# β-Cell Replication Is Increased in Donor Organs From Young Patients After Prolonged Life Support

Peter In't Veld,[1] Neelke De Munck,[1] Kristien Van Belle,[1] Nicole Buelens,[1] Zhidong Ling,[1] Ilse Weets,[1] Patrick Haentjens,[2] Miriam Pipeleers-Marichal,[1] Frans Gorus,[1] and Daniel Pipeleers[1]

**OBJECTIVE**—This study assesses β-cell replication in human donor organs and examines possible influences of the preterminal clinical conditions.

**RESEARCH DESIGN AND METHODS**—β-Cell replication was quantified in a consecutive series of $n = 363$ human organ donors using double immunohistochemistry for Ki67 and insulin. Uni- and multivariate analysis was used to correlate replication levels to clinical donor characteristics and histopathologic findings.

**RESULTS**—β-Cell replication was virtually absent in most donors, with ≤0.1% Ki67-positive β-cells in 72% of donors. A subpopulation of donors, however, showed markedly elevated levels of replication of up to 7.0% Ki67-positive β-cells. β-Cell replication was accompanied by the increased replication of glucagon-, somatostatin-, and CA19.9-positive cells. Prolonged life support, kidney dysfunction, relatively young donor age, inflammatory infiltration, and prolonged brain death before organ retrieval were all found to be significantly associated with an increased level (≥90th percentile) of β-cell replication, with the first three risk factors being independent predictors. Increased β-cell replication was most often noted in relatively young donors (≤25 years) who received prolonged (≥3 days) life support (68%); in contrast, it was rare in donors with a short duration of life support regardless of age (1%). Prolonged life support was accompanied by increased levels of CD68^{+} and LCA/CD45^{+} infiltration in the pancreatic parenchyma.

**CONCLUSION**—These results indicate that preterminal clinical conditions in (young) organ donors can lead to increased inflammatory infiltration of the pancreas and to increased β-cell replication. *Diabetes* 59:1702–1708, 2010

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H uman diabetes is a heterogeneous group of disorders with increased glycemia levels and a decreased functional β-cell mass in common. Type 1A diabetes is characterized by a T-cell–mediated autoimmune destruction of 50–70% of β-cell mass at clinical onset, whereas type 2 diabetes is characterized by a smaller decrease in β-cell mass in association with insulin resistance and loss of β-cell function (1,2). Clinical interventions aimed at restoring a functionally adequate endogenous β-cell mass are therefore of consid-

erable interest, but they are hampered by a relative lack of knowledge about the in vivo conditions that stimulate β-cell replication and neoformation in the adult pancreas (3). Quantification of β-cell replication in the developing human pancreas shows that replication is high in the early fetal pancreas, but decreases rapidly after birth and is only rarely observed in the adult pancreas (4–7). Interestingly, several cases have been described in which patients with a variety of diseases, including lobar pneumonia, hemochromatosis, or acute liver disease, were reported to display prominent mitotic activity in adult islet tissue (8–10). Such chance observations indicate that although replication in the adult pancreas is normally low, adult human islet cells apparently do retain a capacity for replication that can be activated under selected clinical conditions. To characterize such conditions we investigated β-cell replication in a large consecutive series of human organ donors and correlated our findings to the preterminal clinical characteristics of the patients involved.

## RESEARCH DESIGN AND METHODS

**Collection of pancreatic tissue.** Pancreas biopsy specimens were obtained from the Beta Cell Bank in Brussels, which operates for a clinical trial on islet cell transplantation in Belgium (11). The biopsy specimens were taken as part of a quality control procedure that was approved by the medical ethics committee of our university. A single biopsy specimen of ~0.5 cm³ was taken from the body region of the cold-preserved (University of Wisconsin preservation solution flushed) donor pancreas immediately before the remaining tissue was digested for islet isolation. Biopsy specimens were fixed in 4% (v/v) phosphate-buffered formaldehyde, pH 7.4, and embedded in paraffin for routine histopathologic examination. Tissue blocks from 363 of 500 consecutive donors fulfilled all inclusion criteria (minimal biopsy surface area >0.25 cm²; minimal clinical data including age, sex, BMI, time in hospital, cause of death; and availability of a serum sample) and were analyzed by immunohistochemistry.

**Immunohistochemistry.** Consecutive 4-μm paraffin sections were immunohistochemically double stained for the replication marker Ki67 (mouse anti-Ki67; Dako, Glostrup, Denmark) and insulin (guinea pig anti-insulin; a gift of Dr. Van Schravendijk, Brussels Free University, Brussels, Belgium), glucagon (rabbit anti-glucagon; Dr. Van Schravendijk), somatostatin (rabbit anti-SRIF; a gift of Dr. De Mey, Brussels Free University) or synaptophysin (rabbit anti-synaptophysin; Dako). Rabbit anti-Ki67 (Acris Antibodies, Hiddenhausen, Germany) was used in conjunction with mouse anti-carbohydrate antigen-19.9 (Novocastra Laboratories, Newcastle upon Tyne, U.K.) and with mouse anti-LCA/CD45 (Clones 2B11 plus PD7/26; Dako). Double stainings were also performed using rabbit anti-phosphohistone H3 (Upstate Biotechnology, Lake Placid, NY), mouse anti-LCA (Dako), mouse anti-CD68 (clone KP1; Dako) or mouse anti-CD3 (Novocastra Laboratories), and guinea pig anti-insulin. Binding of primary antibodies was detected with biotinylated anti-mouse or anti-rabbit Ig (Amersham, Little Chalfont, U.K.) or biotinylated anti-guinea pig Ig (Vector Laboratories, Burlingame, CA) in combination with streptavidin horseradish peroxidase or alkaline phosphatase complex (both from Dako). For immunofluorescence microscopy the following second antibodies were used: FITC anti-rabbit Ig, AMCA anti-guinea pig Ig, FITC anti-guinea pig Ig, Cy3 anti-mouse Ig, Cy3 anti-rabbit Ig (all from Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa Fluor 488 anti-guinea pig and anti-rabbit Ig, and Alexa Fluor 647 anti-rabbit and anti-mouse Ig (all from Invitrogen, Carlsbad, CA).

