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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). *Taq*I 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^+i). In rat islets, fNa^+i increases within minutes of IL-1 exposure, due

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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 (Klempner *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 inter-individual 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: *Acc*I, *Ava*I, *Ava*II, *BamH*I, *Bcl*I, *Bgl*II, *EcoR*I, *Hind*III, *Hinf*I, *Kpn*I, *Msp*I, *Nco*I, *Pst*I, *Pvu*II, *Rsa*I, *Sac*I, *Ssp*I, *Stu*I, *Taq*I, or *Xba*I. 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 *Nde*I-*Sal*I fragment isolated from pcMnHSOD1lacI[2]. The *Nde*I-*Sal*I 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 *Taq*I revealed a polymorphic pattern. The MnSOD cDNA probe used in this study identified 7 fragments, termed 1 to 7, after digestion with *Taq*I, 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 diallelic 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 *Taq*I 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, non 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 *Taq*I revealed a polymorphic pattern with a double diallelic 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.

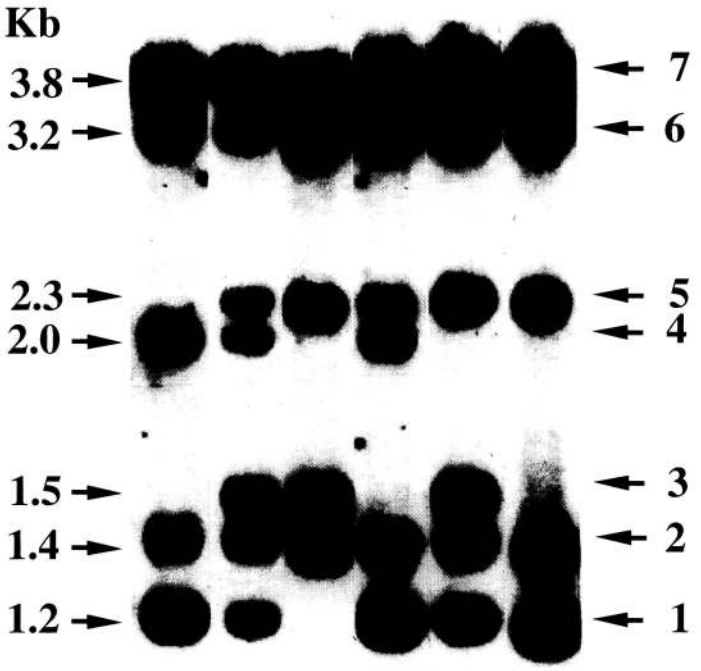

Figure 1. DNA from six healthy individuals digested with the restriction enzyme *Taq*1 and hybridized with a ^{32}P-labelled MnSOD cDNA probe. Six different patterns were identified. Fragments are numbered after molecular size. Fragments 2, 6, and 7 were found in all tested individuals. Fragments 1 and 3 comprises one diallelic RFLP (B) and fragments 4 and 5 another, RFLP A.

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

	RFLP B		**RFLP A**	
Allele #	**1**	**3**	**4**	**5**
IDDM ptt. (n=154)	0,75	0,25	0,61	0,39
Controls (n=178)	0,72	0,28	0,64	0,36

Table 2. MnSOD *Taq*I genotype frequencies in random IDDM patients and control individuals.

Fragment #	1,3,4,5	1,3,4	1,3,5	1,4,5	1,4	1,5	3,4	3,4,5	3,5
IDDM ptt (n=154)	42	0	21	38	33	13	0	0	7
Controls (n=178)	46	0	20	38	52	6	0	0	16

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 *Taq*I-site identifying RFLP B was deleted so was the *Taq*I-site identifying RFLP A, and if the *Taq*I site identifying RFLP A was preserved so was the *Taq*I site identifying RFLP B. Since the two polymorphic *Taq*I-sites are separated by at least one constant *Taq*I-site, this observation suggests that nucleotide changes may occur simultaneously in different regions of the gene. In contrast, deletion of the *Taq*I-site identifying RFLP A was not necessarily associated with deletion of the other polymorphic *Taq*I-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 *Taq*I-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.

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