A MANGANESE SUPEROXIDE DISMUTASE (SOD2) GENE POLYMORPHISM IN INSULIN-DEPENDENT DIABETES MELLITUS

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SUMMARY

Interleukin 1 (IL-1) is selectively cytotoxic to the insulin producing beta cell of pancreatic islets. This effect may be due to IL-1 induced generation of reactive oxygen species and nitric oxide. Since beta cells contain low amounts of the superoxide radical scavenger enzyme manganese superoxide dismutase (MnSOD), this may leave beta cells more susceptible to IL-1 than other cell types. Genetic variation in the MnSOD locus could reflect differences in scavenger potential. We, therefore, studied possible restriction fragment length polymorphisms (RFLPs) of this locus in patients with insulin-dependent diabetes mellitus (IDDM) (n=154) and control individuals (n=178). TaqI revealed a double diallelic RFLP in patients as well as in controls, No overall difference in allelic or genotype frequencies were observed between IDDM patients and control individuals (p=0.11) and no significant association of any particular RFLP pattern with IDDM was found. Structurally polymorphic MnSOD protein variants with altered activities have been reported. If genetic variation results in MnSOD variants with reduced activities, the MnSOD locus may still be a candidate gene for IDDM susceptibility. Whether the RFLPs reported in this study reflects differences in gene expression level, protein level and/or specific activity of the protein is yet to be studied.

KEYWORDS MnSOD RFLP Free oxygen radicals NO Susceptibility genes

INTRODUCTION

The cytokine interleukin 1β (IL-1), mainly produced by the monocyte (Mo)/macrophage (Mø) cell lineage, is selectively cytotoxic to beta-cells in isolated pancreatic islets (Mandrup Poulsen et al. 1986, Bendtzen et al. 1986, Sandler et al. 1987). The effects of IL-1 include decreased insulin biosynthesis and release, decreased glucose oxidation, oxygen uptake, protein synthesis and islet content of insulin and DNA (Sandler et al. 1987, Sandler et al. 1989). Since the first cells to appear in the insulitis process are Mo/Mø (Vorbij et al. 1989, Hanenberg et al. 1989, O’Reilly et al. 1991), IL-1 may be responsible for the initial beta-cell damage eventually resulting in insulin-dependent diabetes mellitus (IDDM) (Nerup et al. 1988). The detailed mechanisms behind the cytotoxic effect of IL-1 to beta-cells are not fully clarified.

The parameter which is first influenced by IL-1, is the cytosolic free sodium concentration (fNa+). In rat islets, fNa+ increases within minutes of IL-1 exposure, due
to an activation of the Na\(^+\)/H\(^+\) exchange over the plasma membrane (Helqvist et al. 1990). IL-1 induces oxygen-derived free radicals (OFR) in other cells (Klemper et al. 1979, Matsubara et al. 1986) and nitric oxide (NO) in islets (Southern et al. 1990, Welsh et al. 1991, Andersen et al. 1993), and it has been shown that an activation of the Na\(^+\)/H\(^+\) increases OFR production in neutrophil granulocytes (Simchowitz et al., 1985). The beta cell cytotoxic agent alloxan induces production of OFR (Malaisse et al. 1982). Beta cells have been suggested to be particularly sensitive to the toxic effect of OFR due to a limited repertoire of O\(_2^-\) scavengers like the enzyme manganese superoxide dismutase (MnSOD). The role of SODs is to remove damaging OFR from the cell by reducing them to hydrogen peroxide, which in term is removed by other enzymes. IL-1 is a major inducer of MnSOD (Wong et al. 1988, Masuda et al. 1988) and also increases the transcription (Cuartero et al. 1992) and the activity of the enzyme in rat pancreatic islets (Borg et al. 1992). Very recent data have shown IL-1 to induce nitric oxide synthase (iNOS) in a number of cell types (Busse and Mülsch 1990, Stadler et al. 1991, Nussler et al. 1992) including islets (Karlsen et al. 1993) resulting in higher intracellular levels of NO. Data suggest also that part of the NO cytotoxicity may derive from its combining with superoxide, leading to the formation of peroxynitrite anion, which decomposes when protonated into the toxic hydroxyl radical (Beckman et al. 1990). Thus, if IL-1 induces formation of OFR and NO in beta-cells, the beta-cell specific cytotoxicity may reflect insufficient capability of these cells to produce protective proteins, e.g. MnSOD, that are normally constitutively expressed or inducible in other cell types. Furthermore, peripheral blood mononuclear cells (PBMC) from diabetic individuals showed decreased mitochondrial SOD-activity, though the total amount of MnSOD was not reduced (Nath et al. 1984). We hypothesize that genetic variation(s) at the MnSOD locus reflect interindividual differences in scavenger potential, which might render some individuals more susceptible to IL-1 mediated beta cell destruction and IDDM.

