β-Cell Proliferation and Apoptosis in the Developing Normal Human Pancreas and in Hyperinsulinism of Infancy

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Hyperinsulinism of infancy (HI), also known as persistent hyperinsulinemic hypoglycemia of infancy, is a rare genetic disorder that occurs in ~1 of 50,000 live births. Histologically, pancreases from HI patients can be divided into 2 major groups. In the first, diffuse HI, β-cell distribution is similar to that seen in normal neonatal pancreas, whereas in the second, focal HI, there is a discrete region of β-cell adenomatous hyperplasia. In most patients, the clinical course of the disease suggests a slow progressive loss of β-cell function. Using double immunostaining, we examined the proportion of β-cells undergoing proliferation and apoptosis during the development of the normal human pancreas and in pancreases from diffuse and focal HI patients. In the control samples, our findings show a progressive decrease in β-cell proliferation from 3.2 ± 0.5% between 17 and 32 weeks of gestation to 0.13 ± 0.08% after 6 months of age. In contrast, frequency of apoptosis is low (0.6 ± 0.2%) in weeks 17–32 of gestation, elevated (1.3 ± 0.3%) during the perinatal period, and again low (0.08 ± 0.3%) after 6 months of age. HI β-cells showed an increased frequency of proliferation, with focal lesions showing particularly high levels. Similarly, the proportion of apoptotic cells was increased in HI, although this reached statistical significance only after 3 months of age. In conclusion, we demonstrated that islet remodeling normally seen in the neonatal period may be primarily due to a wave of β-cell apoptosis that occurs at that time. In HI, our findings of persistently increased β-cell proliferation and apoptosis provide a possible mechanism to explain the histologic picture seen in diffuse disease. The slow progressive decrease in insulin secretion seen clinically in these patients suggests that the net effect of these phenomena may be loss of β-cell mass. *Diabetes* 49: 1325–1333, 2000

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AEC, aminoethylcarbazole; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; HI, hyperinsulinism of infancy; SUR1, sulfonylurea receptor; TUNEL, tranferase-mediated dUTP-X3′ nick end-labeling.

Hyperinsulinism of infancy (HI), also known as persistent hyperinsulinemic hypoglycemia of infancy, is a rare genetic disorder, the molecular basis of which was recently elucidated. Most cases are caused by mutations in either the sulfonylurea receptor (SUR1) or the inward rectifying potassium channel (Kir6.2), 2 subunits of the β-cell K_{ATP} channel (1–5). A minority of patients have glucokinase or glutamate dehydrogenase mutations, whereas in 40–50% of the patients, the genetic cause of the disease is still not known (4–7).

The histologic appearance of the pancreases from affected children can be subdivided into 2 major forms: diffuse HI and focal HI (8–10). The former is more common and bears some characteristics of nesidioblastosis, a phenomenon observed in the healthy fetus and newborn but which normally evolves during the first year of life into the adult-type architecture (9,11,12). In diffuse HI, the neonatal-type β-cell distribution persists (8,13).

Focal HI is generally easily recognized as a discrete region of adenomatous hyperplasia (8), whereas the rest of the pancreas appears normal for its age. Focal HI is caused by the somatic loss of part of the short arm of maternal chromosome 11 in a β-cell precursor of a patient carrying a mutant SUR-1 on the paternal allele (14–16). Two genes that are important in cell cycle regulation, $p57^{KIP2}$ and H19, are located in this region of chromosome 11p, and both are imprinted, with only the maternal allele expressed (17–19). Therefore, the β-cells developing from this mutant precursor will have only one mutant SUR-1 allele, and lack expression of both $P57^{KIP2}$ and H19.

Patients with genetic evidence of diffuse or focal HI who do not undergo surgery appear to enter clinical remission over a period of months to years. Patients with diffuse HI progress to diabetes, whereas patients with suspected focal HI glucose and insulin dynamics normalize (20,21). Increased β-cell proliferation has been reported in focal lesions (22), which is in apparent contradiction to these clinical observations. Preliminary data suggested that β-cells in focal lesions also undergo increased apoptosis (21).

In the current study, we determined the frequency of proliferating and apoptotic β-cells in both diffuse and focal HI. To determine the normal dynamics of β-cell turnover, we examined pancreases obtained during autopsy from human fetuses and infants. Our observations suggest that age-specific changes in β-cell proliferation and apoptosis can explain the pancreatic remodeling normally seen in the postnatal period. In HI pancreases, both of these processes persist, thus

explaining the fetal-type β-cell distribution seen in older patients with diffuse HI. The increased frequency of apoptotic β-cells after 3 months of age may explain the long-term clinical course of the HI.

RESEARCH DESIGN AND METHODS

Patients. Archival tissue from 23 pancreatectomized HI patients was obtained from 3 clinical centers (Table 1). In all, the diagnosis of HI was made according to accepted criteria (23,24). Eleven boys and 12 girls, age range 2 weeks to 41 months, were included in the study. Nine patients had focal disease and 14 had a diffuse form of HI (9). One patient with diffuse disease underwent 3 separate surgeries and a tissue sample was obtained from each.

