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Neonatal hyperinsulinism (HI) is a genetic disorder of pancreatic β-cells characterized by failure to suppress insulin secretion in the presence of hypoglycemia, resulting in brain damage or death if not adequately treated. Germline mutations in four genes have been associated with HI. Some patients have focal regions of β-cell proliferation (focal HI). Seventy HI probands in whom at least one SUR-1 mutation was identified were studied. Clinical data from patients with two SUR-1 mutant alleles were compared with those from patients with single paternally inherited mutations. Thirty-seven probands were homozygous or compound heterozygous for SUR-1 mutations. In 33 probands, only a single mutation was identified, and in 31, the parental origin of the proband could be determined; in 29, the mutation was on the paternal allele (P < 0.0002). For three of these, pancreatic tissue was available and showed focal β-cell hyperplasia. DNA extracted from the focal lesion and adjacent normal pancreas revealed loss of the maternal chromosome 11p15, resulting in reduction to homozygosity for the SUR-1 mutation within the focal lesion only. Using the Tdt-mediated dUTP nick end labeling (TUNEL) reaction, apoptotic β-cells were identified exclusively within the focal region. At diagnosis, disease severity was similar in patients with paternally inherited mutations and those with two mutations. For patients who did not undergo surgery, those with only paternal mutations entered clinical remission within 16 ± 6.2 months, compared with 48 ± 23 months for those with two SUR-1 mutations (P = 0.001). In conclusion, we identified a novel mechanism to explain the pathophysiology of focal HI and provide evidence to suggest that this entity may be self-limiting, since affected β-cells undergo apoptosis. Diabetes 48:1652–1657, 1999
RESEARCH DESIGN AND METHODS

Patients. HI was diagnosed using established criteria based on the concomitant finding of hypoglycemia, inappropriately elevated insulin levels, inappropriately low ketone bodies, and abnormally high glucose requirement to prevent hypoglycemia (10,11).

Focal HI patients with histologic evaluation. Three patients with proven focal HI were studied. The first, a non-Jewish Caucasian patient diagnosed with severe hypoglycemia at 42 days of age, was previously reported (8). Due to lack of response to medical treatment, surgery was recommended. A discrete region of adenomatous b-cell hyperplasia was identified, and a 40% pancreatectomy was performed. The second case was Ashkenazi Jewish, had severe hypoglycemia immediately after birth, and was large for gestational age (5.36 kg), evidence of intrauterine HI. Having failed to respond adequately to medical management, he underwent surgery at the age of 15 days. Because the focal lesion could not be identified during surgery, a 95% pancreatectomy was performed. The third patient, a non-Jewish Caucasian, underwent partial pancreatectomy at age 8 weeks. In all cases histologic examination revealed a discrete region of adenomatous hyperplasia, the remainder of the pancreas being normal for age. In all, surgery was curative.

HI cohort. DNA from 170 HI probands and their families was contributed by physicians from North America, Europe, and Israel. Forty-five probands previously underwent comprehensive mutation analysis of all 30 SUR-1 exons using single-strand conformational polymorphism (SSCP) analysis and direct sequencing. The results of this study were published separately (12). The remaining probands were screened for 24 previously reported SUR-1 mutations (1,12–15) and for novel mutations in the 2nd nucleotide binding domain. Because of the difficulty in obtaining comparable clinical data from all centers, the clinical comparisons reported here were made on a subgroup of 22 patients treated by one of us (H.L.). All ascertainment and treatment decisions were made before the mutation status of any patient was known.

Laboratory methods. DNA was extracted from peripheral lymphocytes using standard methods. Genomic DNA was screened for 24 previously published HI-associated SUR-1 mutations using published materials and methods (1,12–15) and for novel mutations in the 2nd nucleotide binding domain using SSCP as previously described (12).

