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Transgenic Copper/Zinc-Superoxide Dismutase Ameliorates Caerulein-Induced Pancreatitis in Mice

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The role of oxidative stress in acute pancreatitis was investigated by comparing the pathological features of caerulein pancreatitis between transgenic mice that overexpress human Cu/Zn-superoxide dismutase (SOD) and nontransgenic littermates. Both the elevation of serum amylase and the formation of pancreatic edema during the pancreatitis were significantly reduced in the transgenic mice compared with the nontransgenic littermates. In the transgenic mice, the pancreatitis-associated reduction of Cu/Zn-SOD activity in the pancreatic tissues was significantly smaller than that in the nontransgenic mice. These results provide direct evidence that the elevation of intracellular oxygen radicals is an important factor for the progress of acute edematous pancreatitis.

Since Sanfey et al. first demonstrated that exogenously administered oxygen radical scavengers or inhibitors of superoxide generation ameliorate experimental acute pancreatitis, several reports have supported the view that oxygen free radicals may play an important role in the pathophysiology of acute pancreatitis [1-6]. The superoxide dismutases (SODs) catalyze the dismutation of superoxide radicals, O_2^-, to H_2O_2, which is then metabolized to water by glutathione peroxidase or catalase. The SODs in eukaryotes are classified into two types: Cu/Zn-SOD which is constitutively expressed in cytosol, and Mn-SOD which is induced transcriptionally in mitochondria by exposing cells to endotoxins or cytokines [7]. In the previous report, we have shown that the Mn-SOD is induced in the pancreas by administering very low doses of lipopolysaccharide (LPS) to rats, and the increased activity of this enzyme in the pancreas correlated with the decreased severity of caerulein pancreatitis [8]. To elucidate further the role of intracellular oxygen radicals in the pathogenesis of pancreatitis, we investigated whether a genetic variation in oxygen radical metabolism can modulate the pathological features of caerulein pancreatitis. To accomplish the purpose simply and directly, a transgenic mouse strain overexpressing Cu/Zn-SOD was employed [9-11].

MATERIALS AND METHODS

Animals and detection of human Cu/Zn-SOD cDNA. Transgene heterozygotes and nontransgenic controls were generated by crossing TgHS-SF218/10 mice with CD1, and the F1 mice were used for the experiment [9]. Animals were maintained under specific pathogen-free conditions and fed standard mouse chow ad libitum. Transgenic and nontransgenic animals were segregated at 4 weeks of age by PCR analysis of the genomic DNA prepared from the whole blood by proteinase K digestion. Synthesized oligomers were used as the PCR primers [12, 13]. The primer set (5′-TTTGGGTATTGTTGGGAGGA-3′ and 5′-CAGCCTATTTGTCTAAGCAG-3′) amplifies 700 bp of the human SOD gene fragment containing exon 5. The final volume of the amplification cocktail was 40 μl and contained 20 pmol of each primer. The PCR profiles were one cycle at 94°C for 30 sec, at 64°C for 30 sec, at 72°C for 30 sec × 35 times and then cooling to 4°C. An aliquot (18 μl) of the amplification cocktail was loaded onto a 0.8 % TBE gel containing 0.45 μg of ethidium bromide per ml and developed at 100 V/8 cm for 20 min. The image was photographed with Polaroid type 55 film [Fig. 1].

Experimental protocols. Male mice 6-8 weeks old (body weight 20-30 gr) were used after an overnight fast. Every experiment in this study was carried out using 10 mice. Acute interstitial edematous pancreatitis was produced by single intraperitoneal (ip) injection of caerulein (100 μg/kg). For evaluating the pancreatitis and measuring the SOD activity in the pancreas, the mice were anesthetized with an ip injection of pentobarbital (20 mg/kg) before and 6 hr after the injection of caerulein. The blood was drawn by puncturing the axillary artery, and thereafter the mice were killed by exsanguination. The pancreata were removed quickly, trimmed free of adhering fat and lymph nodes and weighed. The blood samples were centrifuged at 10,000 g for 5 min to obtain the sera for assays. Serum amylase activity was measured by the blue-starch method using the Phadebas amylase test. The SOD activities and the protein content in the pancreas were measured by the method described below. Pancreatic