**Quantification of replication and relative β-cell area.** Islet cell replication was assessed in slides double stained for the replication marker Ki67 and

for insulin, glucagon, somatostatin, and the panendocrine marker synaptophysin. Ductal cell replication was assessed in slides double stained for the replication marker Ki67 and for the ductal marker carbohydrate antigen-19.9. All quantitative analyses were performed by transmitted light microscopy on coded slides at a final magnification of ×400 by two independent observers. Minimally 1,000 cells per case were evaluated, except for glucagon and somatostatin for which 400 and 100 cells per case were evaluated, respectively. Relative β-cell area was determined according to Rahier et al. (12) on immunostained sections using a 180-point counting grid at a final magnification of ×140. The number of points overlaying insulin immunoreactive cells (Ni) and parenchyma (Np) was counted in 20 random microscope fields per case. Grid points overlaying the lumen of ducts, arteries, connective tissue, or fatty tissue were not included in the analysis. Relative β-cell area was calculated as (Ni/Np) × 100 and expressed as a percentage. All morphometric analyses were carried out in a blinded fashion on coded slides by two independent observers.

**Quantification of leukocytic infiltration.** Leukocytic infiltration was assessed in slides double stained for insulin and leukocyte common antigen (LCA/CD45), CD68, or CD3. The number of infiltrating CD68+ and LCA/CD45+ cells was quantified at a final magnification of ×400 and is expressed as mean ± SE of cell numbers per 10 high power microscope fields (corresponding to a total area of 2.83 mm²).

**Clinical and histopathologic parameters.** Selected clinical parameters were obtained from the donor information forms and from the Eurotransplant Foundation (Leiden, the Netherlands). Because of the anonymity of the organ donors, clinical data could not always be verified and were sometimes incomplete or not available for all donors. We tested whether the following 13 clinical (13) and histopathologic risk factors were associated with increased β-cell replication: young donor age (≤25 years), high BMI (>30 kg/m²), prolonged time in the intensive care unit (≥3 days), prolonged duration of mechanical respiration (≥3 days), increased infiltration of CD68+ monocytic cells in the pancreatic parenchyma (defined as >90th percentile in organ donors with a minimal [<1 day] stay in the intensive care unit, corresponding to a level of ≥175 CD68+ cells per 10 high power microscope fields), increased infiltration of LCA/CD45+ leukocytes in the pancreatic parenchyma (defined as >90th percentile in organ donors with a minimal [<1 day] stay in the intensive care unit, corresponding to a level of ≥115 LCA/CD45+ cells per 10 high power microscope fields), hypotensive periods (systemic blood pressure <100 mmHg), prolonged duration of brain death (≥12 h to start of cold perfusion as defined by the third quartile), kidney damage (serum creatinine ≥150 μmol/l), liver damage (bilirubin ≥2 mg/dl combined with aspartate aminotransferase ≥25 units/l), pancreas damage (amylase >200 units/l, defined as twice the reference value), hyperglycemia (glucose ≥200 mg/dl), and the reported use of steroid hormones during the stay in the intensive care unit. Serum fructosamine and C-peptide levels were tested retrospectively on samples stored at −80°C.

**Statistical analysis.** Differences between groups were assessed by a Mann-Whitney $U$ test for continuous variables and by a $\chi^2$ test with Yates' correction, or Fisher's exact test, whenever appropriate, for categorical variables. Clinical and biologic characteristics of organ donors were studied after stratification for β-cell replication level (donors with β-cell replication level below percentile 90 [P90] [<0.6%] vs. donors with levels above P90 [≥0.6%]). Logistic regression analysis was performed with β-cell replication level as dependent variable, with inclusion of all variables with $P \leq 0.10$ in univariate analysis (i.e., time in the intensive care unit, kidney damage, age, monocytic infiltration, leukocytic infiltration, duration of brain death, reported use of steroid hormones, and pancreas damage). Duration of mechanical ventilation was not included as a variable because of the number of missing data and the good correlation with time in the intensive care unit (correlation coefficient 0.63 [95% CI 0.56–0.71]; $P < 0.001$). Statistical tests were performed two-tailed using SPSS for Windows PASW 17 (SPSS, Chicago, IL) for personal computers and considered significant at $P < 0.05$ (multivariate analysis) or, in the case of k-independent univariate comparisons, at $P < 0.05/k$ (Bonferroni correction). Only risk factors with an odds ratio (OR) 95% CI that excluded 1.0 were considered predictive.