DNA was isolated from fixed paraffin-embedded pancreatic tissue using standard procedures. Mutations were detected by restriction fragment length polymorphism (RFLP) analysis as previously described (1). To confirm loss of the entire terminal segment of chromosome 11p, two informative polymorphic markers were genotyped in each case. Tyrosine hydroxylase (TH), which is located at the p terminus of chromosome 11, was used for all three cases. One additional informative marker located centromeric to TH was genotyped for each of the three cases (GATA17G04, GATA17G04, and GGA17G05). Microsatellite analysis was performed using AmpliTaq Gold (Perkin-Elmer, Norwalk, CT) for polymerase chain reaction (PCR) and analyzed on a 9% denaturing long-range gel electrophoresed in a Pharmacia ALF (Uppsala, Sweden) automated sequencing system.

Apoptotic b-cells were identified by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique (16). Paraffin sections were deparaffinized, rehydrated, and incubated with 20 µg/ml proteinase K (Boehringer-Mannheim, Mannheim, Germany) for 15 min at 37°C. TUNEL staining was accomplished using the In Situ Cell Death Detection kit (Boehringer-Mannheim) according to the manufacturer’s directions. Sections were then incubated for 30 min at 37°C with guinea pig anti-human insulin antibody diluted 1:50 (Dako, Glostrup, Denmark) followed by detection using a streptavidin-biotin-peroxidase complex developed with aminoethylcarbazole (Zymed, San Francisco, CA). Finally, sections were counterstained with hematoxylin and embedded in Kaiser’s glycerol gelatin (Merck, Darmstadt, Germany). Automated sequencing system.

In all three cases, genotyping was performed on informative polymorphic markers on chromosome 11p as described in methods. A and B: Representative ALF traces of TH and GATA17B04 microsatellite analysis for index case 2. C: PCR products; internal size marker. Genotypes are shown for DNA extracted from peripheral blood lymphocytes (PBL) from the father, the mother, and the proband. Similarly, the DNA extracted from the normal and focal regions of the pancreas was genotyped, and the results are shown. For the proband’s PBL and tissue DNA, maternal and paternal alleles are labeled “p” and “m.” C: Composite results from all three cases. The relative quantity of maternal and paternal alleles was determined by calculating the area under the curve for each allele. The maternal/paternal (M/P) ratio is shown. In each case, two informative markers were used, TH, which is near the p terminus of the chromosome, and another informative marker centromeric to TH (see methods). The expected ratio of 1 is seen in DNA extracted from peripheral blood and from the “normal” pancreas. In contrast, a low ratio indicative of a marked preponderance of paternal allele is seen in the adenomatous (focal) region.

RESULTS

Focal HI cases. Focal lesions were reported in six patients in whom at least one SUR-1 mutation was identified. Three of these were from the group that underwent mutation screening of all SUR-1 exons and three were from the group that was screened for both known mutations and for novel mutations in the 2nd SUR-1 nucleotide binding domain. In all six, the proband inherited the SUR-1 mutation on the paternal allele of chromosome 11p. Histologic samples were available for confirmation and further study only from the latter three cases, and the further analysis described here was performed only on these three confirmed focal cases. In each, a different mutation was identified, nt1630 g→a, nt3992 g→a, and R1494Q. The first two of these mutations have previously been reported in patients with classical autosomal recessive HI in whom mutations identified on both SUR-1 alleles (12,17). DNA extracted from the hyperplastic region of the pancreases contained almost exclusively the mutant allele as demonstrated both by the analysis of informative flanking polymorphic markers (Fig. 1) and by analysis of the mutation itself (Fig. 2).
The surgical specimens were evaluated for evidence of apoptosis (Fig. 3). Seven, three, and five apoptotic cells per 1,000 $\beta$-cells were seen in 10 random high-power fields scanned from the focal lesions of the three cases (see METHODS). In contrast, no apoptotic $\beta$-cells were identified in the normal regions of any of the pancreases, or partially digested, indicating heterozygosity. In sharp contrast, DNA extracted from the focal lesion was almost completely digested, demonstrating that it contained almost exclusively the mutant allele. B: Histogram showing the ratio of relative densitometric values of similar DNA analysis on all three families. No mutant allele was identified in any of the maternal DNA samples, whereas the ratio of mutant/wild-type allele in genomic DNA from the fathers and the probands as well as DNA extracted from the normal pancreases (NI) showed a mutant/wild-type ratio of -1 (range 0.6–1.3). In sharp contrast, DNA from the focal lesion showed a marked preponderance of mutant DNA (ratio 7.0 ± 3.5, $P = 0.002$).