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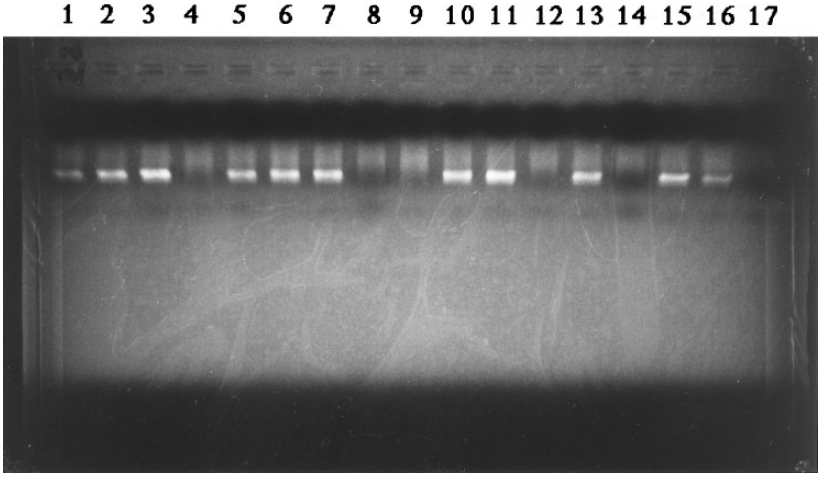

FIG. 1. Identification of the mice with human Cu/Zn-SOD transgene and nontransgenic littermates by PCR analysis of the genomic DNA prepared from the whole blood. The lanes 1-3, 5-7, 10, 11, 13, 15 and 16 show a clear band, indicating the presence of human Cu/Zn-SOD gene in these mice. The mice in the other lanes are judged to be nontransgenic littermates.

edema was evaluated by the ratio of pancreatic wet weight to body weight (P/B: %). For light microscopic observations, a portion of the pancreas close to the spleen was fixed in 10 % formalin and embedded in paraffin wax. Tissue sections 4 μm thick were prepared, deparaffinized, and stained with hematoxylin and eosin. The histological grade of pancreatitis was scored according to the method described by Niederau et al. [14]

Measurements of SOD activity and protein content in the pancreas. A block of fresh pancreatic tissue was immersed in 6 ml of potassium phosphate buffer (0.05 M), and was disrupted for 60 sec by a Polytron homogenizer. The SOD activity was measured by the xanthine oxidase-nitroblue tetrazolium (NBT) method according to Oberley et al. [15] First, total SOD was measured, and then the assay was repeated in the presence of NaCN (5 mM), an inhibitor of Cu/Zn-SOD, to obtain the specific activity of Mn-SOD. The activity of Cu/Zn-SOD was calculated by subtracting the activity of Mn-SOD from the total SOD activity. The protein content of the pancreas was determined by the Lowry method on the same samples used for the measurements of the SOD activity.

Chemicals and assay kits. Caerulein (Ceosunin) was supplied by Kyowa Hakko Co., Ltd., Tokyo, Japan. The Phadebas amylase test was purchased from Pharmacia Diagnostic, Uppsala, Sweden, and xanthin oxidase from Sigma Chemical Co., St. Louis, MO, U.S.A. The PCR kit was purchased from Perkin-Elmer Japan, Urayasu, Japan. Pentobarbital (Nembutal) was purchased from Tokyo-Kasei, Tokyo, Japan, and all other chemicals were from Wako Pure Chemical Industries, Osaka, Japan.

Data analysis. All data are expressed as mean ± standard error of the mean (M ± SEM). For the statistical evaluation of pancreatitis in the transgenic and nontransgenic mice, the differences between the two experimental groups were evaluated by the two-tailed unpaired Student's *t*-test. A *p*-value less than 0.05 was considered to be statistically significant.