## RESULTS

**Increased β-cell replication is found in a subset of organ donors.** In a consecutive series of $n = 363$ donor pancreata (donor age 2–75 years), immunohistochemical double staining for the replication marker Ki67 and insulin indicated for most organs (72%) a low level of replication (≤0.1%) in the 1,000 β-cells that were evaluated for each organ. The remaining donors (28%) presented levels of β-cell replication between 0.2 and 7.0% (Table 1). Donors

TABLE 1
Frequency distribution of patients with different levels of β-cell replication

| β-Cell replication level | $n$ | % |
| --- | --- | --- |
| ≤0.1% | 262 | 72.2 |
| 0.2–0.5% | 65 | 17.9 |
| 0.6–1.0% | 23 | 6.3 |
| >1.0% | 13 | 3.6 |
| Total | 363 | 100 |

with a high level of replication (defined as ≥90th percentile; $n = 36$ patients) were between 6 and 66 years of age, with different causes of death (Table 2). The replicating β-cells were found scattered throughout the islets (Fig. 1A–D); they were only infrequently found to be associated with ducts. Donors who showed increased levels of Ki67-positive β-cells also presented with mitotic figures and cells that costained for insulin and the $G_2$ to M transition marker phosphohistone H3 (Fig. 1E and F). When the $n = 36$ donors with a high level of β-cell replication were compared with the $n = 327$ donors with a lower level of β-cell replication, they were found to be of significantly younger median age (29 vs. 48 years; $P < 0.0001$; Table 2), whereas BMI in the two groups was comparable. A high male-to-female ratio (24 male vs. 12 female donors) in the group of donors with high β-cell replication was most probably caused by the high number of male donors in the youngest age group (37 male vs. 14 female donors in the age-group ≤25 years vs. 189 male and 172 female donors in the total study population; sex data on two patients were missing).

**Relative β-cell area in organ donors with a high level of β-cell replication.** When the relative β-cell area was determined in the group with high replication (≥90th percentile; 36 patients) a mean relative β-cell area of $1.78 \pm 0.18\%$ was found. This mean relative area was higher, but not statistically different, from that found in the $n = 36$ matched (age, sex, and BMI) controls from the group with a low level of β-cell replication ($1.28 \pm 0.05\%$; $P = 0.085$). To assess whether severe β-cell degranulation could have influenced quantification of β-cell area, we performed double labeling for the β-cell transcription factor Nkx6.1 and insulin in a subset of donors from both groups. It was observed that most (>90%) Nkx6.1-positive cells also showed cytoplasmic insulin immunoreactivity, indicating that the majority of β-cells were insulin-positive and that the fraction of severely degranulated "hidden" β-cells was relatively low (Fig. 1G and H).

**Increased β-cell replication is accompanied by an increase in both endocrine and exocrine cell replication.** Immunohistochemical double labeling for Ki67 and the islet cell markers glucagon and somatostatin showed that a 20-fold higher mean level of β-cell replication in the ≥90th percentile group was accompanied by a 52-fold higher level of α-cell replication and a fivefold higher level of δ-cell replication (Figs. 1I–L and 2). Virtually all replicating islet cells showed positivity for the panendocrine marker synaptophysin (Fig. 1M and N). Replication was not only observed in islet cells but also throughout the pancreatic parenchyma outside the islets where it was observed in acinar cells and ductal cells (Fig. 1O and P). Ductal replication was quantified on Ki67/CA19.9 coimmunostained slides and a 3.7-fold higher level of ductal cell

TABLE 2
Characteristics of donors with a high level of β-cell replication (≥90th percentile)