FIG. 2. PCR amplification of DNA followed by restriction-enzyme digestion and ethidium bromide–stained agarose-gel electrophoresis was performed with DNA from all three patients. A: Representative electrophoretic gel demonstrating detection of R1494Q mutation in index case 3. The first three lanes from the left show genomic DNA extracted from peripheral blood leukocytes (PBL) from the father, mother, and proband. The lanes labeled “NI” and “Focal” show DNA extracted from fixed, paraffin-embedded normal and focal-lesion pancreatic tissue. When applied to mutant SUR-1, the restriction endonuclease Stu 1 digests the 233-bp PCR product to 115- and 118-bp fragments. The wild-type allele is not digested. Genomic DNA from the mother is not digested at all, showing that no mutant allele is present. In contrast, genomic DNA from the father and proband, as well as DNA extracted from the normal pancreas, is partially digested, indicating heterozygosity. In sharp contrast, DNA extracted from the focal lesion was almost completely digested, demonstrating that it contained almost exclusively the mutant allele. B: Histogram showing the ratio of relative densitometric values of similar DNA analysis on all three families. No mutant allele was identified in any of the maternal DNA samples, whereas the ratio of mutant/wild-type allele in genomic DNA from the fathers and the probands as well as DNA extracted from the normal pancreases (NI) showed a mutant/wild-type ratio of -1 (range 0.6–1.3). In sharp contrast, DNA from the focal lesion showed a marked preponderance of mutant DNA (ratio 7.0 ± 3.5, $P = 0.002$).

FIG. 3. Photomicrographs of pancreatic tissue double stained for the TUNEL reaction (alkaline phosphatase) and insulin (peroxidase) from all three patients. For patient 1, two examples of the focal lesion are given (A and B) since no normal tissue was available for study. For patients 2 (C and D) and 3 (E and F), normal tissue (C and E) is shown alongside the focal lesions (D and F). The arrows mark typical apoptotic nuclei.

The surgical specimens were evaluated for evidence of apoptosis (Fig. 3). Seven, three, and five apoptotic cells per 1,000 $\beta$-cells were seen in 10 random high-power fields scanned from the focal lesions of the three cases (see METHODS). In contrast, no apoptotic $\beta$-cells were identified in the normal regions of any of the pancreases using identical scanning techniques ($P < 0.004$). Apoptosis was not seen in normal or pathologic tissue obtained from a patient with benign insulinoma (data not shown).

**HI patients with SUR-1 mutations.** One hundred seventy HI probands were screened for SUR-1 mutations as described in METHODS. Thirty-seven probands were found to have two mutant alleles (2MUT), being either homozygotes ($n = 23$) or compound heterozygotes ($n = 14$). In 33 probands, only a single mutation was identified. One of these had a new mutation not found in either parent (12), and for one, DNA was not available from either parent. Therefore, parental origin of the mutation could be determined in 31 probands. In 29, the mutant allele was inherited from the father (1MUT-PAT, $P = 0.0002$).

To exclude abnormal chromosome segregation in these families, the segregation pattern of the SUR-1 alleles was determined using haplotype analysis in siblings of heterozygous probands with paternal mutations. Nineteen (48%) of the 40 unaffected siblings carried the paternal mutation, and 8 of these (42%) also carried the same maternal allele as the affected sibling (Table 1). Only 1 (2.5%) of 40 siblings had HI, however, and this sibling was haploidentical with the proband at the SUR-1 locus. Thus, although both the paternal and maternal alleles segregated normally, the clinical disease did not ($P = 0.004$). A similar analysis of the families of probands.
with two mutant SUR-1 alleles revealed that both allele segregation and affection status segregated normally (Table 1). Furthermore, heterozygous siblings in the two-mutation group were always unaffected regardless of whether the mutant allele was inherited from the mother or the father.

Twenty-three 2MUT and 12 1MUT-PAT patients underwent surgery, but pathology reports are available for only 9 and 8, respectively. Focal lesions were identified in six of the 1MUT-PAT patients, but in none of the 2MUT group (Table 1, \( P = 0.002 \)).