Control subjects. Archival pancreatic tissue samples were obtained from 44 autopsies carried out between 1988 and 1998, and included 25 boys and 19 girls, ranging in age from 17 weeks of gestation to 6 years (Table 2). All fetuses and infants died as a result of diseases not related to the pancreas, and in all, autopsies were done for medical reasons according to accepted procedures at each individual institution. All dysmorphic subjects were excluded, as were subjects with known chromosomal abnormalities. Only subjects in whom the autopsy was performed within 24 h of death were included. All samples were stained with hematoxylin and eosin and screened for adequate quantity of tissue, normal morphology, and good tissue preservation.

Immunohistochemistry. Five micron sections were prepared from archival paraffin-embedded tissue, placed on SuperFrost Plus glass slides (Menzel-Glaser, Germany), and left to dry at 37°C overnight. Slides were deparaffinized in xylol, rehydrated in serial concentrations of alcohol (100, 90, and 80%) and double-distilled water. Proliferating β-cells were identified by double staining for insulin and Ki-67 antigen, a nuclear marker of cells that are in the replicative cycle (25). Antigen retrieval was carried out as described by Cattoretti et al. (26). Briefly, slides were microwaved in 0.01 mol/l citrate buffer (pH 6) for 3 min at full power until boiling and for 15 min at 20% power. Slides were left to cool at room temperature for 20 min, blocked with nonimmune serum for 10 min, and incubated for 1 h at 37°C with mouse monoclonal anti-Ki-67 antibody at the dilution supplied (Zymed cat. no. 08-0156). Bound antibody was detected using the streptavidin biotin-peroxidase kit (Zymed cat. no. 85-9943) and developed by 3,3′-diaminobenzidine tetrachloride nickel – cobalt (DAB-Black; Zymed cat. no. 00-2013). Avidin/biotin-blocking kit (Zymed cat. no. 00-4303) was used before incubation with guinea pig anti-insulin antibody (Dako, Denmark) for 30 min at 37°C, dilution 1:100. Detection of insulin binding was accomplished using the streptavidin biotin-alkaline phosphatase kit and developed with fast red (Zymed cat. no. 85-9942).

Apoptotic β-cells were identified by the terminal deoxynucleotidyl transferase–mediated dUTP-X 3′ nick end-labeling (TUNEL) technique (27). Slides were incubated with 20 µg/ml proteinase K (Boehringer-Mannheim) for 15 min at 37°C. TUNEL stain was carried out using an alkaline phosphatase in situ cell-death detection kit (Boehringer-Manhnheim, Germany) according to the manufacturer's instructions and developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma, St. Louis, MO). As a positive control, a slide of a normal adult pancreas was preincubated in 100 µg/ml DNase I (Boehringer-Mannheim) and stained as previously described. The same tissue was used as a negative control by staining as previously described but without terminal transferase. Control slides were included in each batch stained. Insulin was stained using the streptavidin biotin-peroxidase kit (Zymed cat. no. 85-9943) and developed with aminoethylcarbazole (AEC).

All slides were lightly counterstained with Meyer's hematoxylin.

Quantification. All slides were coded, and at least 1,000 β-cells were counted under high magnification (×400, Nikon, L-300). When assessing the frequency of apoptotic cells, we excluded regions with tissue damage or necrosis, as evidenced by large numbers of positively stained endocrine and exocrine cell nuclei. Such regions were frequently seen at the periphery of the tissue sample. Only cells with clear double stain for TUNEL or Ki-67 and insulin were counted. At final assess-

TABLE 1
Clinical characteristics of HI patients

Sex	Birth weight (kg)	Age of onset (months)	Age at surgery (months)	Postoperative glycemic status	Paternal mutation	Maternal mutation	Ref.
Diffuse disease							
F	—	Birth	0.6	Hypoglycemic	delcP317	delcP317	5
F	4.5	Birth	0.75	Hypoglycemic	delcP317	delcP317	5
F	3.4	Birth	1.25	Euglycemic	None found	None found	
F*	4.1	1.25	1.5	Hypoglycemic	3992 –3c to g	N188S	5
M	3.6	Birth	1.6	Diabetes	delcP317	delcP317	5
F	—	0.5	2.25	—	No DNA		
M	4.1	Birth	2.25	Hypoglycemic	3992 –9 g to a	3992 –9 g to a	3
M	5.04	Birth	3.25	Hypoglycemic	Kir6.2 Y12X	Kir6.2 Y12X	4
F	—	Birth	3.5	—	No DNA		
F	2.4	0.5	6.5	Hypoglycemic	None found	None found	
F	5	Birth	10.5	Euglycemic	3992 –9 g to a	delF1388	3
M	4.4	Birth	13	Hypoglycemic	3992 –9 g to a	delF1388	3
F	5	0.25	13.25	—	No DNA		
F*			19.25	Hypoglycemic			
F*			29.25	Diabetes			
M	3.8	Birth	41	Hypoglycemic	A1493T	None found	†
Focal disease							
M	5.36	Birth	0.5	Euglycemic	3992 –9 g to a	None found	3
F	3.25	Birth	0.833	Euglycemic	None found	None found	
M	4.18	Birth	1.25	Diabetes	None found	None found	
M	3.91	Birth	2	Euglycemic	R1494Q	None found	‡
M	3.65	1.5	3	Euglycemic	1630 +1 g to t	None found	5
M	3.61	Birth	5.5	Euglycemic	None found	None found	
F	3.3	5	6	Euglycemic	No DNA		
M	3.63	10	11	Euglycemic	A1493T	None found	†
F	3	Birth	12	—	No DNA		