| Donor | β-Cell replication level (%) | Age (years)/Sex | BMI (kg/m$^2$) | Cause of death |
|---|---|---|---|---|
| 1 | 7.0 | 18/M | 22 | Cerebrovascular accident |
| 2 | 5.8 | 18/M | 24 | Craniocerebral trauma |
| 3 | 5.5 | 19/M | 26 | Polytrauma, brain edema |
| 4 | 3.2 | 6/M | 13 | Inhalation trauma |
| 5 | 3.2 | 55/F | 21 | Subarachnoid bleeding |
| 6 | 2.6 | 13/F | 14 | Cerebellar infarction |
| 7 | 2.5 | 30/M | 23 | Craniocerebral trauma |
| 8 | 2.5 | 42/M | 27 | Craniocerebral trauma |
| 9 | 1.8 | 36/M | 26 | Craniocerebral trauma |
| 10 | 1.8 | 19/M | 23 | Medulloblastoma |
| 11 | 1.6 | 39/F | 24 | Aneurysm |
| 12 | 1.2 | 30/M | 22 | Craniocerebral trauma |
| 13 | 1.2 | 26/F | 23 | Hypoxia |
| 14 | 1.0 | 20/M | 31 | Cerebral trauma |
| 15 | 1.0 | 23/M | 22 | Craniocerebral trauma |
| 16 | 1.0 | 25/M | 22 | Hanging (suicide) |
| 17 | 1.0 | 25/M | 30 | Craniocerebral trauma |
| 18 | 1.0 | 53/M | 27 | Intracranial bleeding |
| 19 | 0.9 | 18/M | 23 | Craniocerebral trauma |
| 20 | 0.9 | 38/M | 28 | Strangling (suicide) |
| 21 | 0.8 | 27/M | 26 | Polytrauma |
| 22 | 0.7 | 66/M | 28 | Subarachnoid bleeding |
| 23 | 0.7 | 24/M | 24 | Craniocerebral trauma |
| 24 | 0.7 | 46/F | 31 | Intracranial bleeding |
| 25 | 0.7 | 41/M | 29 | Hypoxia brain damage |
| 26 | 0.7 | 19/F | 26 | Craniocerebral trauma |
| 27 | 0.7 | 30/F | 23 | Brain edema |
| 28 | 0.7 | 19/M | 24 | Craniocerebral trauma |
| 29 | 0.7 | 36/F | 24 | Subarachnoid bleeding |
| 30 | 0.7 | 44/F | 28 | Intracranial bleeding |
| 31 | 0.6 | 59/F | 25 | Cerebrovascular (ischemia) |
| 32 | 0.6 | 54/F | 31 | Subarachnoid bleeding |
| 33 | 0.6 | 42/M | 25 | Subarachnoid bleeding |
| 34 | 0.6 | 22/F | 20 | Craniocerebral trauma |
| 35 | 0.6 | 63/M | 32 | Subarachnoid bleeding |
| 36 | 0.6 | 20/M | 20 | Brain edema (hanging) |
| All donors with high replication ($n = 36$) | 1.0 (0.7–1.8) | 29 (20–42) 24M/12F | 24 (22–28) | |
| Matched controls with low replication ($n = 36$) | 0 (0–0.1) | 28 (21–42) 24M/12F | 24 (22–26) | |
| All donors with low replication ($n = 327$) | 0 (0–0.1) | 48 (37–54) 165M/160F | 24 (22–26) | |

Data are medians (interquartile range) unless otherwise specified. F, female; M, male.

replication was found in the group with high β-cell replication (Fig. 2).

**Increased β-cell replication is associated with prolonged duration of life support and young donor age.** Multi- and univariate analysis was performed to test 13 clinical parameters (risk factors) for their association with β-cell replication. Thirty-six donors with a high level of β-cell replication (≥90th percentile) were compared with the $n = 327$ patients with low replication (<90th percentile). Seven risk factors showed a significant association in univariate analysis: prolonged duration of mechanical respiration (OR31.1), prolonged duration of stay in an the intensive care unit (24.5), kidney dysfunction (7.1), young donor age (6.7), increased CD68$^+$ monocytic infiltration (6.2), increased LCA/CD45$^+$ leukocytic infiltration (3.7), and a prolonged duration of brain death (3.0). Three of these parameters were found to be independent predictors of β-cell replication in multivariate analysis: young donor

