The Effect of Human SOD on the Survival Rate in Rats with Temporary Splanchnic Ischemia

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The accumulation of oxygen free radicals is reported to occur in the organs subjected to temporary ischemia followed by reperfusion, resulting in the fatal outcome of the animals. The effects of human SOD, a representative scavenger of oxygen free radicals, on the survival rates were investigated in the rats with temporary splanchnic ischemia. The temporary ischemia was induced by the occlusion of anterior mesenteric and celiac arteries for 30 min under anesthesia. Prior and after treatment with 2 mg/100 g of human SOD, iv or sc, produced significant improvements in survival rates. Human SOD, cloned from human placenta DNA and expressed in microorganisms, has extreme homogeneity. The results suggest the possible introduciton of human SOD into clinical field as an effective scavenger of oxygen free radicals. (Key words: splanchnic ischemia, survival rate, oxygen free radicals, lipid peroxidation, superoxide dismutase)

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It's well known that a complete ischemia followed by reperfusion induces severe cellular injury in organs such as skin, heart, intestine, kidney, and pancreas[1–5]. There is increasing evidence that oxygen free radicals generated from xanthine oxidase in the ischemic cell and/or NAD(P)H oxidase in the activated phagocytes are responsible for these injuries[6,7]. If the excessive presence of oxygen free radicals can be effectively eliminated by the exogenous supply of enzymatic scavenger such as superoxide dismutase (SOD), the lipid peroxidation and the ensuing cellular injuries would be prevented in the laboratory animals, and the survival rate should be improved[8].

Recently, the technic has been established to produce human SOD in microorganisms by the cloning of human gene, allowing the application for therapeutic purpose. However, there are controversies in the benefit of human SOD to the prevention and treatment of ischemia and reperfusion injury[9]. The present study attempts to clarify the effects of human SOD applied on the splanchnic ischemia from the point of view of the prevention of lipid peroxidation in rats.

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Methods

The present study was composed of two experiments. The first was the observation of the effects of exogenous human SOD on the survival rate of rats with temporary splanchnic ischemia. The second was the determination of blood and hepatic lipoperoxide (LPO) as the indices of activity of oxygen free radicals in rats treated with human SOD at 3 h after reperfusion.

Surgical procedure

Male Wister rats, weighing 250 g, were used for the study. They were fed with the usual pellets and water ad libitum. Following

6 h starvation, anesthesia was induced with 36 mg/100 g chloral hydrate ip. Twenty four G teflon mantled needles were placed into a tail vein and left carotid artery, for the drug administration and recording arterial blood pressure. The abdomen was incised for the access to the splanchnic arteries. After administration of 100 units/100 g of heparin, anterior mesenteric and celiac arteries were occluded using soft clips. The clips were removed after fixed time and the abdominal incision was closed.

Experimental protocol

Rats were divided into 8 groups. The first was a sham operated control group of 6 rats, receiving 1 ml of saline and laparotomy. The second was a sham operated control group of 5 rats, receiving 2 mg/100 g of human SOD and laparotomy. The third of 5 rats was a control group, receiving saline and splanchnic occlusion. The 4th of 7 rats was an experimental group with prior treatment of human SOD. The animals received 2 mg/100 g of human SOD iv 5 min prior to the occlusin of splanchnic arteries. The 5th of 5 rats was an experimental group with prior treatment of SOD. The animals received 2 mg/100 g of SOD sc 30 min prior to the arterial occlusion. The 6th of 6 rats was an experimental group with after treatment of SOD. The animals received 2 mg/100 g of human SOD iv immediately after the release of splanchnic vessel occlusion. The 6th of 6 rats was an experimental group with pre-treatment of a chemical quencher, α-tocopherol. They received 50 mg/100 g of α-tocopherol ip 30 min prior to the occlusion of splanchnic vessels. The 8th of 5 rats was an experimental group with a large amount of fluid after release of splanchnic vessel occlusion. The animals received 10 ml/100 g of Ringer's lactate solution for 5 h via tail vein after splanchnic reperfusion. The rates of fluid infusion were 5 ml/100 g for first one h, 2 ml/100 g for second one h, and 3 ml/100 g for following 3hr.

Observatin of survival rates

As a preliminary study, duration of splanchnic ischemia was changed to 10, 20, 30, 40, 50, and 60 min. Each group consisted of 10 rats. The animals received splanchnic vessel occlusion after the prior infusion with 1 ml of saline. After recovery from anesthesia, they were allowed to take food and water freely in an air conditioned cage. The number of rats surviving longer than 24 h was scored. The preliminary experiment showed low survival rates in rats with splanchnic ischemia exceeding 30 min. Therefore, the duration of splanchnic vessel occlusion was fixed at 30 min in the present study.

Determination of blood and hepatic LPO levels

In the second experiment, the blood sample and liver specimen were taken at 3 h after reperfusion. The blood was centrifuged for the separation of plasma. A small portion of the excised organ was honogenized with a 10 fold-diluted saline using a Polytron high-speed homogenizer. After centrifugation at 1,000 g for 15 min, the supernatant was collected. LPO level was measured with Yagi's method[10]. The amount of LPO was expressed as manomoles of malondialdehyde per gram of wet weight.

