curative grafts might explain this difference, as β-cell overstimulation was presumably lower in FEP than in PEP grafts. Less severe β-cell overstimulation may also explain why, in curative grafts, proinsulin to insulin ratios were significantly lower in the recipients of 2,000 islets than in mice transplanted with 1,000 islets (Table 2). However, the recipients of curative grafts that returned to normoglycemia immediately after the transplantation (data not shown) still showed severe disproportionate hyperproinsulinemia 1 month later (Table 2). The presence of disproportionate hyperproinsulinemia in long-standing normoglycemic mice implies intrinsic defects in insulin processing by transplanted β-cells.

The expression of the enzymes that convert proinsulin into insulin (PC1, PC2, and CPE) was different in native, cultured, and transplanted islets. CPE was abundantly expressed by either native, cultured, or transplanted islets. Conversely, PC1 and PC2 were clearly detectable only in the islet grafts. This does not mean that PC1 and PC2 are not expressed by cultured islets. Perhaps the culture condition may interfere with these stainings. CPE staining was intense in islet grafts (Fig. 1C) while PC1 was scant in both insulin- and proinsulin-positive cells (Fig. 1F, Fig. 2C) and abundant in the α-cells (Fig. 2F). Staining for PC2 was more intense than that of PC1 in islet grafts but localized exclusively in the α-cells (Fig. 2L). Selective PC2 downregulation has been reported in the islets of diabetic Goto-Kakizaki rats, which do not show hyperproinsulinemia (19). Therefore, the combination of PC2 and PC1 deficiency, as we observed in transplanted β-cells, may be necessary to determine the abnormally high proinsulin levels detected in our recipients.

Ultrastructural analysis of the islet grafts showed degranulated and preapoptotic β-cells with intra- and extracellular deposition of IAPP-positive fibrils (Fig. 3). Human IAPP (hIAPP) is the major constituent of pancreatic amyloid deposits found in type 2 diabetic subjects (5) and insulinomas (35). hIAPP is synthesized as a propeptide (prohIAPP) that colocalizes with insulin granules where it is converted to IAPP by the same endopeptidases (PC1/2 and CPE) that convert proinsulin into insulin (3,20,30). A particular proIAPP cleavage product has the potential for "seeding" amyloid deposition, a process hindered by insulin but much less efficiently by proinsulin (52). In overstimulated β-cells, simultaneous depletion of insulin stores and/or defective proIAPP processing may determine a favorable condition for the precipitation of fibrillogenic proIAPP products (37) leading to cell death (27,38). The lack of PC2 in transplanted β-cells is particularly relevant in this regard because impaired NH_2-terminal processing of hIAPP, as occurs in absence of PC2, determines amyloid formation and cell death in vitro (29). Studies in mice overexpressing hIAPP further support that β-cell overstimulation can induce islet amyloidosis (21,45,48) and that transplantation is another amyloidogenic procedure (49).

