Hyperinsulinism of infancy (HI) is a rare genetic disorder with a prevalence in outbred populations of ~1/50,000 live births (1,2). An incidence as high as 1/2,500 has been reported in inbred populations (2,3). The molecular basis of the disease was recently elucidated, and most cases are caused by mutations in either the sulfonylurea receptor-1 (SUR1) gene ABCC8 or the inward rectifying K+ channel Kir6.2 gene KCNJ11, the two subunits of the β-cell ATP-sensitive K+ channel (K$_{ATP}$ channel) (4–7). A minority of patients have glucokinase or glutamate dehydrogenase-1 mutations, whereas in 40–50% of the patients, the genetic cause of the disease is still not known (5,8–10). The clinical presentation of HI can be variable, ranging from mild disease to severe, life-threatening hypoglycemia that, if not adequately treated, causes irreversible neurological damage (11,12).

The histological appearance of the pancreases from affected children is heterogeneous and can be subdivided into two major forms: diffuse HI and focal HI (13–15). The diffuse form is more common and bears some histological characteristics of nesidioblastosis, a normal phenomenon observed in the fetus and newborn that includes poorly defined islets, small clusters of endocrine cells scattered throughout the exocrine tissue, and a high frequency of endocrine cells interposed between ductular cells (15–17).

Focal HI can generally be recognized as a discrete region of adenomatous hyperplasia, often too small to be identified macroscopically. Histologically, the lesion is comprised of nodules of endocrine and exocrine elements. The β-cells are pleomorphic, some having giant nuclei and abundant cytoplasm (13). The rest of the pancreas has normal endocrine architecture for age, with β-cells containing small nuclei and shrunken cytoplasm (18).

We have previously reported increased frequencies of proliferating β-cells in pancreases from HI patients and in pancreases in early stages of human development. Focal HI presented the highest proliferation frequency compared with diffuse HI and control subjects (19). The mechanisms regulating the rate of β-cell proliferation are not known; however, the genetic alteration in focal HI may provide an insight into the control of β-cell turnover.

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 gene on the paternal allele (20,21). In all cases, it is the paternal allele that carries the mutation and the maternal allele that is somatically lost, suggesting that the gene(s) responsible for controlling β-cell proliferation is carried on the paternal allele.
TABLE 1
Clinical characteristics of HI patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Birth weight (kg)</th>
<th>Age of onset (months)</th>
<th>Age at surgery (months)</th>
<th>Postoperative status</th>
<th>Paternal mutation</th>
<th>Maternal mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffuse HI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>F</td>
<td>4.1</td>
<td>1.25</td>
<td>1.5</td>
<td>Hypoglycemic</td>
<td>3992–3 c to g</td>
<td>N188S</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>3.6</td>
<td>Birth</td>
<td>1.6</td>
<td>Diabetes</td>
<td>delcP317</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>5.04</td>
<td>Birth</td>
<td>3.25</td>
<td>Hypoglycemic</td>
<td>Kir Y12X</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>4.4</td>
<td>Birth</td>
<td>13</td>
<td>Hypoglycemic</td>
<td>3992–9 g to a</td>
<td>delF1388</td>
</tr>
<tr>
<td>Focal HI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>5.36</td>
<td>Birth</td>
<td>0.5</td>
<td>Euglycemic</td>
<td>3992–9 g to a</td>
<td>None found</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>3.19</td>
<td>Birth</td>
<td>2</td>
<td>Euglycemic</td>
<td>R1494Q</td>
<td>None found</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>3.3</td>
<td>5</td>
<td>6</td>
<td>Euglycemic</td>
<td>None found</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>3.25</td>
<td>Birth</td>
<td>0.833</td>
<td>Euglycemic</td>
<td>None found*</td>
<td>None found</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>4.18</td>
<td>Birth</td>
<td>1.25</td>
<td>Diabetes</td>
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<td>None found</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>3.61</td>
<td>Birth</td>
<td>5.5</td>
<td>Euglycemic</td>
<td>None found</td>
<td>None found</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>3</td>
<td>Birth</td>
<td>12</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>4</td>
<td>Birth</td>
<td>1.5</td>
<td>Diabetes</td>
<td>None found</td>
<td>None found</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>3.9</td>
<td>Birth</td>
<td>2</td>
<td>Euglycemic</td>
<td>None found</td>
<td>—</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>3.8</td>
<td>Birth</td>
<td>3</td>
<td>Diabetes</td>
<td>None found</td>
<td>—</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>3.63</td>
<td>10</td>
<td>11</td>
<td>Euglycemic</td>
<td>A1493T</td>
<td>None found</td>
</tr>
</tbody>
</table>