Clinical data are presented for all HI patients included in the study. *The patient underwent 3 pancreatic surgeries, and specimens from each were studied and included according to the patient's age at the time of surgery; †Kutchinski et al., accession no. H971606, Human Genetics electronic mutation submission; ‡Kutchinski et al., accession no. H971605, Human Genetics electronic mutation submission.

ment, 2 control samples were excluded from the apoptosis studies, one because of an insufficient number of β-cells and the other because of poor tissue preparation. Similarly, 2 focal HI samples were excluded from the apoptosis study and one from the proliferation study because of poor tissue preparation.

Statistical analysis. The frequencies of apoptotic and proliferating cells are presented as percent of insulin positive cells (mean ± SE). For analysis, the perinatal period was defined as 2 months before and after birth. Groups were compared using the Mann-Whitney *U* test. A 1-tailed analysis was used to compare patient- and age-matched control groups because the a priori hypothesis based on previous studies was that both apoptosis and proliferation are increased in diseased tissue. A 2-tailed analysis was used to compare different control age-groups because there was no a priori hypothesis.

RESULTS

β-Cell proliferation. Proliferating β-cells were readily identified by double immunostaining in red for insulin and dark brown for Ki-67 (Fig. 1*A*, *C*, and *D*). In the control pancreases, the proportion of β-cell proliferation was significantly correlated with age ($P = 0.0001$, Spearman correlation test). Proliferation decreased from 3.2 ± 0.5% during weeks 17–32 of gestation to 0.13 ± 0.08% after 6 months of age. In the perinatal period, intermediate levels of proliferation were seen (1.1 ± 0.2%, Figs. 2*A* and 3*A*).

TABLE 2
Clinical characteristics of autopsy specimens from which control pancreases were obtained

Age (months)	Sex	Cause of death
–5.75	M	Induced abortion
–5.25	M	Induced abortion
–5	F	Induced abortion
–4.25	M	Acute chorioamnionitis
–4.25	F	Induced abortion
–3.75	M	Induced abortion
–3.5	M	Omphalocele, hypoplastic lungs
–3.5	M	Intraventricular hemorrhage
–2.75	F	Prematurity, hyaline membrane disease, multiple anomalies
–2.75	M	Prematurity, hypoplastic lungs
–2.5	M	Intracranial hemorrhage
–2	F	Hyaline membrane disease
–1.5	M	Hyaline membrane disease, multiple anomalies
–1.25	F	Congenital heart disease
–1.25	M	Prune-belly syndrome, intra-alveolar hemorrhage
–1	F	Renal agenesis, pulmonary hemorrhage
–0.75	M	Central nervous system and muscular/skeletal anomalies
–0.75	F	Congenital heart disease
–0.25	M	Massive amniotic fluid aspiration
0	F	Meconium aspiration, hyaline membrane disease
0	M	Acute placental abruption
0	M	Thanatophoric dwarfism
0	F	Pulmonary hemorrhage
0.5	M	Achondroplasia, megalencephaly
0.5	M	Hydrocephalus
1	F	Congenital heart disease
1	M	Congenital heart disease
1	M	Neonatal hepatitis
1.75	M	Sudden infant death syndrome
2	M	Recurrent apnea
3	M	Bronchopneumonia
3.5	F	Nezelof syndrome
4.5	F	β-Cell deficiency, pneumonia
5.5	F	Septicemia
6	F	Combined immune deficiency, bronchopneumonia
6	F	Sudden infant death syndrome
6	M	Gastroenteritis
6	M	Hydrocephalus
8	F	Hydrocephalus
11	F	Microcephaly, bronchopneumonia
24	M	Sudden infant death syndrome
36	M	Tetrology of fallot, postoperative complications
36	F	Cardiac hypertrophy, bronchopneumonia
72	F	Bronchopneumonia

Data are shown describing the clinical characteristics of the 44 subjects from whom the autopsy specimens were taken. Ages are given in months, with fetal age given as negative value relative to 40 weeks' gestation. For cause of death, relevant data are given, including clinical diagnosis and/or autopsy findings. All patients with known or suspected pancreatic abnormalities were excluded.