RESULTS

The level of serum amylase before the induction of pancreatitis was not significantly different between the human Cu/Zn-SOD transgenic mice [SOD(+)] and the nontransgenic littermates [SOD (−)] [5996 ± 393.7 and 6414 ± 380.0 IU/l in the SOD (+) and SOD (−) mice, respectively]. Six hr after the injection of caerulein, the serum amylase of SOD (−) mice increased to a 3.6-fold higher level (22790 ± 3322 IU/l) than that before the induction of pancreatitis. The serum amylase of SOD (+) mice was also increased by pancreatitis (13615 ± 1165 IU/l), but the increase was inhibited by 54.5 % [Fig. 2A]. Similarly, the P/B ratio of the two groups were not significantly different before the induction of pancreatitis [0.86 ± 0.03 % and 0.83 ± 0.04 % in the SOD (+) and SOD (−) mice, respectively]. The P/B ratio of SOD (−) mice was increased to 1.086 ± 0.04 % at 6 hr after the induction of pancreatitis, but that of SOD (+) mice was only 0.946 ± 0.04 %. Accordingly, the human Cu/Zn-SOD transgene inhibited the pancreatitis-caused elevation of the P/B ratio by 66.4 % [Fig. 2B]. The histological changes of caerulein pancreatitis are characterized by interstitial edema, necrosis, vacuole formation in acinar cells and mild infiltration of inflammatory cells. These histological changes were milder in the SOD (+) mice, and the amelioration of intra-acinar vacuoles was the strongest [Table 1].

The activity of Cu/Zn-SOD in the pancreas was 1.7-fold higher in the human Cu/Zn-SOD transgenic mice than in the nontransgenic littermates [124 ± 16.2 and 72.6 ± 9.5 U/mg protein in the SOD (+) and SOD (−) mice, respectively], whereas no significant difference was found in the Mn-SOD activity between the two groups [9.6 ± 1.7 and 9.4 ± 1.4 U/mg protein in the SOD (+) and SOD (−) mice, respectively]. The activity of Cu/Zn-SOD in the pancreas was 12.9-fold higher than that of Mn-SOD in the transgenic mice and 7.7-fold higher in the nontransgenic littermates. With pancreatitis, the Cu/Zn-SOD activity of SOD (−) mice decreased to 43.5 % of the enzyme level before the induction of pancreatitis (31.6 ± 3.5 U/mg protein). In the