As shown by M30 and apoptag staining, and in agree-

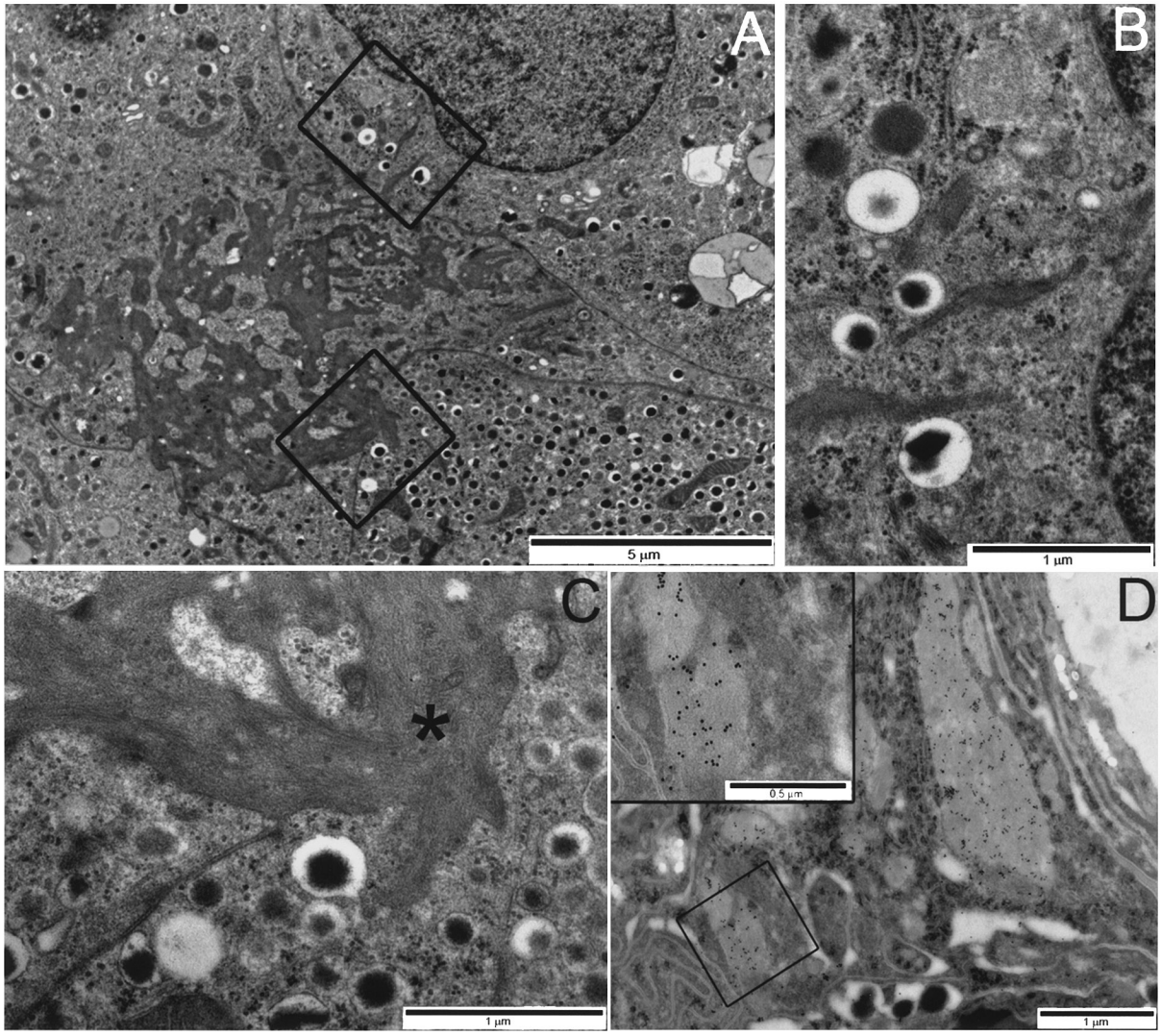

Figure 3. Amyloid deposition in transplanted β-cells. (A) In transplanted islets, densely packed fibrillar deposits are found in both β-cells (right bottom and right top) and in the extracellular space. (B) Corresponds to the superior rectangle of (A); fibrillar material is present in reticulum cysternae among typical secretory granules, characterized by a crystalline central core surrounded by a clear halo. (C) Corresponds to the inferior rectangle of (A); fibrillar material is present in the extracellular space (asterisk), close to a β-cell, containing typical secretory granules, present in the right bottom. (D) Immunogold technique demonstrates that fibrillar deposits are intensely IAPP positive, as better shown in the inset.

ment with previous data (15), we observed massive β-cell apoptosis in human islets cultured in vitro (Fig. 4A). M30 was negative in the islet grafts (Fig. 4B), but the occurrence of sporadic β-cell apoptosis was detected with electron microscopy. Negative Ki67 staining confirmed lack of β- and α-cell replication in both cultured and transplanted islets (Fig. 4 G, H), and indicates that the inverted β-/α-cell ratio of human islet grafts is exclusively due to the loss of the β-cells. The cell cycle inhibitor $p16^{INK4}$ was positive in both the β- and α-cells and more abundant than in the exocrine pancreas. This was particularly evident in cultured and transplanted islets (Table 3), where $p16^{INK4}$ was expressed in both nuclei and cytoplasm (Fig. 5I, N). Noteworthy is that the expression pattern of $p16^{INK4}$, $p21^{WAF1}$, and $p27^{Kip1}$ was similar in grafts harvested from either normoglycemic or hyperglycemic mice (not shown). It has been recently shown that $p16^{INK4}$ increases with age, and limits β-cell regenerative potential of rodent islets (25). Therefore, abundant $p16^{INK4}$ expression in stabilized grafts may account for a lack of replication of the engrafted β-cells. Interestingly, $p27^{Kip1}$ and $p21^{WAF1}$ were positive in the cytoplasm of native islets, but were negative in cultured islets when several β-cells undergo apoptosis. Downreg-