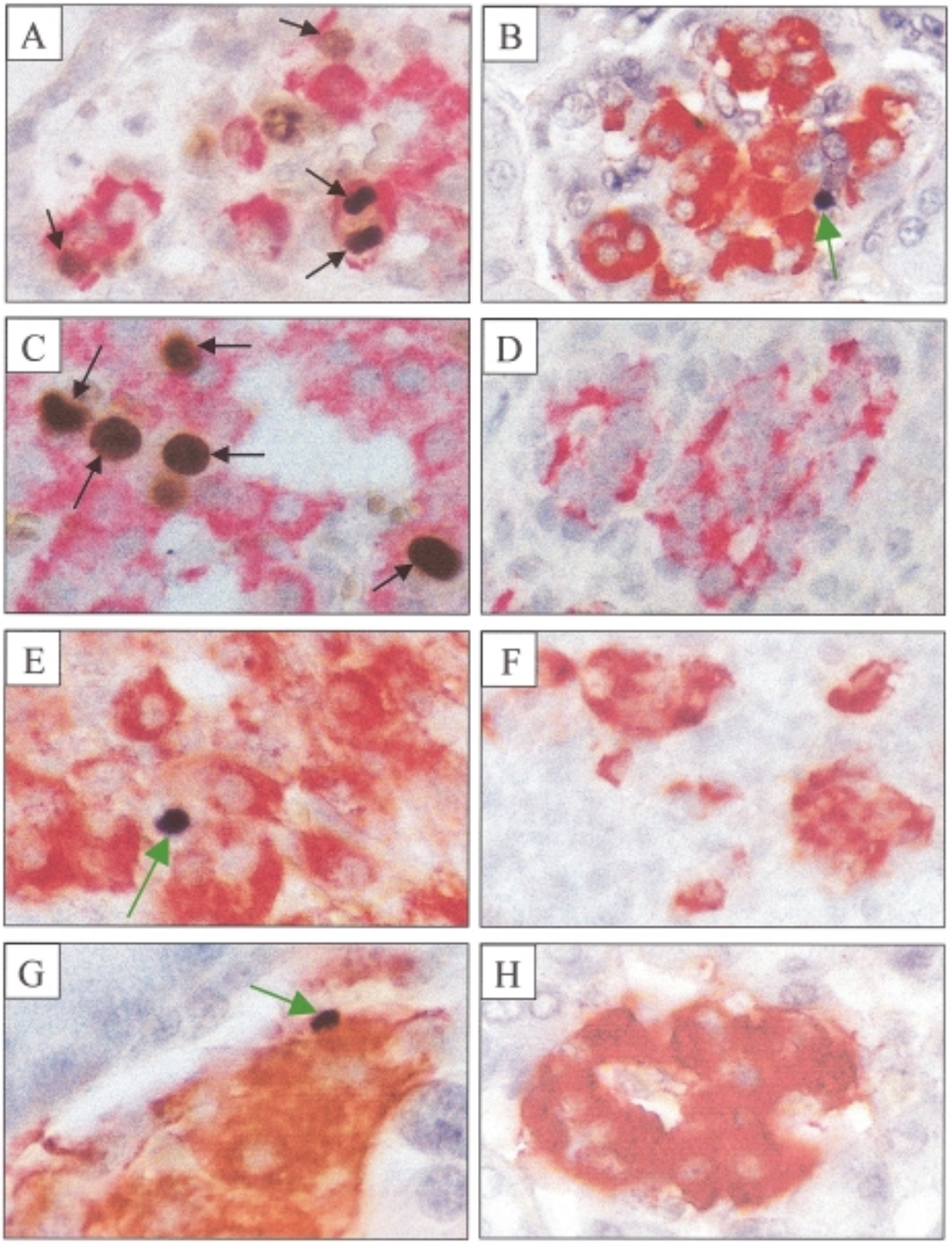

FIG. 1. Double immunostaining for Ki-67, visualized with 3,3′-diaminobenzidine tetrachloride nickel-cobalt (DAB-Black), and insulin, visualized with fast-red, of pancreases from a 19-week gestation human fetus (*A*); a focal lesion from a 1.25-month HI patient (*C*); and the nonaffected area outside the lesion from the same patient (*D*). Double immunostaining for terminal deoxynucleotidyl transferase–mediated dUTP-X 3′ nick end-labeling visualized with BCIP/NBT (black) and insulin with AEC (red-brown) of pancreases from a full-term control neonate (*B*); the focal lesion from the same HI patient as above (*E*); the nonaffected area outside the lesion from the same patient (*F*); a 6.5-month-old patient with diffuse HI (*G*); and an age-matched control subject (*H*). The black arrows indicate the double-positive cells for Ki-67 and insulin, and the green arrows indicate double-positive cells for TUNEL and insulin. Original magnification ×400.