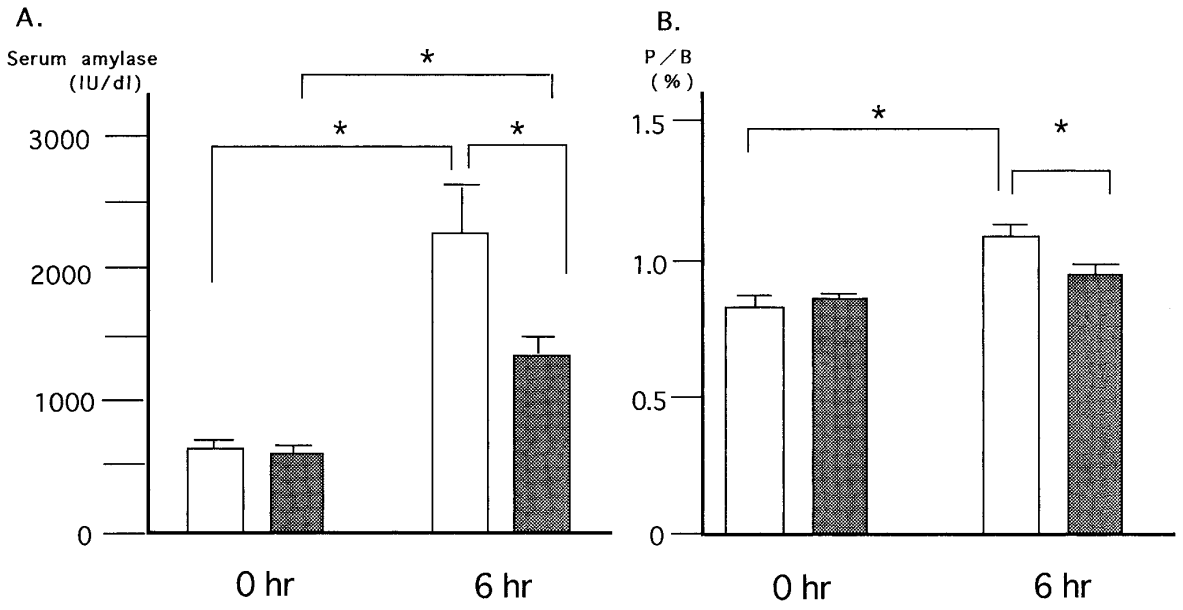

FIG. 2. Serum amylase concentration (A) and pancreatic edema (B) before (0 hr) and 6 hr after the induction of caerulein pancreatitis in the human Cu/Zn-SOD transgenic mice (open bars) and the nontransgenic littermates (closed bars). The stars indicate that the differences are statistically significant ($p < 0.05$).

SOD (+) mice, pancreatitis also decreased the activity of Cu/Zn-SOD in the pancreas to 51.5% of the basal activity (63.8 ± 4.6 U/mg protein), which was, however, still at a level comparable with the basal activity of SOD (−) mice [Fig. 3A]. In contrast with Cu/Zn-SOD, the Mn-SOD activity of SOD (+) and that of SOD (−) mice were reduced to a similar degree by the pancreatitis [4.0 ± 0.44 and 4.8 ± 1.0 U/mg protein in the SOD (+) and SOD(−) mice, respectively] [Fig. 3B].

DISCUSSION

The Cu/Zn-SOD transgenic mouse strain, TgHS-SF218/10, carries a stable multiple copy insertion of a native human Cu/Zn-SOD gene. It is known that the expression of this transgene supplements endogenous Cu/Zn-SOD activity in a variety of tissues from 1.5 to 5 times the normal levels. Kubisch et al. have examined the pancreas of these mice by quantification of Cu/Zn-SOD mRNA and have shown that the ratio of human to mouse mRNA in the pancreas is slightly greater than 1 [10]. Therefore, the level of total Cu/Zn-SOD in the transgenic pancreas was expected to be about twice normal. In the present study, we determined that the activity of Cu/Zn-SOD in the basal state pancreas of the transgenic mice is 1.7-fold higher than that of the nontransgenic littermates, but the activity of Mn-SOD is not different between the two groups. These findings are in agreement with the results of Kubisch et al., and indicate that the human Cu/Zn-SOD gene is expressed in the transgenic pancreas and that the human transcripts generate functional Cu/Zn-SOD subunits that increase total SOD activity. From the rationale of transgenic animal technology, it is considered reasonable that the human Cu/Zn-SOD gene may be expressed in all constituent cells of the pancreas including acinar cells, duct cells, vascular endothelial cells, and endocrine cells.

In the present study, we have demonstrated that pancreatic edema, elevation of serum amylase and histological findings of caerulein pancreatitis were all ameliorated in the transgenic mice compared with the nontransgenic littermates. There is evidence that the pathogenesis of caerulein pancreatitis is intra-acinar activation of pancreatic enzymes as a consequence of a cholecystokinin receptor-mediated sorting disturbance

TABLE 1

Histological Changes of Caerulein Pancreatitis in the Human Cu/Zn-SOD Transgenic Mice and the Nontransgenic Littermates

	Necrosis	Vacuolization	Inflammation
SOD (−)	0-1	2-3	2-4
SOD (+)	0-±	1-2	2-3

SOD (+): Cu/Zn-SOD transgenic mice, SOD (−) : nontransgenic littermates. The pancreas was examined at 6 hr after the injection of caerulein. The histological grading of necrosis and vacuolization refers to the approximate percentage of cells involved. 0; absent, ±; <5%, 1-5; 15%, 2; 15-35%, 3; 35-50%, 4; >50%.

The grading of the inflammatory cell infiltration (inflammation) refers to a scale ranging from 0 as minimal to 4 as maximal alteration.