age, prolonged duration of stay in an intensive care unit, and kidney dysfunction (Table 3). High β-cell replication levels were observed in 15 of 22 (68%) donors who combined prolonged life support with young donor age (≤25 years), which is significantly more frequent than in donors of the same age with shorter duration of life support (1 of 28 or 4%; $P < 0.001$, $\chi^2$ test) (Table 4). Comparison with the older age-group shows that in older patients, the prevalence of high replication levels is less and occurs after a more prolonged stay in an intensive care unit. Regardless of donor age, the average frequency of high replication was 1% in donors with <3 days stay in an intensive care unit. Average duration of mechanical ventilation was $6 \pm 3$ days in the ≥90th percentile group vs. $2 \pm 2$ days for the <90th percentile group ($P < 0.001$). Six clinical parameters, including hyperglycemia and use of steroid hormones, showed no significant association with increased β-cell replication. As blood glucose values
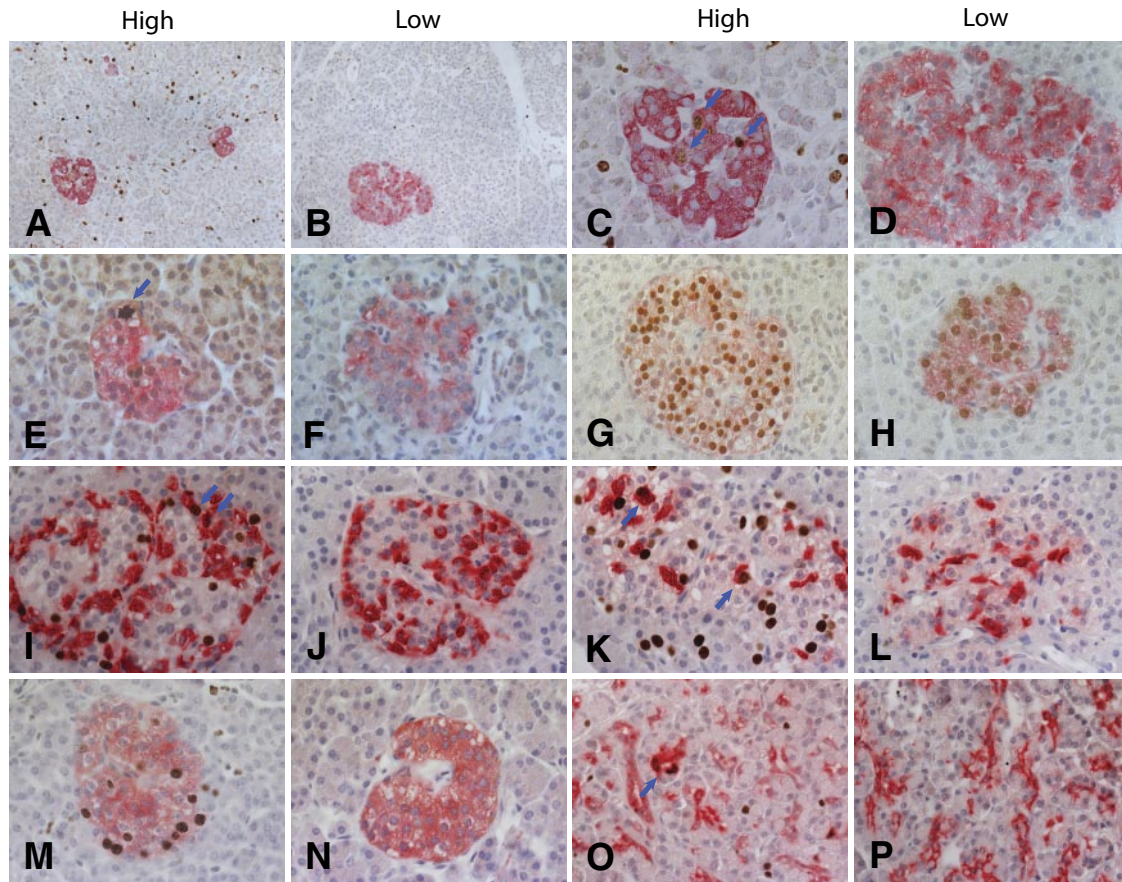
**FIG. 1. Imunohistochemical double staining for replication markers and pancreatic cell type–specific markers in paraffin sections of human donor pancreas.** Donors with a relatively high level of β-cell replication (≥90th percentile) (high) show increased replication in endocrine and ductal cell types compared with donors with a low level of β-cell replication (<90th percentile) (low). Representative images are shown. Staining for Ki67 (brown) and insulin (red) shows replicating cells in and around islets of Langerhans (*A* and *B*; ×70); part of the replicating islet cells correspond to insulin-positive β-cells (*C* and *D*; blue arrows; ×200). Staining for the $G_2$ to M transition marker phosphohistone H3 (brown) and insulin (red) shows an immunopositive mitotic β-cell (*E* and *F*; blue arrow; ×200). Staining for the transcription factor Nkx6.1 (brown) and insulin (red) shows colocalization of the two markers (*G* and *H*; ×200). Staining for Ki67 (brown) and glucagon (red) shows replicating α-cells (*I* and *J*; blue arrows; ×200). Double staining for Ki67 (brown) and somatostatin (red) shows replicating δ-cells (*K* and *L*; blue arrows; ×200). Double staining for Ki67 (brown) and the panendocrine marker synaptophysin (red) shows replicating endocrine islet cells (*M* and *N*; ×200). Double labeling for Ki67 (brown) and the ductal marker CA19.9 (red) shows replicating ductal cells (*O* and *P*; blue arrow; ×200). (A high-quality digital representation of this figure is available in the online issue.)

may change rapidly, we also tested serum fructosamine as a surrogate marker for prolonged hyperglycemia and compared levels of circulating C-peptide. We found no significant difference in the level of serum fructosamine between the 36 donors with high replication (152 ± 31 μmol/l) and their 36 matched controls (175 ± 24 μmol/l), nor did we find significant differences in the level of circulating C-peptide (1.75 ± 3.22 vs. 1.35 ± 2.43 μg/l).

**Prolonged duration of life support is associated with increased inflammatory infiltration.** Immunohistochemical staining for CD68 showed a diffuse infiltration of positive cells throughout the pancreatic parenchyma. The infiltration was variable between patients but was most pronounced in the group with high replication and in patients on prolonged life support (Fig. 3*A* and *B*). Small numbers of CD68$^+$ cells were observed in the islet interstitium, but no apparent colocalization with islet cell replication was found. Immunohistochemical staining for LCA/CD45 and CD3 showed focal areas of infiltration around the vasculature, in the interstitial connective tissue, and in the parenchyma, but was rare in islets (Fig. 3*C–F*). Quantification showed that prolonged life support was accompanied by a significantly increased infiltration of CD68$^+$ and LCA/CD45$^+$ cells in both young and older

donors, with the increase in CD68 positivity preceding the increase in LCA/CD45 positivity (Table 5). Donors with high β-cell replication (≥90th percentile; $n = 36$ patients) showed a significant ($P < 0.001$) 1.7-fold increase in CD68 positivity (274 ± 26 vs. 161 ± 18), and a significant ($P < 0.001$) 2.0-fold increase in LCA/CD45 positivity (85 ± 12 vs. 43 ± 6) when compared with $n = 36$ matched patients with low replication.