Subjects 1–13 were evaluated for p57KIP2 expression, whereas subjects 8–15 were evaluated for IGF-II expression. All patients except for nos. 12–14 were previously reported (19). *For samples in which no mutation was found, only part of the coding sequence of SUR1 and Kir6.2 were sequenced. It is likely, therefore, that mutations will be identified in the future.

for the focal proliferation is imprinted. A large number of genes are located in the lost portion of Ch11p, including p57KIP2, H19, IGF-II, and a p53-induced protein with a death domain (Pidd). Pidd is a 910–amino acid protein induced by a tumor suppressor (p53) that promotes apoptosis (22). It is not known whether this gene is imprinted or whether it is expressed in β-cells. IGF-II is imprinted, with only the paternal allele expressed, and increased expression of this gene has been associated with increased β-cell proliferation and overgrowth syndromes (23,24). Both p57KIP2 and H19 are paternally imprinted, with only the maternal allele expressed, and thus are candidate genes for enhanced cell proliferation (25–28). H19 is an untranslated RNA molecule thought to be an important regulator of IGF-II mRNA levels (29). p57KIP2 (CdkN1C) is a 1.5-kb gene encoding a 335-amino acid protein that belongs to the cyclin-dependent kinase (Cdk) inhibitor family. It is an important inhibitor of several G1 cyclin/Cdk complexes, causing cell cycle arrest in terminally differentiated cells (26,28); loss or underexpression of p57KIP2 has been related to several malignancies (30–32). It is not known whether p57KIP2 is expressed or imprinted in human β-cells.

We examined pancreases from patients with diffuse and focal HI and normal pancreases from different developmental stages, using immunohistochemistry to test for p57KIP2 expression. Using immunofluorescence and computerized imaging, we developed a method to quantify IGF-II staining in β-cells.

Our observations in pancreatic tissue suggest a cellspecific localization of p57KIP2 and IGF-II in β-cells. A stable fraction of β-cells expressed p57KIP2 during different developmental stages. We demonstrated a loss of p57KIP2 inside lesions of focal HI, a finding consistent with the increased rates of proliferation previously demonstrated. IGF-II expression inside the focal lesions was mildly increased when compared with the β-cells in the unaffected surrounding tissue.

RESEARCH DESIGN AND METHODS

Archival tissues from 15 pancreactectomized HI patients were obtained from five clinical centers (Table 1). In all, the diagnosis of HI was made according to accepted criteria (11,12). A total of 11 male and 4 female patients, age range 2 weeks to 13 months, were included in the study. Of these patients, 11 had focal disease; for these patients, analyses compared tissue inside and outside the lesion in each patient. Four patients had the diffuse form of HI. Of the 15 subjects, 12 were previously reported (Table 1).

Controls. A total of 15 control pancreatic samples were included in the study; 12 were obtained from autopsies carried out between 1988 and 1998 in seven male subjects and five female subjects aged 17 weeks gestation to 3 years. These samples consist of a random subgroup of the previously published control population (19). 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 or genetic syndromes. We only included subjects in whom the autopsy was performed within 24 h of death. Adult control pancreas samples were obtained from two pancreas donors and from a patient who underwent partial pancreatectomy for insulinoma. All samples were stained with hematoxylin and eosin and screened for adequate quantity of tissue, normal morphology, and good tissue preservation.

Immunohistochemistry. Sections (5 μm thick) were prepared from archival paraaffin-embedded tissue, placed on SuperFrost Plus glass slides (Menzel-Glaser, Germany), and left to dry at 37°C overnight. Slides were deparaffinized in xylene and then rehydrated in serial concentrations of alcohol (100, 90, and 80%) and double-distilled water. Antigen retrieval was carried out as described by Cattoretti et al. (33). Briefly, slides were microwaved in 0.01 mol/l citrate buffer (pH 6) for 3 min at full power until boiling and then for 15 min at 20% power. Slides were left to cool at room temperature (RT) for 30 min. They were then blocked by nonimmune serum for 10 min at RT before application of each primary antibody.

p57KIP2 Hormone double-staining. Slides were double-stained for p57KIP2 and each of the four major pancreatic hormones (insulin, glucagon, somatostatin, and pancreatic polypeptide). Antibodies, incubation times, detection systems, and substrates are listed in Table 2. To prevent cross-reactivity of the two detection systems, avidin-biotin blocking kit (catalogue no. 00-4303; Zymed) was used before incubation with anti-hormone antibody. As a negative control, slides underwent the same procedure but were incubated with phosphate-buffered saline without anti-p57KIP2 antibody. Each batch included a negative control.