In the present study we, therefore, searched for restriction fragment length polymorphisms (RFLPs) of the human MnSOD gene and subsequently evaluated the allele and genotype distribution in IDDM patients and healthy control individuals.

**MATERIALS AND METHODS**

**Subjects**

A panel of 10 IDDM patients and 10 control individuals were screened for RFLPs using different restriction enzymes. For further analysis an independent sample of 154 unrelated randomly selected IDDM patients and 178 healthy, unrelated randomly selected control individuals were studied. In addition, 4 two-generation families (22 individuals) and 1 three-generation family (8 individuals) were typed in order to determine segregation of the different MnSOD alleles. All individuals studied were of Caucasoid origin.

The study was approved by the Ethics Committee of the County of Copenhagen.

**Restriction fragment length polymorphism (RFLP) studies**

DNA was extracted from peripheral blood mononuclear cells by standard procedures and digested with the following restriction enzymes: AccI, Avai, AvaiII, BamHI, BclI, BglII, EcoRI, HindIII, Hinfl, Kpnl, MspI, NcoI, PstI, PvuII, Rsal, SacI, SspI, SstI, TaqI, or XbaI. After electrophoresis of 10 µg DNA in a 0.8% agarose gel and blotting to a nylon
filter (GeneScreen Plus), DNA was hybridized with a human cDNA probe. The probe was an 588 bp NdeI-SaII fragment isolated from pcMnHSOD1lacI2. The NdeI-SaII fragment was derived from the MnSOD cDNA and encodes the mature protein but not the mitochondrial targeting sequence. The probe was kindly provided by Dr. R. Hallewell, Chiron Corporation, Emeryville, CA.

**Statistical analysis**

Genotype- and allele frequencies were compared using Fisher’s exact test or chi-square with Yates correction where appropriate. Five per cent (two-sided) was chosen as level of significance unless otherwise stated. Linkage was estimated according to Mattiuz et al (Mattiuz et al. 1970).

**RESULTS**

Only the restriction enzyme TaqI revealed a polymorphic pattern. The MnSOD cDNA probe used in this study identified 7 fragments, termed 1 to 7, after digestion with TaqI, where fragment 1 had the lowest molecular weight (MW) and fragment 7 the highest MW (Fig. 1). Three of the fragments were constant, i.e. found in all individuals, with sizes of 3.8, 3.2, and 1.4 kb, and two di allelic polymorphisms consisted of fragments of 2.3 kb/2.0 kb (RFLP A) and 1.5 kb/1.2 kb (RFLP B), respectively, (Fig. 1).

Table 1 shows the frequencies of the 4 polymorphic alleles in 154 randomly selected IDDM patients and 178 healthy individuals. No differences between IDDM patients and control individuals were observed. No deviation from Hardy-Weinberg equilibrium was observed.

Table 2 shows the frequencies of the MnSOD TaqI RFLP genotypes in patients and control subjects. Only 6 of 9 theoretical genotypes were found in IDDM patients as well as in control individuals.

No significant difference in overall genotype frequency between patients and controls was observed (p=0.11). The most obvious differences were in frequencies of the homozygous genotypes, where homozygosity for fragment 1 and 5 was more frequent in diabetic individuals. However, none of these differences were significantly when corrected for number of comparisons. The difference in frequencies were reflected by significantly stronger linkage of fragment 1 with fragment 4 in controls compared to patients (p=0.0003 and p=0.087, respectively).

The family analysis demonstrated codominant segregation of the polymorphic fragments. In families where it was possible to define haplotypes derived from heterozygous individuals, allele 1 and 4 co-segregated and so did allele 3 and 5, reflecting the strong linkage of these alleles.

**DISCUSSION**

Only the restriction enzyme TaqI revealed a polymorphic pattern with a double di allelic RFLP. These RFLPs were originally reported by Xiang et al (Xiang et al. 1987), who studied small groups of healthy individuals of Caucasian and Chinese origin. In this larger study we used a probe which identified 3 constant fragments, including a 1.4 kb fragment not previously reported. The frequencies of the polymorphic alleles were close to that reported by Xiang et al (Xiang et al. 1987) for Caucasoids.
Figure 1. DNA from six healthy individuals digested with the restriction enzyme *TaqI* and hybridized with a $^{32}$P-labelled MnSOD cDNA probe. Six different patterns were identified. Fragments are numbered after molecular size. Fragments 2, 6, and 7 were found in all tested individuals. Fragments 1 and 3 comprises one diallelic RFLP (B) and fragments 4 and 5 another, RFLP A.

Table 1. Allelic frequencies of polymorphic MnSOD *TaqI* alleles in IDDM patients and controls.