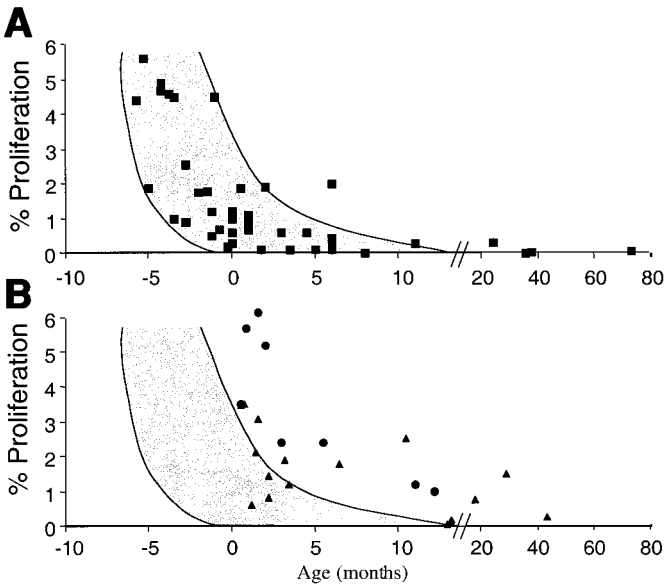

FIG. 2. *A*: Percent β-cell proliferation in control pancreases. Individual results are shown (■) and the normal range is demonstrated by the shaded area. *B*: Percent β-cell proliferation in patients with diffuse (▲) and focal (●) HI. The shaded area represents the normal range as shown in panel *A*.

In HI patients, proliferation was generally greater than expected from the control data (Fig. 2*B*). In patients with diffuse disease who were younger than 3 months of age at the time of surgery, the incidence of Ki-67–positive β-cells was significantly greater than in the age-matched control group (2.13 ± 0.46 vs. 0.88 ± 0.19, respectively; $P < 0.05$; Fig. 4). In focal HI patients, the proportion of proliferating β-cells within the lesion was even higher (5.48 ± 0.82%) and was significantly greater than in control pancreases ($P < 0.01$) or in HI patients with diffuse disease ($P < 0.01$). The uninvolved regions of the pancreas from these patients showed a frequency of proliferation (0.88 ± 0.17%) that was similar to that seen in the control group.

Patients and control subjects older than 3 months of age had β-cell proliferation lower than that found in the younger group. However, here too the proportion of proliferating β-cells seen in the pancreases from patients with diffuse disease and within the focal lesions was significantly greater than in the age-matched control group ($P < 0.02$ and $P < 0.01$, respectively; Fig. 4).

β-Cell apoptosis. Apoptotic β-cells were readily identified by double immunostaining for insulin, stained in red, and the TUNEL reaction, which stains fragmented nuclear DNA black (Fig. 1*B* and *E–H*). In the normal pancreas, changes in the frequency of β-cell apoptosis during the fetal, perinatal, and postnatal periods were markedly different from those of proliferation (Fig. 5*A*). During weeks 17–32 of gestation and after the first 6 months of life, β-cell apoptosis was relatively rare (0.6 ± 0.2 and 0.08 ± 0.03%, respectively). In contrast, during the perinatal period, defined here as 2 months before and after birth, there was an increase in the frequency of β-cell apoptosis (1.3 ± 0.3%; Fig. 3*B*).

In the older HI patients, the frequency of apoptosis was greater than expected from the control data (Fig. 5*B*). Figure 6 compares levels of apoptosis in different age-groups of HI patients and control subjects. During the first 3 months of life, the frequency of β-cell apoptosis was not significantly different in patients with diffuse HI (1.67 ± 0.33%) or focal HI (1.2 ± 0.1%) when compared with each other or with control subjects (1.09 ± 0.43%, $P > 0.1$). However, after the age of 3 months, apoptosis in control subjects decreased markedly to 0.13 ± 0.03%, whereas in diffuse and focal HI patients it remained unchanged (1.76 ± 0.62 and 1.77 ± 0.5%, respectively; $P < 0.005$ compared with the age-matched control group). In focal HI patients, the frequency of apoptosis in the uninvolved portion of the pancreas (0.43 ± 0.033%) was significantly less than that of the focal lesion ($P = 0.05$).

DISCUSSION

In this study, we demonstrated that in the normal neonatal pancreas, there is a steady decline in the frequency of β-cell proliferation that approaches 0 by the age of ~6 months. In contrast, β-cell apoptosis is rare in the early fetal period and

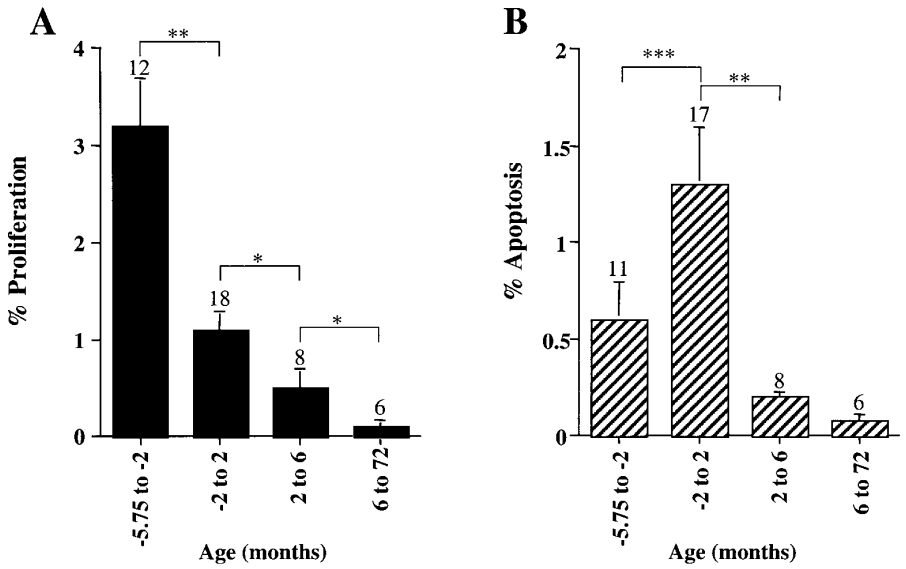

FIG. 3. Mean (%) of proliferating (*A*) and apoptotic (*B*) β-cells in control pancreases presented according to age-group. The number of subjects in each group is given immediately above each column. **P* < 0.05; ***P* < 0.001; ****P* = 0.07.