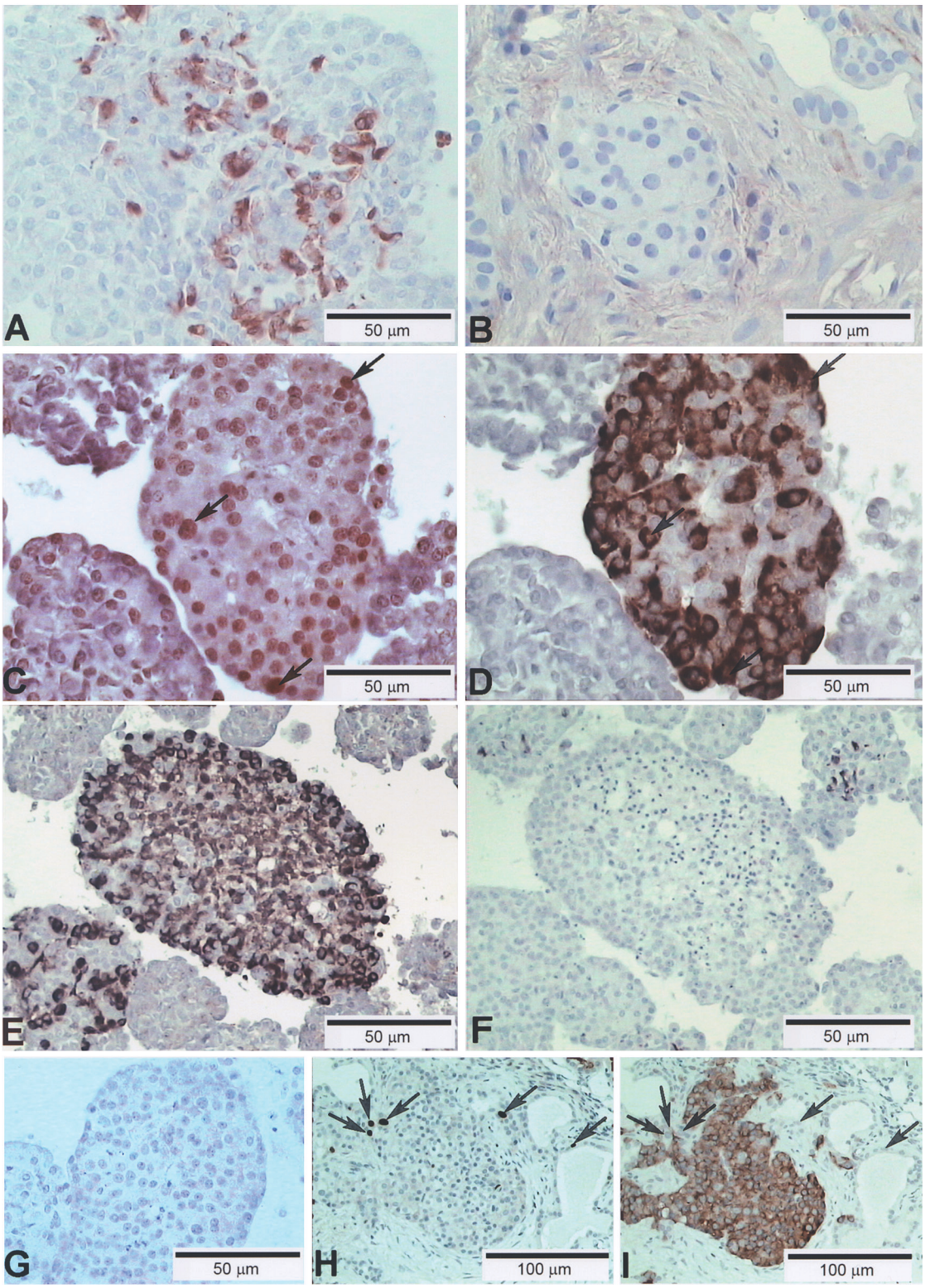

Figure 4. Apoptosis and replication in cultured and transplanted islets. M30 immunoreactivity in 5-day cultured islets (A) indicated apoptosis, which was not the case for transplanted islets (B). Apoptag staining confirmed M30 results. In serial sections of 5-day cultured islets, apoptag staining (C) colocalizes with insulin (D) (arrows indicated some of the cells with colocalization). On the contrary, in 5-day cultured islets glucagon-producing β-cells (E) did not show apoptosis, as indicated by negative staining for M30 (F). Cultured islets were negative for Ki67 (G), while rare Ki67-positive cells were present in the islet grafts (H). However, these cells were not of endocrine origin as shown by the negative synaptophysin staining (arrows indicated Ki67-positive, synaptophysin-negative cells) (I).