IGF-II/insulin. Sections were double-stained for IGF-II and insulin. Antibodies, incubation times, detection systems, and substrates are listed in Table 2. Cross-reactivity of the anti-IGF-II antibody with proinsulin or insulin was excluded by preabsorbing the antibody with the two peptides overnight, a
Because the increased proliferation was observed in all slides, although this did not reach statistical significance (Fig. 4).

**DISCUSSION**

We have shown that in the pancreas, p57 expression is almost exclusively in the endocrine cells, and that within the islets, expression is primarily localized to β-cells. During development, the proportion of β-cells expressing p57 does not appear to vary, and in diffuse HI, the proportion of β-cells expressing the protein is not different from control subjects of a similar age group. In contrast, p57 expression is not expressed by β-cells within the focal HI lesion. IGF-II expression was also seen primarily in β-cells, and staining was increased within the lesion of focal HI when compared with that of β-cells in the unaffected region from the same patient.

p57 was originally described in 1995; it is a Cdk inhibitor causing cell cycle arrest and accumulation of cells in the G1 phase. It has been shown to bind to cyclin/Cdk complexes in a cyclin-dependent manner and inhibit their activity (26,28). The gene is located within a cluster of imprinted genes in humans and mice, with the maternal allele primarily expressed (27,34).

**IGF-II expression.** Because the increased proliferation previously documented in focal HI could be caused by increased expression of the maternally imprinted IGF-II gene, we quantitated the IGF-II protein content of β-cells inside and outside of the lesion in eight patients. IGF-II staining was identified exclusively in β-cell cytoplasm, both inside and outside the focal lesion (Fig. 1–L). Outside the lesion, all cells stained with IGF-II also stained for insulin; however, only ~27% of the insulin-stained area also stained for IGF-II. In normal β-cells from the same age group, a similar IGF-II distribution was seen (data not shown). To quantify the amount of IGF-II within the β-cell mass, the intensity of IGF-II staining was expressed as the ratio of IGF-II IOD to insulin-stained area. In focal HI, IGF-II staining within the focal lesion was slightly increased when compared with that outside of the lesion in the same patient (7.5 ± 0.9 vs. 5.7 ± 0.6 arbitrary units; P < 0.04) (Fig. 5).

**RESULTS**

**p57 expression.** p57 was demonstrated as dark brown nuclear staining, whereas pancreatic hormones were stained red in cell cytoplasm (Fig. 1A–H). Within the pancreas, p57 was specifically localized to the endocrine cells. Very few p57 positive cells were seen in the acinar tissue, and none were found among the duct cells (Fig. 1A). In the control pancreas, β-cells demonstrated the highest frequency of p57 expression (34.9 ± 2.7%), whereas other islet cell types stained for p57 with much lower frequency (~1–3%) (Figs. 1B–D and 2). No significant change in p57 positive β-cell proportion was observed during the different developmental stages of the human pancreas (Figs. 1E and 3).

The percentage of p57 positive β-cells in diffuse HI was similar to that in the control subjects (Fig. 1F and 4). Complete loss of p57 staining was clearly demonstrated inside the affected area of focal HI (Fig. 1G–H). Interestingly, a tendency toward increased p57 expression was observed in β-cells outside the affected area of focal HI compared with diffuse HI and control subjects, although this did not reach statistical significance (Fig. 4).

**IGF-II expression.** Because the increased proliferation previously documented in focal HI could be caused by increased expression of the maternally imprinted IGF-II gene, we quantitated the IGF-II protein content of β-cells inside and outside of the lesion in eight patients. IGF-II staining was identified exclusively in β-cell cytoplasm, both inside and outside the focal lesion (Fig. 1–L). Outside the lesion, all cells stained with IGF-II also stained for insulin; however, only ~27% of the insulin-stained area also stained for IGF-II. In normal β-cells from the same age group, a similar IGF-II distribution was seen (data not shown). To quantify the amount of IGF-II within the β-cell mass, the intensity of IGF-II staining was expressed as the ratio of IGF-II IOD to insulin-stained area. In focal HI, IGF-II staining within the focal lesion was slightly increased when compared with that outside of the lesion in the same patient (7.5 ± 0.9 vs. 5.7 ± 0.6 arbitrary units; P < 0.04) (Fig. 5).
FIG. 1. A: Low-power (100×) image of adult pancreas stained for p57KIP2 (brown nuclear stain) and insulin (red cytoplasmic stain). B–D: Adult islets (400×) stained for p57KIP2 (brown nuclear stain) and insulin, glucagon, and somatostatin, respectively (red cytoplasmic stain). E–H: Islets stained (400×) for p57KIP2 and insulin from a 26-week-old gestation fetus (E), a 6-week-old patient with diffuse HI (F), and a 5-week-old patient with focal HI showing staining outside the lesion (G) and inside the focal lesion (H). p57KIP2-Positive nuclei are indicated with arrows. I–L: Immunofluorescent staining (400×) for insulin (I and K) and IGF-II (J and L) in focal HI outside (I and J) and inside (K and L) the lesion.
hyperlipidemia, a phenomenon that may have important implications in the pathogenesis of type 2 diabetes. The low expression in other islet cells compared with $\beta$-cells suggests that the latter represent a higher differentiation stage. The very low $p57\text{KIP}2$ expression in acinar and ductular cells may provide a possible explanation for the proliferative capacity those cells retain (35).