The genetic and clinical data from the 1MUT-PAT and 2MUT patients were compared. The incidence of specific mutations could only be compared in Ashkenazi Jewish patients since most SUR-1 mutations are rare, usually occurring only in single families (12). In the Ashkenazi Jewish population >85% of the HI-associated alleles carry either the nt3992 g→a mutation or the delF1388 mutations (14). One of these two mutations was present on 98% (\( n = 58 \)) of the disease-associated alleles in 2MUT Ashkenazi patients (77% nt3992 g→a; 23% delF1388) and on 100% (\( n = 17 \)) of disease-associated chromosomes in 1MUT-PAT patients (76% nt3992 g→a; 24% delF1388).

The clinical phenotype and natural history of the disease were also compared. Birth weight was similar in the two groups: 4.04 ± 0.21 and 3.81 ± 0.16 kg for the 2MUT and 1MUT-PAT groups, respectively (\( P = 0.31 \)). Other, more subjective clinical data were also compared. However, clinical assessment and treatment approaches differ widely among medical centers, detailed clinical data on many patients referred from abroad was not available, and most centers favor early surgical intervention. Therefore, for the study of the natural history of the disease, we only evaluated patients treated by one of us (H.L.) who, for the last 10 years, has advocated medical treatment whenever possible for all patients, since a substantial percentage of operated patients develop severe diabetes at puberty (18). Thirteen patients with two mutations and nine patients with paternal mutations, none of which underwent pancreatectomy, were evaluated. Both groups had patients with clinically very severe disease (7 of 13 and 3 of 9, respectively, \( P = 0.41 \)). Age at diagnosis was similar, with 11 of 13 and 6 of 9 patients in each group, respectively, presenting with hypoglycemia within the first 24 h of life (\( P = 0.61 \)). Interestingly, 8 of the 9 patients with only a single, paternal mutation entered clinical remission, and drug therapy could be withdrawn after 16 ± 6.2 months (range 1–56). The ninth patient is still receiving octreotide treatment at the age of 7 months. In contrast, in the group with two mutations, 9 of 13 patients still required drug therapy after 48 ± 23 months (range 14–58, \( P = 0.001 \)). The other four became drug independent after 15–107 months. These findings suggest that patients with a single paternal mutation who did not undergo surgery enter clinical remission much earlier than those with two mutations.

**DISCUSSION**

In three patients with focal HI, we demonstrated that somatic loss of maternal chromosome 11p15 in the focal lesion results in reduction to homozygosity for an inherited SUR-1 mutation and causes severe disease, unresponsive to diazoxide treatment.

DeLonlay et al. (7) documented loss of maternal ch11p in 10 patients with focal HI. However, genomic loss of the same region of the maternal chromosome results in Beckwith-Wiedemann syndrome, a syndrome associated with usually mild, transient, diazoxide-responsive hyperinsulinemic hypoglycemia in only about 30% of cases (19). Since patients with focal HI have severe HI unresponsive to diazoxide, loss of maternal chromosome 11p alone is not sufficient to cause the focal HI phenotype. Diazoxide binds to the sulfonylurea receptor causing the \( K_{\text{ATP}} \) channel to increase the mean open time, thereby inhibiting glucose-induced depolarization. Complete loss of \( K_{\text{ATP}} \) function caused by homozygous SUR-1 mutations results in severe HI, unresponsive to diazoxide (20). Therefore, reduction to homozygosity of a paternal SUR-1 mutation within the focal lesion fully explains the phenotype seen in focal HI patients. We previously reported one focal HI patient with reduction to homozygosity of the paternal SUR-1 mutation in the focal lesion (8), and in a follow-up study of DeLonlay’s findings, Verkarre et al. (9) reported similar findings in four patients. It is therefore likely that the combination of a paternally inherited SUR-1 mutation along with somatic loss of the maternal allele of chromosome 11p is the genetic etiology of most, if not all, cases of focal HI.