## DISCUSSION

In the present study we investigated β-cell replication levels in the normal human pancreas. We identified a subgroup of organ donors who presented with increased levels of β-cell replication and correlated our findings to the periterminal clinical conditions of the patients involved. We report that a prolonged period on life support (≥3 days), kidney dysfunction, a relatively young donor age (≤25 years), inflammatory infiltration, and a prolonged period of brain death before organ retrieval were all found to be significantly associated with an increased level (≥90th percentile) of β-cell replication. The effect was most pronounced in young donors who received prolonged life support; in contrast, it was rare in donors with
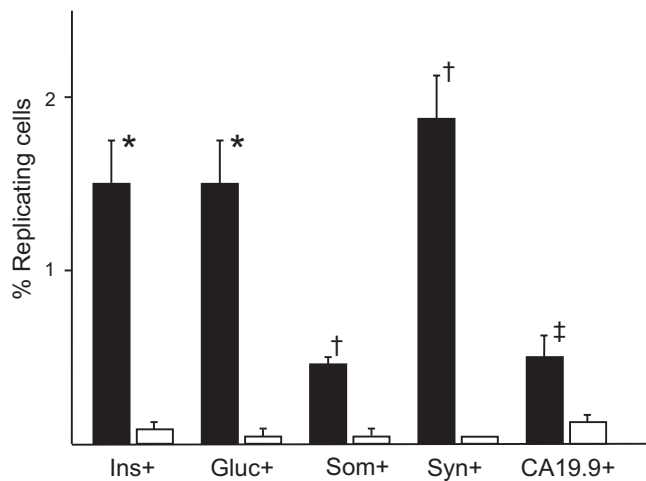
**FIG. 2. Replication levels in insulin-positive β-cells (Ins+), glucagon-positive α-cells (Gluc+), somatostatin-positive δ-cells (Som+), and carbohydrate antigen 19.9–positive ductal cells (CA19.9+) as determined by double immunohistochemistry for Ki-67. Donors with a high level of β-cell replication (≥90th percentile; n = 36; ■) are compared with matched controls (<90th percentile; n = 36; □). Results are expressed as means ± SE; significance of difference with the control condition was calculated with a nonparametric Mann-Whitney U test: \*P < 0.001; †P = 0.002; ‡P = 0.016.**

a short duration of life support regardless of age. The increase in replicative rate was not limited to β-cells but was also observed in α-cells, δ-cells, and ductal cells. We found that prolonged life support was associated with increased pancreatic infiltration of both $CD68^+$ monocytic cells and $LCA/CD45^+$ leukocytes. The increase in CD68 positivity appeared to precede the increase in LCA/CD45 positivity by several days. These results indicate that preterminal clinical conditions in organ donors can lead to both increased inflammatory infiltration of the pancreatic parenchyma and to an activation of β-cell replication that

TABLE 4
Donors with a high level of β-cell replication (≥90th percentile) stratified according to donor age and duration of stay in the intensive care unit

| | Time in intensive care unit | | | |
|---|---|---|---|---|
| Age | <3 days | 3–5 days | ≥6 days | Total |
| ≤25 years | 1/28 (3.6) | 8/13 (61)\*† | 7/9 (78)\*† | 16/50 (32)† |
| >25 years | 1/164 (0.6) | 2/76 (2.6) | 17/68 (25)\* | 20/308 (6.5) |
| Total | 2/192 (1.0) | 10/89 (11)\* | 24/77 (31)\* | 36/358 (10) |

Data are fraction (%). \*P < 0.001 vs. <3 days in the intensive care unit. †P < 0.001 vs. equivalent condition >25 years.

is most pronounced in patients in the younger age category.

Replication of adult human β-cells is a rare finding in histopathologic studies of human pancreas. In a study of 327 autopsy cases, only 14 patients were found to express one or more mitotic figures in the islets of Langerhans (9). In a similar study of 174 autopsy cases, only 18 patients showed one or more islet cell mitoses (10). Both studies were limited by the lack of specific immunohistochemical techniques to identify islet cell types and by the lack of sensitive techniques for the detection of islet cell replication. More recent studies, using immunohistochemistry for the nuclear marker Ki67 that is expressed during late $G_1$, S, M and $G_2$ phases of the cell cycle, found that β-cell replication decreased progressively from 3.2% at 17–32 weeks of gestation to 1.1% perinatally (5). After birth, the degree of β-cell replication was found to drop further, with initial levels being sufficient to account for the expansion of β-cell mass from birth to adulthood, but with β-cell replication levels decreasing hyperbolically with age to reach levels that are generally <0.1% in young adults (6,7). The low level of β-cell replication in the adult pancreas is supported by our present studies in which 72% of donors

TABLE 3
Clinical and histopathologic parameters associated with a high level of β-cell replication