The finding that $p57\text{KIP}2$ expression does not change during different stages of development is unexpected, because the proportion of $\beta$-cells undergoing proliferation does change during fetal development, as we previously reported (19). However, the proliferation frequency ranged from $\sim5\%$ at gestational week 17 to 0% in the adult pancreas. Because only 30–40% of $\beta$-cells are $p57\text{KIP}2$ positive, it is likely that the methods used are not sufficiently sensitive to detect small absolute differences in the low proportion of cells undergoing proliferation at the different developmental stages. In two samples triple-

FIG. 2. Percentage of different islet cell types positive for $p57\text{KIP}2$ in control pancreases. The number of samples in each group is given above each column. Pancreatic polypeptide cells were very rare, and only in two samples was it possible to count 1,000 PP $p57\text{KIP}2$-positive cells.

FIG. 3. Percentage of $\beta$-cells staining positive for $p57\text{KIP}2$ in different age groups. Each column represents the mean of three samples.

FIG. 4. Percent of $\beta$-cells staining positive for $p57\text{KIP}2$ in control pancreases and diffuse and focal HI pancreases. In focal disease, $\beta$-cells were evaluated separately outside and within the lesion from the same pancreas. The number of samples in each group is given above each column.

FIG. 5. IGF-II expression inside and outside the lesion from eight patients with focal HI, expressed as the ratio of IGF-II staining IOD to insulin-stained area. AU, arbitrary units.
expressing p57KIP2 do not undergo proliferation (data not shown).

Focal HI is caused by specific loss within affected β-cells of a portion of the maternal allele of Ch11p, which contains the p57KIP2 gene. p57KIP2 has been shown to be paternally imprinted in several tissues (27). Our finding of loss of p57KIP2 expression within the focal HI lesion suggests that the gene is also imprinted in human β-cells. This relatively simple immunohistologic stain can be used to confirm loss of heterozygosity (LOH) of the maternal allele in these lesions and may be of use in differentiating focal HI from other forms of hyperinsulinism. Similarly, the same stain may be useful in confirming LOH for this region in other tissues in diseases such as BWS and in certain tumors, such as Wilms' tumor (37), adrenocortical tumors (31), and lung cancers (30), as long as normal expression and imprinting are confirmed for each tissue type.

The finding of loss of p57KIP2 expression in focal HI may explain the increased β-cell proliferation in the adenomatous portion compared with unaffected pancreas and diffuse HI (19,38). Other adjacent genes, some imprinted, located in the chromosomal region lost in focal HI may play an additive or synergistic role in inducing β-cell proliferation. Furthermore, other mechanisms must also regulate β-cell proliferation because even within the focal lesion, proliferation rates are only ∼6% (19).

p57KIP2-positive cells tended to be more frequent outside the focal lesion compared with cells from control and diffuse HI subjects of the same age group, although this difference did not reach statistical significance. β-Cells outside the lesion are exposed to hypoglycemia and high insulin concentration released from the lesion. This leads to suppressed metabolic activity and decreased cytoplasmic volume (18) and may also result in decreased proliferation mediated by high p57KIP2 expression.

IGF-II is located in the same region on chromosome 11 but is maternally imprinted and has been associated with increased β-cell proliferation (23). Others have demonstrated that pancreatic IGF-II expression is limited to β-cells (39). In our samples, positive staining was also seen exclusively in β-cells, and a similar staining pattern was found in pancreases from all age groups (data not shown). To estimate the IGF-II content of affected and unaffected -cells in focal HI, we developed a method using quantitative image analysis of immunofluorescence. By comparing IGF-II IOD to insulin area, we obtained an estimate of the quantity of IGF-II protein as a function of -cell area. This expression and imprinted in human pancreatic β-cells. Levels of expression do not appear to parallel changes in rates of β-cell proliferation during development, whereas decreased expression in focal HI is associated with increased rates of proliferation and increased IGF-II expression. Manipulation of p57KIP2 expression in β-cells may provide a mechanism by which the rate of proliferation can be modulated, and thus this gene may be a potential therapeutic target for reversing the β-cell failure observed in diabetes.

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