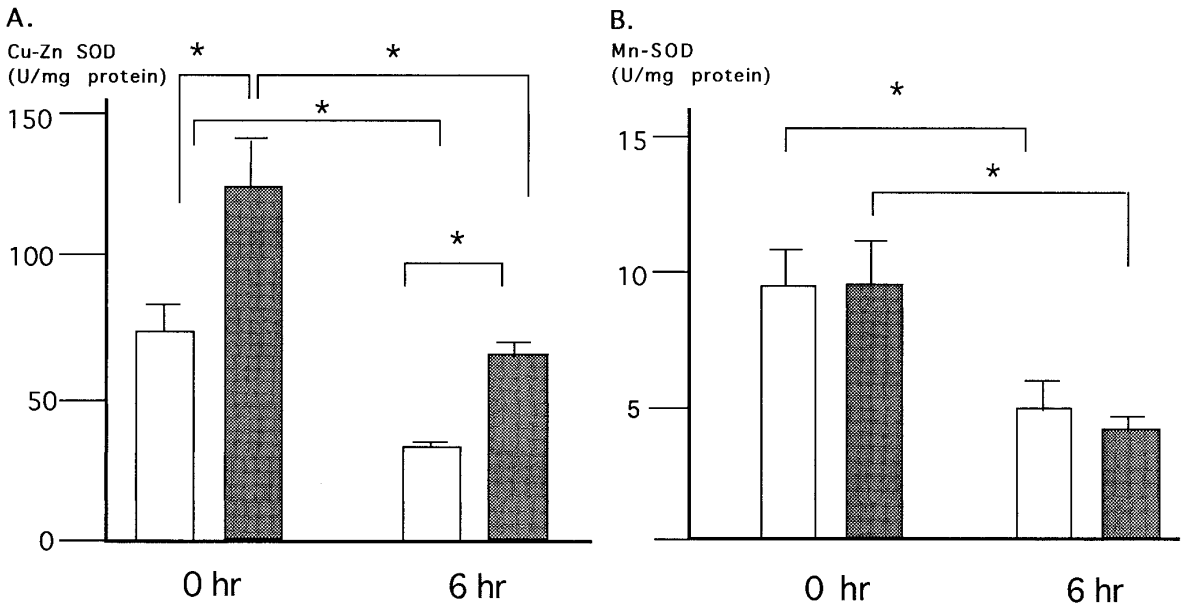

FIG. 3. The activity of Cu/Zn-SOD (A) and that of Mn-SOD (B) in the pancreas before and 6 hr after the induction of caerulein pancreatitis in the human Cu/Zn-SOD transgenic mice (open bars) and the nontransgenic littermates (closed bars). The stars indicate that the differences are statistically significant ($p < 0.05$).

of zymogen granules and lysosomes, which results in the formation of acinar cell vacuoles (crinophagy) [16]. Several previous studies have shown that exogenously administered SOD reduces the pancreatic edema of caerulein pancreatitis [4,5]. However, the beneficial outcome cannot be explained by its effects on the pathogenic events within acinar cells, because the large molecular size makes it impossible for SOD to pass through the cell membrane of acinar cells. We believe that the present study is the first to demonstrate directly the importance of intracellular oxygen radicals in the pathogenesis of acute pancreatitis.

Studies of mitochondria from rat lung, bovine heart, and rat heart have shown that the ubiquinone-cytochrome b complex of the inner mitochondrial membrane transport chain is a major site of O_2^- production in the cell [17]. Oxygen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) inhibit cellular enzymes, and shift the cellular redox state toward oxidation and impair energy production [18]. Previously, we have reported that a low dose LPS can induce Mn-SOD in the pancreas, wherein both the pancreatic edema and elevation of serum amylase during caerulein pancreatitis are strongly inhibited [8]. However, as shown in the present study, a similar degree reduction of the Mn-SOD activity occurred during caerulein pancreatitis in the Cu/Zn-SOD transgenic mice and in the nontransgenic littermates, but a supplement of Cu/Zn-SOD by transgene mitigated clearly the pancreatitis. These results suggest that in addition to mitochondrial Mn-SOD, cytosolic Cu/Zn-SOD may be an important safeguard against the development of acute edematous pancreatitis.

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