| | β-Cell replication level | | Univariate | | Multivariate | |
|---|---|---|---|---|---|---|
| Risk factors | <90th percentile | ≥90th percentile | OR (95% CI) | P value | OR (95% CI) | P value |
| n | 327 | 36 | | | | |
| Mechanical respiration ≥3 days | 54/183 | 13/14 | 31.1 (4.0–243.3) | <0.001 | — | — |
| Intensive care unit ≥3 days | 132/322 | 34/36 | 24.5 (5.8–103.6) | <0.001 | 16.2 (3.2–88.2) | 0.001 |
| Kidney dysfunction | 11/324 | 7/35 | 7.1 (2.6–20.0) | <0.001 | 10.9 (2.3–52.6) | 0.003 |
| Age ≤25 years | 35/326 | 16/36 | 6.7 (3.2–14.0) | <0.001 | 17.4 (5.3–57.1) | <0.001 |
| Increased CD68 infiltration | 97/327 | 26/36 | 6.2 (2.9–13.3) | <0.001 | 2.0 (0.6–6.0) | 0.240 |
| Increased LCA infiltration | 42/327 | 13/36 | 3.9 (1.8–8.1) | <0.001 | 3.2 (1.0–9.9) | 0.046 |
| Brain death ≥12 h | 91/321 | 19/35 | 3.0 (1.5–6.1) | 0.002 | 2.8 (1.0–7.8) | 0.043 |
| Use of steroids | 28/327 | 7/36 | 2.6 (1.0–6.4) | 0.042 | 3.8 (0.9–15.8) | 0.062 |
| BMI >30 kg/m² | 19/327 | 4/36 | 2.0 (0.6–6.3) | 0.224 | — | — |
| Hyperglycemia | 71/300 | 7/36 | 0.8 (0.3–1.9) | 0.572 | — | — |
| Hypotensive periods | 93/203 | 14/31 | 1.0 (0.5–2.1) | 0.946 | — | — |
| Liver damage | 1/30 | 1/36 | 0.8 (0.5–13.8) | 0.896 | — | — |
| Pancreas damage | 64/237 | 4/36 | 0.3 (0.1–1.0) | 0.049 | 0.5 (0.1–1.8) | 0.287 |

Uni- and multivariate analysis were performed to test the association between 13 clinical and histopathologic parameters (risk factors) and high levels of β-cell replication (≥90th percentile) including prolonged duration of mechanical respiration (≥3 days), prolonged time in the intensive care unit (≥3 days), kidney dysfunction (serum creatinine ≥150 μmol/l), young donor age (≤25 years), increased $CD68^+$ cell infiltration (≥90th percentile), increased $LCA/CD45^+$ cell infiltration (≥90th percentile), prolonged duration of brain death (≥12 h to start of cold perfusion), the use of steroid hormones (yes/no), high BMI (>30 kg/m²), hyperglycemia (glucose ≥200 mg/dl), hypotensive periods (systemic blood pressure <100 mmHg), liver damage (bilirubin ≥2 mg/dl combined with aspartate aminotransferase ≥25 units/l), and pancreas damage (amylase >200 units/l). Logistic regression analysis was performed with β-cell replication level as a dependent variable, with inclusion of all variables with P ≤ 0.10 in univariate analysis. Duration of mechanical ventilation was not included as a variable because of the number of missing data and the good correlation with time in the intensive care unit.
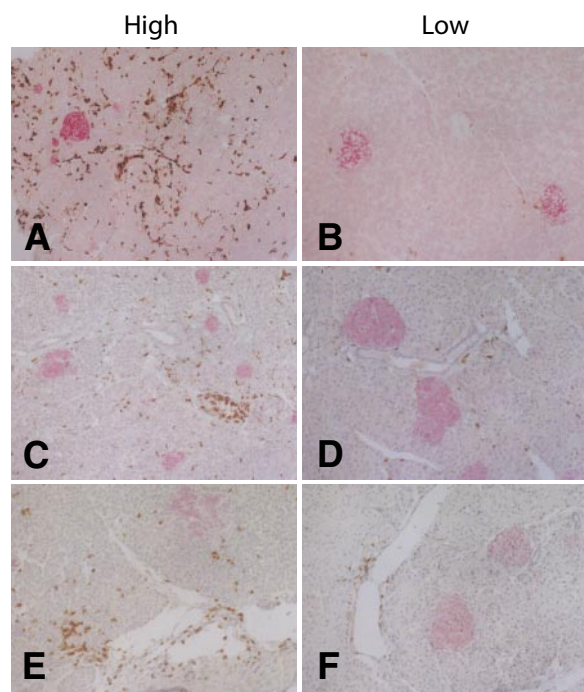
FIG. 3. Immunohistochemical staining for monocytic and leukocytic infiltration markers in paraffin sections of human donor pancreas. Donors with a relatively high level of β-cell replication (≥90th percentile) (high) have increased inflammatory infiltration compared with donors with low level of β-cell replication (<90th percentile) (low). Representative images are shown. Double staining for CD68 (brown) and the panendocrine marker synaptophysin (red) (*A* and *B*; ×70), LCA/CD45 (brown) and synaptophysin (red) (*C* and *D*; ×70), and CD3 (brown) and synaptophysin (red) (*E* and *F*; ×70). (A high-quality digital representation of this figure is available in the online issue.)

show a replication level in this range. However, the remaining donors presented with replication levels that were significantly higher (0.2–7.0%), reaching levels normally found only in early fetal pancreas (4–6).