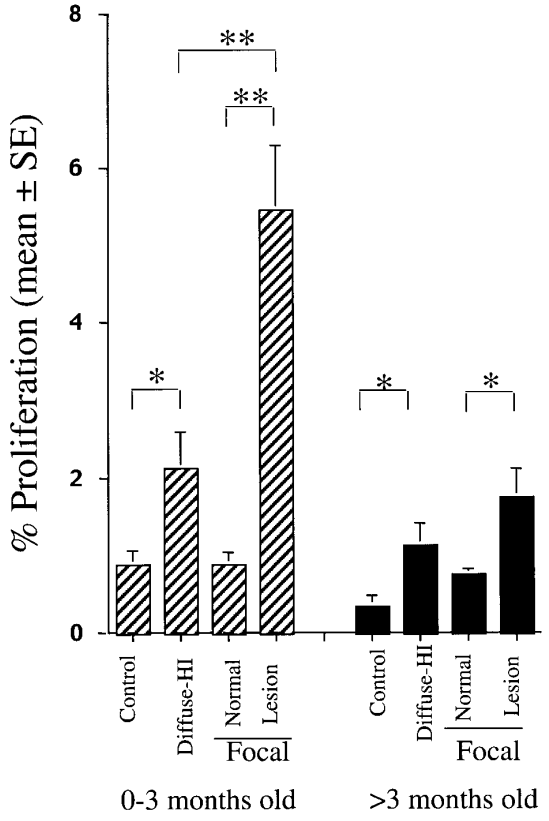

FIG. 4. Mean ± SE of percent proliferation in control and HI patients according to age-group. The striped columns represent data from infants 0–3 months of age, whereas the black columns represent data from infants older than 3 months. Statistical comparisons were made as indicated by the brackets above the columns. Significance between groups is indicated by *($P < 0.05$) or **($P < 0.01$).

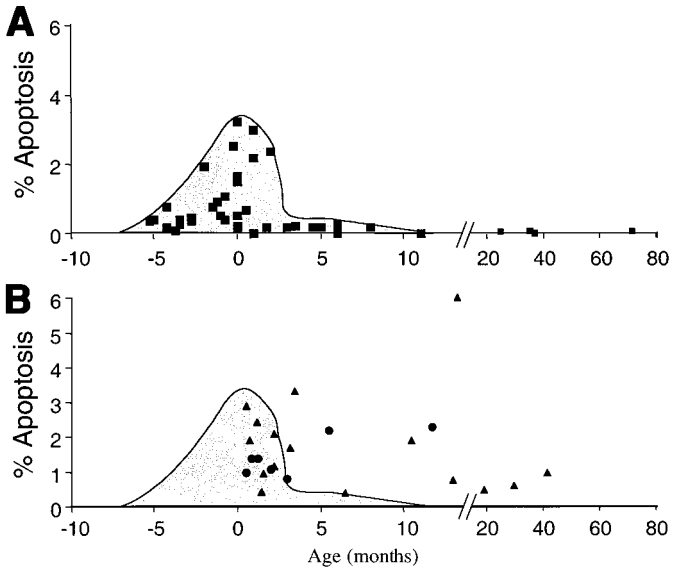

FIG. 5. *A*: Percent β-cell apoptosis in control pancreases. Individual results are shown (■) and the normal range is demonstrated by the shaded area. *B*: Percent β-cell apoptosis in patients with diffuse (▲) and focal (●) HI. The shaded area represents the normal range as shown in panel *A*.

after 6 months of age, with increased levels during the perinatal period. In HI patients, both proliferation and apoptosis are increased when compared with age-matched control groups and both persist beyond the perinatal period.

The use of archival pathological samples has obvious limitations. However, it is the only practical way of making the observations of the kind presented here in human subjects (22). To decrease the probability of spurious results, we used very strict criteria to determine which samples qualify for inclusion in the study. Only cells that stained for insulin were counted, to guarantee that the frequency of apoptosis and proliferation measured related specifically to β-cells and not to other endocrine or nonendocrine cell types. Therefore, it is likely that both of these were underestimated because cells that have not yet started to produce insulin, as well as those that are completely degranulated, were not counted. By using these strict criteria, we believe that we succeeded in obtaining results that are reliable within the limitations of the method.