<table>
<thead>
<tr>
<th>Allele #</th>
<th>RFLP B</th>
<th>RFLP A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>IDDM ptt. (n=154)</td>
<td>0.75</td>
<td>0.25</td>
</tr>
<tr>
<td>Controls (n=178)</td>
<td>0.72</td>
<td>0.28</td>
</tr>
</tbody>
</table>
Table 2. MnSOD TaqI genotype frequencies in random IDDM patients and control individuals.

<table>
<thead>
<tr>
<th>Fragment #</th>
<th>1,3,4,5</th>
<th>1,3,4</th>
<th>1,3,5</th>
<th>1,4,5</th>
<th>1,4</th>
<th>1,5</th>
<th>3,4</th>
<th>3,4,5</th>
<th>3,5</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDDM ptt</td>
<td>42</td>
<td>0</td>
<td>21</td>
<td>38</td>
<td>33</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>(n=154)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>46</td>
<td>0</td>
<td>20</td>
<td>38</td>
<td>52</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>(n=178)</td>
<td></td>
<td></td>
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</tbody>
</table>

In both IDDM patients and control individuals 6 of 9 theoretical genotypes were identified. Allele 4 and 5 comprise one diallelic RFLP (RFLP A), whereas allele 1 and 3 comprise another diallelic RFLP (RFLP B). Combinations with allele 3 and 4 on the same haplotype were not observed.

No fragment pattern occurring only in IDDM patients was observed. Unexpectedly, only 6 different genotypes were observed. The reason for this was that fragment 3 (RFLP B) always occurred with fragment 5 (RFLP A), and fragment 4 (RFLP A) with fragment 1 (RFLP B), indicating that if the TaqI-site identifying RFLP B was deleted so was the TaqI-site identifying RFLP A, and if the TaqI site identifying RFLP A was preserved so was the TaqI site identifying RFLP B. Since the two polymorphic TaqI-sites are separated by at least one constant TaqI-site, this observation suggests that nucleotide changes may occur simultaneously in different regions of the gene. In contrast, deletion of the TaqI-site identifying RFLP A was not necessarily associated with deletion of the other polymorphic TaqI-site. However, 26.5% (88/332) of all tested individuals were heterozygous for both RFLPs, thus not allowing exact haplotype assignment of the polymorphic fragments. Since the genomic sequence of the human MnSOD has not been reported the exact position of the polymorphic TaqI-sites cannot be assigned.

No significant differences in allelic or overall genotype frequencies were observed between patients and controls. A difference in linkage of the alleles of the two RFLP’s was found between controls and patients. The implication of this is not clear.

We have proposed IDDM to be a polygenic disease in which MHC class II specificities confer a major part of the genetic susceptibility, but other genes, including non-MHC genes, must be involved (Spielman et al. 1989, Pociot et al. 1990, Pociot et al. 1992, Pociot et al. 1993a). Though the present data do not support a role of the present MnSOD polymorphisms as genetic markers, recent preliminary data demonstrated a difference in the MnSOD allelic frequency between familial and sporadic IDDM cases (p=0.06, two-tailed) (Pociot et al. 1993b). Furthermore, linkage studies in NOD backcross mice [(NOD/Uf x C57BL/6)F1 x NOD/Uf] showed that the MnSOD locus (on mouse chromosome 17) was highly associated with overt diabetes (Cheng and Wakeland, personal communication), suggesting a role of MnSOD in the disease process of this animal model. This preliminary observation of the MnSOD locus being associated with overt diabetes and not insulitis supports our hypothesis that beta cells may be susceptible to IL-1 cytotoxicity due to insufficient radical scavenger potential (Mandrup-Poulsen et
al. 1990). To our knowledge, the possible existence of genetic variation within the MnSOD locus of the (NOD) mouse has not been reported.

Recently, structurally polymorphic MnSOD protein variants with altered activities was reported (Borgstahl et al. 1992). Selection for MnSOD variants with reduced activity might be a predisposition for diseases, such as diabetes (Asayama et al. 1986, Oberley et al. 1988), that are associated with oxidative damage. This would be in line with the recent observation that mutation in the Cu/ZnSOD is associated with amyotrophic lateral sclerosis (Rosen et al. 1993). Finally, mitochondrial damage has been implicated in a rare form of insulin-requiring diabetes (Ballinger et al. 1992), SOD is protective of healthy pancreatic islet tissue transplanted into diabetic animals (Nomikos et al. 1989) and a possible role of SOD in the process of diabetic retinopathy development has been suggested (Kernell et al. 1992).

If genetic variation results in MnSOD variants with reduced activities (Borgstahl et al. 1992), the MnSOD locus may still be a candidate gene for IDDM susceptibility. Whether the RFLPs reported in this study reflects differences in gene expression level, protein level and/or specific activity of the protein is yet to be studied.

REFERENCES