In the studied donor organs, high levels of β-cell replication were found to be accompanied by an increased replication of α-cells, δ-cells, and ductal cells. The increase in replication thus appears to be a generalized phenomenon with virtually all pancreatic cell types being induced into a replicative state. In patients with a high replicative activity, we also noted the presence of mitotic figures inside the islets of Langerhans and positivity for the $G_2$ to M transition marker phosphohistone H3, albeit at a much lower frequency than that of Ki67 positivity. These observations indicate that Ki67-positive cells are driven toward a

TABLE 5
Mean number of infiltrating $CD68^+$ monocytes and $LCA/CD45^+$ leucocytes stratified according to donor age and duration of stay in the intensive care unit

| Age | Marker | Time in intensive care unit | | |
| | | <3 days | 3–5 days | ≥6 days |
| --- | --- | --- | --- | --- |
| ≤25 years | CD68 | 116 ± 14 | 152 ± 20* | 343 ± 50† |
| | LCA/CD45 | 34 ± 5 | 34 ± 16 | 116 ± 24† |
| >25 years | CD68 | 115 ± 5 | 168 ± 11† | 281 ± 17† |
| | LCA/CD45 | 57 ± 4 | 60 ± 5 | 93 ± 11* |
| All ages | CD68 | 115 ± 4 | 166 ± 10† | 283 ± 16† |
| | LCA/CD45 | 54 ± 3 | 58 ± 6 | 96 ± 10† |

Data are means ± SE of cell numbers per 10 high power microscope fields. *$P < 0.05$ vs. <3 days in the intensive care unit. †$P < 0.001$.

proliferative pathway, rather than toward a polyploid state that is relatively frequent in normal human pancreas (14).

When the 10% of patients with the highest replication level (P90) were correlated with the available clinical data, a significant association was found with a prolonged duration of life support, kidney dysfunction, relatively young donor age, inflammatory infiltration, and a prolonged period of brain death. A total of 68% of patients with both a prolonged life support and young donor age were found to present with high levels of β-cell replication, in contrast with 1% of patients with a shorter duration of life support, irrespective of age. These observations suggest that β-cell replication was induced only after admission to the hospital and took several days to develop: donors with high levels of replication had a duration of mechanical respiration that on average exceeded 6 days, whereas donors with low replication were on average mechanically respirated for 2 days. The mechanism behind this induction is unknown, but several hypotheses can be proposed.

A first possibility is that it is caused by prolonged treatment with drugs that are known to induce β-cell replication: patients in the intensive care unit often receive treatment with high doses of steroids, which were shown to induce a marked elevation of plasma insulin levels and 20- to 30-fold higher levels of islet cell replication in primates (15). However, we did not find any evidence for this possibility: treatment with steroids was not significantly associated with high β-cell replication, although the duration of drug treatment was not always known and the use of steroids may not always have been registered in the donor file. We therefore also tested for differences in circulating C-peptide levels, as steroid treatment was reported to result in increased circulating insulin levels (15), but no significant differences were observed.

A second possibility is that a prolonged period of hyperglycemia may contribute to the induction of β-cell replication in these patients. Although no significant differences in plasma glycemia could be found between the two groups, it cannot be excluded that such differences did exist before the time point of blood sampling just before death. We therefore measured fructosamine levels as a surrogate marker for prolonged hyperglycemia but did not find any evidence for a significant difference between the two groups.

A third possibility is that β-cell replication is activated by a prolonged period of hypoxia leading to cellular damage as might be the case in the subpopulation of patients with extended life support. The presence of higher numbers of $CD68^+$ macrophages and $LCA/CD45^+$ leukocytes seen dispersed throughout the pancreatic parenchyma in such patients may be indicative of cellular damage, and the macrophages may be involved in clearing cellular debris. It is so far not known which signals are responsible for the proliferative stimulus. Cytokines may be released by pancreatic cells such as ductal cells (16), as well as by the infiltrating monocytes and leukocytes. Release of proinflammatory cytokines and/or leukocytic infiltration has been described in both human donor kidney and liver (17,18) and in rodent islets after brain death (19). Several recent studies suggested that β-cell replication and neogenesis are stimulated by inflammatory lesions induced by autoimmunity (20–23) or injury (24). Follow-up studies will be necessary to dissect the mechanism of the replicative response that is described in the present report. Gene expression analysis of human pan-

creas samples and isolated islet fractions collected during the present study may indicate the nature of the factors involved. Exposure of isolated human islets to proinflammatory cytokines in vitro may help establish their stimulatory effect on β-cell replication.

Our observation of increased inflammatory infiltration throughout the pancreatic parenchyma in organ donors with extended life support is also relevant in the context of islet transplantation. It cannot be excluded that islets isolated from such donors may either contain higher numbers of passenger leukocytes or that islet cells are activated by cytokine exposure leading to changed allograft reactivity. It also warrants caution in the interpretation of histopathologic changes in postmortem pancreas in the context of presumed autoimmune lesions and stresses the importance of obtaining control groups that are adequately matched in terms of clinical history.

The presence of replicating β-cells in adult organ donors indicates that although such cells are rare under normal circumstances, they have retained their potential for growth and can be induced to enter the mitotic cycle upon activation by preterminal clinical conditions. Alternatively, replicating cells may be derived from progenitor cells (24) or from existing adult cells, such as peripheral blood monocytes (25), by a process of transdifferentiation.

In summary, we have quantified β-cell replication in a large consecutive series of human organ donors and found evidence that a subgroup of donors present with high levels of replication in pancreatic endocrine cells, including islet β-cells. Multivariate analysis of clinical data showed that high levels of replication were significantly and independently associated with extended life support, kidney damage, and young donor age. These patients were also characterized by significantly increased levels of inflammatory infiltration in the pancreatic parenchyma. The results indicate that preterminal clinical conditions in organ donors can activate β-cell replication. Elucidation of the cellular and molecular pathways involved in this process may help researchers devise new strategies for stimulating β-cell growth in vivo.

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