Proliferation. The proportion of proliferating β-cells reported here are different from those reported by Bouwens et al. (28), who found similar frequencies of β-cell proliferation in the early fetal period but essentially no proliferation after 24 weeks of gestation. Sempoux et al. (22) found 2% proliferation in control infants, somewhat more than observed in the current study and markedly greater than that reported by Bouwens et al.. The reason for these differences is unclear, because all of the studies used Ki-67 and insulin immunostaining to identify proliferating β-cells. The larger number of samples included in our study may partially explain the difference. Our findings are qualitatively similar to those reported in the neonatal rat in which proliferation decreased from a peak of 4.7% at 2 days of age to a nadir of 1.8 at 31 days (29). In the rat studies, proliferation was measured 6 h after bromodeoxyuridine injection, a method that is clearly not applicable to human studies. Therefore, the absolute frequency of proliferation cannot be compared directly. It is important to note that we report here the percent of insulin-positive cells staining positive for Ki-67 and not the absolute number of cells undergoing proliferation. Because of the methodology used, absolute numbers per pancreas could not be determined.

The mechanisms regulating β-cell proliferation in humans are not known. In the rodent, IGF-II is elevated in the fetus and decreases after birth, which is in parallel with the decrease in proliferation (30), and β-cell overexpression of IGF-II in transgenic animals results in islet hyperplasia (31). Whether the same mechanisms are important in humans is not known; however, in Beckwith-Weidemann syndrome, Chllp15.5 uniparental paternal disomy (32) or relaxation of imprinting, leads to overexpression of IGF-II, which is associated in some cases with hypoglycemia caused by hyperinsulinism and islet-cell hyperplasia (11,33).

The frequency of proliferation observed in the HI patients was higher than in control subjects of the same age-group. This difference is unlikely to be methodological for several reasons. The effect of variations in sample handling on Ki-67 stains has been extensively studied, albeit not in pancreatic tissue (34). A delay of up to 24 h before fixation does not appear to affect Ki-67 staining, and the time of formalin fixation does not appear to affect results as long as the samples are heated for at least 14 min by microwave in a citrate buffer. All of our samples met both of these criteria. An addi-

tional indication that our results in autopsy specimens can be compared with surgical specimens is the observation that in patients with focal lesions, the proportion of β-cell proliferation in the uninvolved region of the pancreas was similar to that seen in the control subjects from the same age-group.

Our findings are qualitatively similar to those reported by Sempoux et al. (22), although in that study, the difference between diffuse HI and the control group did not reach statistical significance. The frequency of Ki-67–positive β-cells of 2.94% in 1.5- to 12-month-old patients and 1.97% in age-matched control subjects is somewhat greater than we found in our samples. This difference may be due to counting criteria because, as previously stated, we used very strict criteria for defining Ki-67–positive β-cells, realizing that ours may be an underestimation.

In patients with focal disease, proliferation was even higher (Fig. 4), again consistent with the findings reported by Sempoux et al. (22). The primary difference between focal and diffuse disease is that in the focal lesion, maternally expressed, imprinted genes on chromosome 11p are deleted (35). There are at least 2 such genes known, H19 and $P57^{kip2}$. The former is an untranslated RNA molecule thought to be an important regulator of IGF-II mRNA levels (36), whereas the latter is an important inhibitor of several G1 cyclin/Cdk complexes causing cell cycle arrest in terminally differentiated cells (17). Thus, a deficiency of these gene products may explain the increased proliferation seen in focal HI and suggests that these or other imprinted genes in the region may be important regulators of β-cell proliferation.

Apoptosis. We observed an apparent increase in the frequency of apoptotic β-cells in the perinatal period, with very low levels before 2 months before birth, peaking at the time of birth, and then decreasing over the following 2 months. After ~2 months of age, very few apoptotic β-cells were seen (Fig. 3*B*). These results are similar to those seen in rodents, although in the rat, apoptosis peaked at the age of 13 days (29). The mechanism that controls apoptosis is complex and has not yet been completely defined. IGF-II has been shown to suppress apoptosis and is elevated in the rat fetus. IGF-II levels decrease after birth, in association with the observed postnatal increase in apoptosis (30). In patients older than 3 months of age with hyperinsulinism, rates of apoptosis were significantly higher than in the age-matched control group (Fig. 6).

The mechanism by which HI causes increased apoptosis is not known. HI is caused by mutations in one of the 2 subunits of the β-cell K_{ATP} channel (1). These mutations result in continuous β-cell membrane depolarization and opening of voltage-gated calcium channels, resulting ultimately in increased intracellular calcium levels (37). This may precipitate activation of an apoptosis cascade. Indeed, in a transgenic model of HI with dominant-negative K_{ATP} channel mutation, increased apoptosis was demonstrated. Mice carrying this mutation had hyperinsulinemic hypoglycemia in the newborn period that progressed to hypoinsulinemic hyperglycemia later in life (38).

Frequency of apoptosis in focal lesions was similar to that seen in diffuse disease suggesting that imprinted genes on chromosome 11p15, such as $P57^{kip2}$ and H19, are not important regulators of β-cell death.