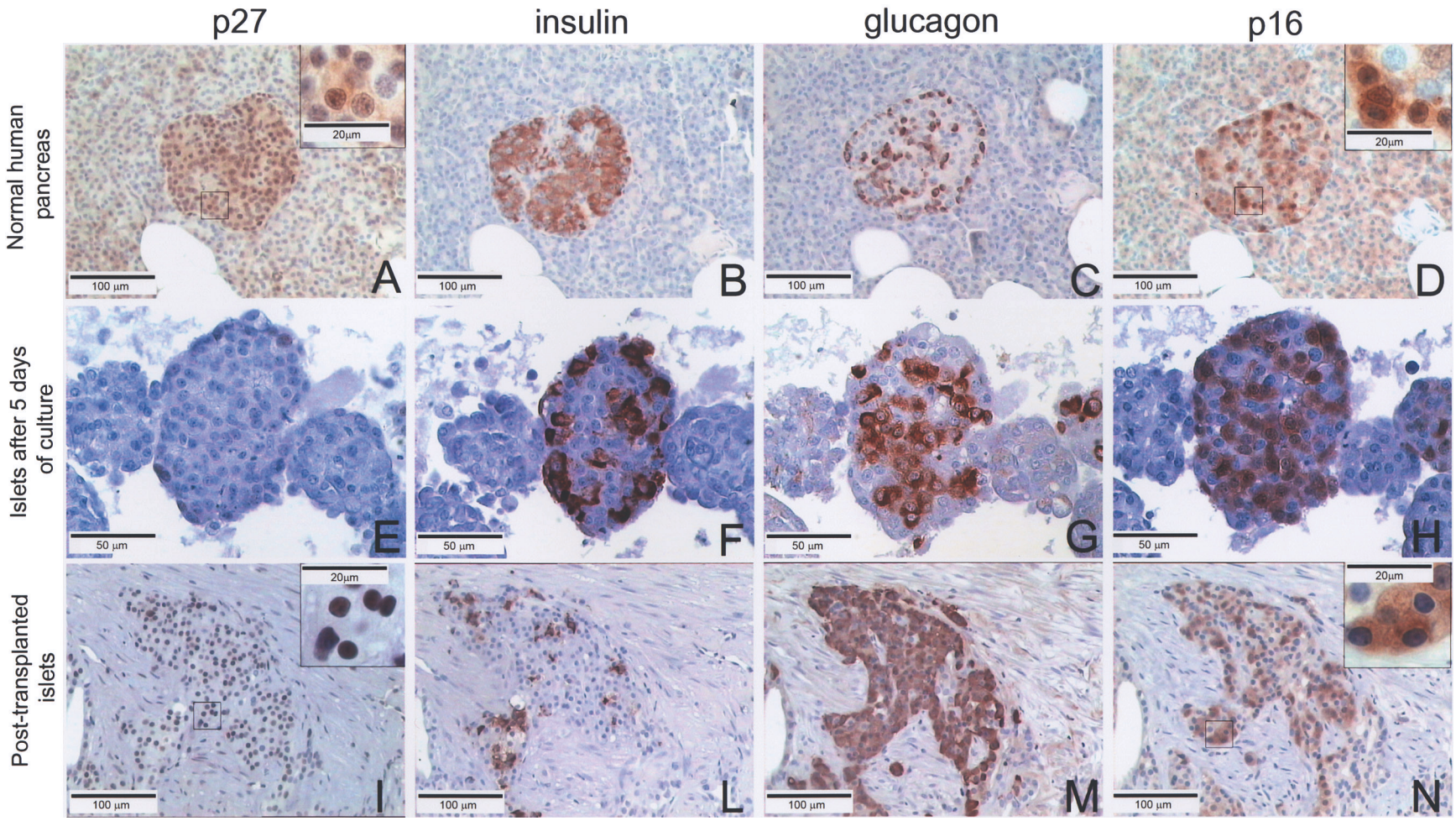

Figure 5. Cell cycle inhibitory proteins expression and subcellular localization in autopsic pancreas, and in cultured and transplanted islets. In normal native islets the majority of the cells were $p27^{Kip1}$ positive, showing both cytoplasmic and nuclear staining (A). In 5-day cultured islets, $p27^{Kip1}$ was completely absent (E), while a strong $p27^{Kip1}$ positivity was detected in stabilized grafts (I) where $p27^{Kip1}$ was exclusively localized to the nucleus (inset). Conversely, $p16^{INK4}$ was expressed by the endocrine cells of normal native (D), cultured (H), and transplanted islets (N). This $p16^{INK4}$ was localized in both the cytoplasm and nuclei (inset). Note that $p16^{INK4}$ was almost absent in the contaminant exocrine tissue (N). A progressive reduction in the β-cells (B, F, L) and a dramatic increase in the relative α-cells numbers (C, G, M) occurs from native to cultured to transplanted islets (original magnification 400×, inset 1,000×).