It is not possible to study the natural history of focal HI directly, since the definitive diagnosis of a focal lesion can only be made at the time of surgical resection. Furthermore, even in surgically treated patients, the histologic diagnosis of focal HI is still not accepted in all centers, and therefore unconfirmed histologic reports of diffuse HI cannot be accepted as definitive evidence. As an alternative approach, we studied 67 HI probands in whom at least one SUR-1 mutation was identified. Patients who inherited mutations on both SUR-1 alleles (2MUT) clearly have autosomal recessive disease (AR HI). Patients who inherited only a single mutation could also have recessive disease if the second mutation was missed by the screening methods used. Alternatively, they could have disease caused by only a single mutation, such as that found in our three index cases.

We tested the hypothesis that focal HI, because of an inherited paternal SUR-1 mutation and somatic loss of chromosome 11p, is a common cause of HI, and that the natural history of this form of HI is different from autosomal recessive HI.

In patients with only a single known mutant allele, highly significant preponderance of paternally inherited mutations was found (\( P = 0.0002 \)). The mutation analysis techniques

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>2MUT</th>
<th>1MUT-PAT</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of probands</td>
<td>37</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Number of siblings</td>
<td>94</td>
<td>40</td>
<td></td>
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<tr>
<td>Haploidentical</td>
<td>25*</td>
<td>8</td>
<td>0.51</td>
</tr>
<tr>
<td>HI-affected</td>
<td>23†</td>
<td>8</td>
<td>0.001</td>
</tr>
<tr>
<td>Surgery performed</td>
<td>23</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Histology report available</td>
<td>9</td>
<td>8</td>
<td>0.16</td>
</tr>
<tr>
<td>Focal lesions identified</td>
<td>0</td>
<td>6</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*Two siblings of one affected individual were found to be homozygous for the nt3992 g→a mutation. Both were reported to be clinically normal, but neither was examined by a pediatric endocrinologist and neither underwent any specific testing for hypoglycemia. †All affected siblings were haploidentical to the proband at the SUR-1 locus.
used are expected to miss a significant number of mutations, since only a sub-group of patients underwent comprehensive SSCP analysis, and even this technique may miss 10–20% of mutations. Furthermore, the introns and the regulatory regions of the gene were not screened in any of the patients, and some patients in this heterogeneous cohort may have disease caused by mutations in different genes. These limitations of methodology readily explain the large number of patients (100) in whom no mutation was found. However, no bias was introduced to favor identification of mutant alleles inherited specifically on the paternal allele, as was found in our patients. Only 3% (n = 1) of the siblings of 1MUT-PAT probands were clinically affected, whereas the expected 24% (n = 8) were haplidential to the proband. This is not consistent with the hypothesis that mutations are in fact present on the maternal allele in all cases but were missed for technical reasons. Similarly, the alternative hypothesis that the unexpected low incidence of disease in siblings is due to increased mortality or decreased ascertainment efficiency is not supported by these findings. Therefore, our finding that the mutation was on the paternal allele in 29 of 31 probands with a single mutation cannot be explained on the basis of any known methodologic issue.

There is no evidence to suggest that the findings reported here can be explained on the basis of imprinting or allel-specific expression of SUR-1 itself. All siblings of 2MUT HI probands who were heterozygous for paternally inherited SUR-1 mutations were clinically unaffected.