Normal β-cell distribution in the pancreas undergoes a progressive change between the early fetal and the adult pancreas. In the fetal and neonatal pancreas, islets are small and poorly formed, and many insulin-positive cells are seen

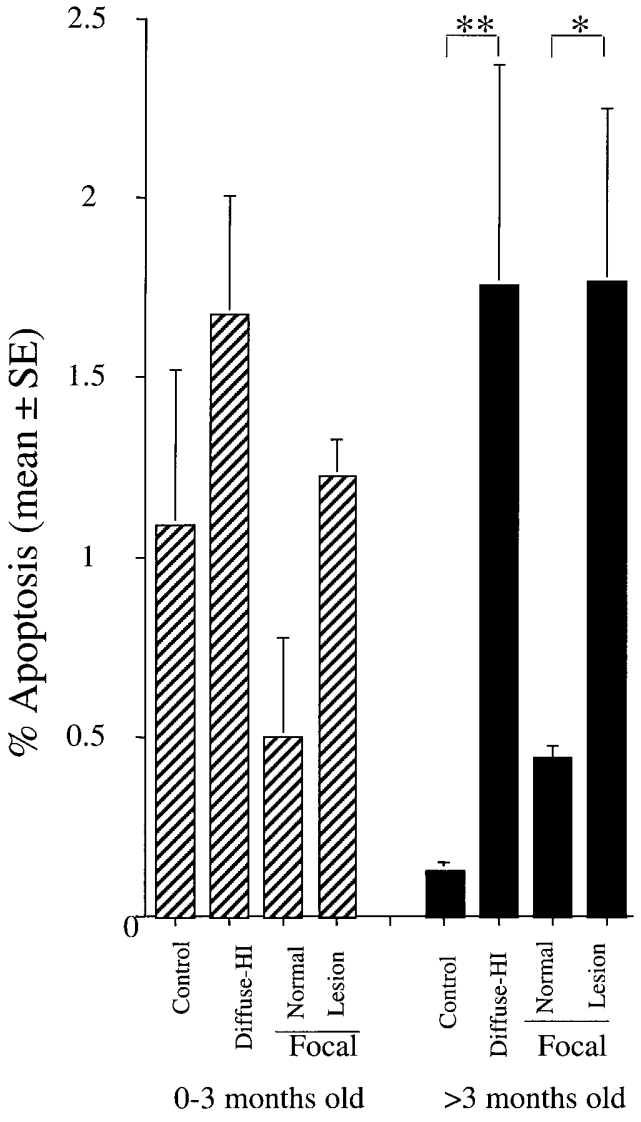

FIG. 6. Mean ± SE of percent apoptosis for control and HI patients according to age-group. The striped columns represent data from infants 0–3 months of age, whereas the black columns represent data from infants older than 3 months. Statistical comparisons were made as indicated by the brackets above the columns. Significance between groups is indicated by * ($P < 0.05$) and ** ($P < 0.01$).

dispersed in the exocrine tissue and budding from ducts. After the age of 6 months, the β-cell distribution approaches the adult-type architecture, with well-formed islets and fewer extra-islet insulin-positive cells (11,12). Our findings demonstrate that β-cell proliferation and apoptosis continue in the early postnatal period and may thus be responsible for this process of islet remodeling (Fig. 3). In diffuse HI, the increase of both proliferation and apoptosis readily explains the persistence of the neonatal-like β-cell distribution seen in these patients if pancreatectomy is delayed beyond the neonatal period. (Updated information on neonatal hyperinsulinism is available on the European Network for Research into Hyperinsulinism website: http://www.phhi.u-net.com.)

Although we found an increase in both β-cell proliferation and apoptosis in HI, the net β-cell turnover cannot be estimated with the methods used here. The Ki-67 stain is positive

during all active parts of the cell cycle, including G1, S, G2, and mitosis (39), whereas the TUNEL reaction detects DNA fragmentation, an early step of apoptosis (27). The time course of these 2 processes has not been adequately described in human β-cells. However, the clinical course of patients with both diffuse and focal HI suggests that the net rate of apoptosis is greater than that of proliferation in this disease (20,21)

The observation that K_{ATP} channel mutations increase the frequency of apoptosis suggests the possibility that pharmacological closure of these channels using sulfonylurea-based drugs may have a similar effect, as recently demonstrated in vitro (40,41). However, further studies are required to determine if indeed sulfonylureas, in the doses used to treat diabetes, have a deleterious effect on net β-cell mass.

In conclusion, we demonstrated that normal human β-cells undergo a wave of increased apoptosis in the perinatal period. We have also demonstrated that in patients with HI, the affected β-cells have increased proliferation and apoptosis that persist after the neonatal period, explaining the persistent neonatal-like β-cell distribution seen in patients with diffuse HI. Although not testable using the methods available, the clinical course of the disease suggests that the net rate of apoptosis is greater than that of proliferation, thus causing the slow progressive decrease in β-cell function seen in these patients. Findings in focal HI suggest that imprinted genes on Ch11p may be important in the regulation of β-cell proliferation but not apoptosis. These observations have obvious clinical relevance for the therapeutic approach to patients with HI and, because of the mode of action of commonly used antidiabetic drugs, may also have relevance for the treatment of type 2 diabetes.

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