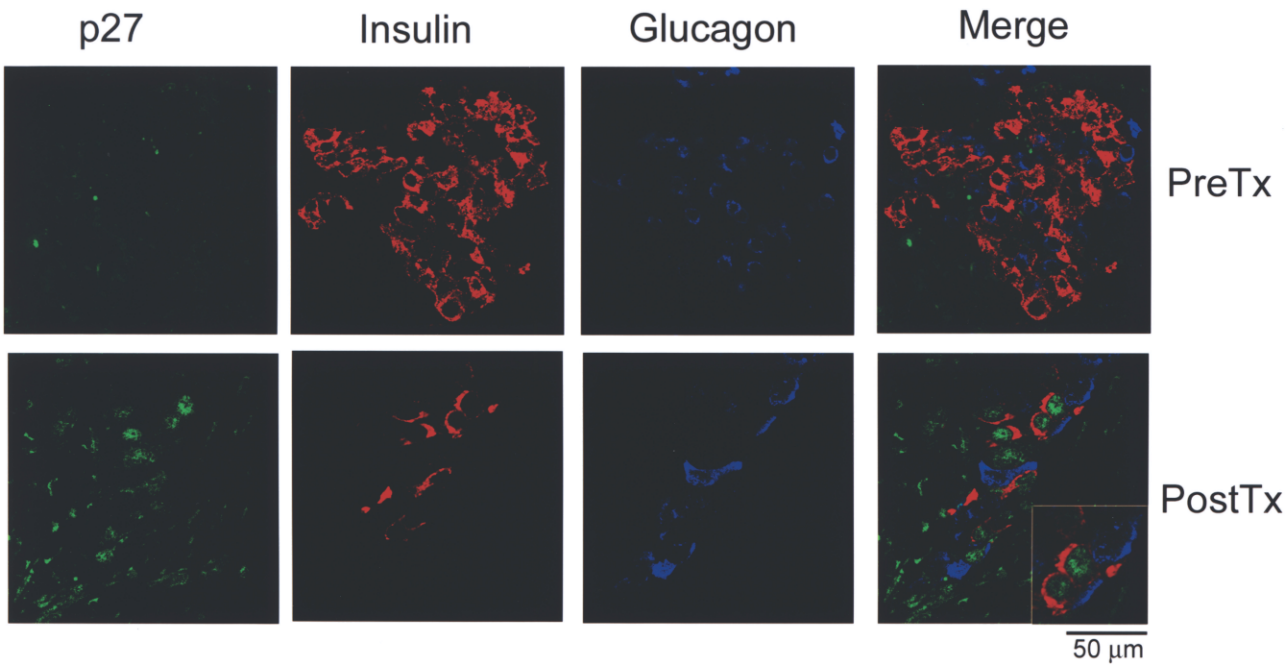

Figure 6. Localization of $p27^{Kip1}$ with confocal microscopy in β- and α-cells of cultured and transplanted islets. Triple label immunostaining with antibodies against $p27^{Kip1}$ (in green), insulin (in red), and glucagon (in blue) shows that $p27^{Kip1}$ is preferentially localized in the nuclei of the β-cells and, at a lower extent, also in the α-cells. Scale bar: 10 μm.

ulation of $p27^{Kip1}$ in cultured islets was confirmed by RT-PCR. Transplanted islets returned to show positive $p27^{Kip1}$ and $p21^{WAF1}$ staining, which, however, were entirely localized to the nucleus. Moreover, $p27^{Kip1}$ was preferentially expressed by the β-cells (Fig. 6). Altogether, these data are consistent with the recent finding that $p27^{Kip1}$ is a negative regulator of β-cell transition from quiescence to proliferation and a potentially important target for β-cell regeneration therapies (18).

In conclusion, the nonimmune-mediated loss of β-cells occurring in human islet grafts recapitulates the pathogenesis of β-cell loss in type 2 diabetes, where a defective β-cell mass with reduced regenerative potential is challenged by chronic overstimulation (39). Lack of replication of the engrafted β-cells would not compensate for continuous β-cell death caused by IAPP fibril deposition due to abnormal proinsulin and proIAPP processing by β-cells deficient in both PC1 and PC2. Further studies are needed to prove this hypothesis and to explore whether selective PC1/2 overexpression and/or downregulation of one or more inhibitory cyclins may increase survival and regenerative potential (47) of transplanted human β-cells.

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