The clinical characteristics of 2MUT and 1MUT-PAT probands were compared. Onset of disease and clinical severity was similar, consistent with the findings of deLonlay et al. and Verkarre et al. (7,9) for patients with proven focal HI. 1MUT-PAT patients entered clinical remission significantly earlier than 2MUT patients, however. We found an increased number of apoptotic cells in the focal lesions when compared with the rest of the pancreas in all our three patients, suggesting that homozygosity for SUR-1 mutations may be associated with increased β-cell apoptosis. Miki et al. (21) reported a transgenic mouse model that overexpresses a mutant Kir6.2 that disrupts KATP channel activity in a manner analogous to the HI-associated SUR-1 mutations. These mice had hyperinsulinemic hypoglycemia (analogous to human HI) in the newborn period, followed by diabetes, associated with β-cell loss attributed to apoptosis. The same group subsequently reported a Kir6.2 knockout mouse model (22). Interestingly, Kir6.2−/− mice lack β-cell KATP channels and have neonatal hypoglycemia and markedly impaired glucose-stimulated insulin secretion, but as adults have only mild glucose intolerance. The reason for the difference in clinical expression of the two mouse models is unclear; however, the later phenotype is similar to that seen in some HI patients after partial pancreatectomy (23). In the Kir6.2−/− mice, the transient nature of the hypoglycemia may be due to decreased β-cell mass, and the minimal glucose intolerance may be due to primary or secondary increased sensitivity to insulin, the mechanism of which has yet to be established. Kagimoto et al. (24) demonstrated apoptotic cell death in β-cells exposed to glibenclamide, a drug of the sulfonylurea class that binds to SUR-1, closing the KATP channel. Efanova et al. (25) demonstrated that either hyperglycemia or tolbutamide, a sulfonylurea, can induce apoptosis in β-cells isolated from ob/ob mice and Wistar rats. Apoptosis could be inhibited by either diazoxide, an antagonist of sulfonylurea effects on SUR-1, or D-600, a blocker of L-type calcium channels, suggesting that opening of L-type calcium channels and resultant increased intracellular calcium concentration played an important role in regulating apoptosis. These findings appear to conflict with those of Zhou et al. (26), who demonstrated apoptosis secondary to depletion of intracellular calcium stores, independent of changes in intracellular calcium concentrations. This apparent conflict may be due to the fact that the later study was carried out primarily in the MIN6 cell line and apoptotic signaling in cell lines may be different from that of primary β-cells in culture. This is suggested by the observation that 10 times higher concentrations of thapsigargin, a drug that depletes intracellular stores, were required in confirmatory experiments on primary cultures of mouse islets.

Recently, IGF-2 was shown to protect against apoptosis of β-cells (27). The IGF-2 gene is imprinted, and only the paternal allele is expressed. Expression is high in the fetal and neonatal period and decreases considerably after birth. It is possible that the normally high prenatal IGF-2 levels explain the prenatal growth of the focal lesion, and the postnatal fall of IGF-2 explains the disappearance of the lesion during the first several months of life. A similar increase in apoptosis may also be present in diffuse HI and may explain the apparent clinical improvement over time seen in these patients. Additional studies are required to confirm these hypotheses.

Our data, when taken together, provide strong evidence to support the hypothesis that as many as 40% of HI patients may have a single, paternally inherited SUR-1 mutation and a second non-Mendelian event (somatic loss of chromosome 11p) causing expression of the recessive disease trait. This series of events fully explains the clinical and genetic data presented here. In the affected clone, all KATP channels are mutated, the maternally expressed tumor suppressor genes are lost, and the paternally expressed growth factor, IGF-2, is expressed. Together, this will result in proliferation of cells unable to turn off insulin secretion in the presence of hyperglycemia. The precise timing of the somatic event will determine the severity and age of onset of clinical disease and the extent of pancreatic involvement. The latter can be limited to a small lesion or to a major portion of the pancreas.

The role of KATP mutations in the genetic susceptibility to type 2 diabetes is still controversial. Several studies have demonstrated associations between common SUR-1 or Kir6.2 polymorphisms and diabetes (28–31). Recently, Goksel et al. (32) reported the association between an apparently silent SUR-1 mutation and hyperinsulinemia in nondiabetic subjects. These polymorphisms themselves do not appear to have major effects on the functional characteristics of the channel, and the association may be due to unidentified mutations in the regulatory regions of the genes or in other, closely linked genes.

Our findings of the association between major defects in KATP channel activity and apoptosis suggest that subtle changes in channel function may have major consequences over the life of a patient. Although speculative at this point, these findings may have implications for the pathogenesis and the treatment of type 2 diabetes. Mutations that cause less dramatic changes in channel activity may modulate apoptosis. Furthermore, sulfonylureas, the most common drugs used for treating non–insulin-requiring diabetic patients, cause KATP channels to close in a manner analogous to that seen in HI.
patients. Our findings, supported by those of others (24,25), raise the possibility that these drugs may be increasing apoptosis of β-cells, thus hastening the deterioration of β-cell function characteristic of patients with this disease. Clearly, this requires direct study before any clinical decisions can be made based on this speculation.

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