SOD and α-tocopherol

Human SOD was offered by Nippon Kayaku Co., Ltd., Tokyo. The SOD was cloned from human placenta DNA library and expressed in Esherichia coli. The enzyme produced in Esherichia coli was extracted and purified to homogenous quality with a single band by SDS-polyacrylamide gel electrophoresis. Alpha-tocopherol was purchased from Yamanouchi Pharmaceutical Co. Ltd., Tokyo.

Statistical analysis

Survival rates were presented as percent and Fisher's exact probability test was used to evaluate significant differences between two groups. LPO concentraiton in blood and liver were expressed as mean $\pm$ standard deviation. The differences among groups were assessed by the analysis of variance (ANOVA) and the difference between 2 groups by Student's unpaired t-test. The differences were considered significant at $P<0.05$.

Table 1. Relationship betwen the duration of occlusion and suvival rates

Duration of occlusion	Survival rates	
(minutes)	%	(survived/total)
10	100	(10/10)
20	90	(9/10)
30	10	(1/10)
40	0	(0/10)
50	0	(0/10)
60	0	(0/10)

Results

Preliminary experiments

Survival rates after various duration of splanchnic vessel occlusion are shown in table 1. Splanchnic ischemia of 30 min duration showed a survival rates of 10%, and the occlusion longer than 30 min produced zero perçent of survival rate.

Survival rates

The rats in sham operated control groups receiving only laparotomy with prior injection of saline or human SOD showed no toxic reactions, and they all survived over 24 hr. The survival rate of animals in the control group receiving splanchnic vessel occlusion with prior administration of saline was zero percent (0/6). The effects of exogenous supply of scavengers and fluid therapy are summarized in table 2.

The pre-treatment with human SOD, iv or sc, improved the survival rate significantly. The administration of human SOD immediately following the release of splanchnic vessel occlusion also improved the survival rate. No significant improvement of survival rate was observed in the group with α-tocopherol. Animals with fluid therapy after reperfusion showed a significant elevation of survival rate.

Blood and liver LPO

Hepatic LPO at 3 h after release of splanchnic vessel occlusion showed a significant difference among 8 groups by ANOVA. There was a significant increase in the hepatic LPO in the control group receiving splanchnic ischemia and prior administraiton of saline in comparison with those of sham operated control groups. Prior administration of human SOD, iv or sc preserved LPO at low levels with no difference between sham operated control group. The administration of human SOD immediatly following the release of splanchnic vessel occlusion reduced the hepatic LPO significantly. Prior treatment with α-tocopherol also reduced the hepatic LPO level. Fluid therapy produced no difference in the hepatic LPO between control group. No difference was produced in the blood LPO concentration by ANOVA.

Table 2. Survival rates in eight groups

Group (No)	Survival rates (%)	Notes
1 (6)	100	sham operated control
2 (5)	100	sham operated control
3 (5)	0**	control
4 (7)	71!!	SOD
5 (5)	100!!	SOD
6 (5)	80!!	SOD
7 (6)	0**	α-tocopherol
8 (5)	80!!	fluid therapy

The simbol, **, shows a significant difference ($P<0.01$) between sham operated control groups. The simbol, !!, shows a significant difference ($P<0.01$) between control group.

Table 3. Hepatic and blood LPO levels in eight groups

groups (No)	Liver (nM/g)	Blood (nM/ml)
1 (5)	54.7± 6.2	4.4±0.5
2 (5)	53.8± 5.8	4.3±0.6
3 (5)	87.9±12.5**	5.1±0.9
4 (7)	59.2± 8.2!!	5.0±1.2
5 (5)	58.5± 7.9!!	4.9±0.8
6 (5)	61.9± 8.3!!	5.2±1.2
7 (6)	68.9± 9.1**!	5.1±0.9
8 (5)	79.8±10.0**	4.9±0.8

The simbol, **, shows a significant difference ($P<0.01$) between sham operated control groups. The simbols, ! or !!, shows significant differences ($P<0.05$ or $P<0.01$) between control group.

Discussion

The occlusion of anterior mesenteric and celiac vessels followed by the release of

occlusion after 30 min produced a survival rate of 10 percent within 24 h in the present study. Dawidson et al.[11] reported that the survival rate was markedly reduced when the duration of ischemia exceeded 50 min, showing the maximum of 90 min for the survival without treatment.

The discrepancy in survival rates between Dawidson's and our results may be referred to the difference of the operative procedures. Anterior mesenteric and celiac vessels were occluded in the present study, while only anterior mesenteric artery in the Dawidson's study. Occlusion of two main vessels induced an almost complete ischemia in the splanchnic area including the liver.

The mechanisms by which the animals die after temporary occlusion of splanchnic vessels are thought to be circulatory derangement due to the depletion of plasma fluid after recirculation and/or oragn failure due to cellular necrosis. It was reported that hematocrit increased to 60 percent as a result of plasma depletion after 75 min of mesenteric arterial occlusion[12]. The present sutdy showed an improvement of the survival rate by the fluid administration of 10 ml/100 g for 6 h, supporting the hypothesis of plasma depletion.

Many substances, such as histamine, kinin, prostanoids, eicosanoids, lysosomal hydrolases, proteases and activated complement, are proposed to be responsible for the rise in vascular permeability. There is increasing evidence that oxygen free radicals play a majar role in producing vascular permeability by acting directly on the vascular endothelium and/or by inducing the release of vasoactive substances as cited above[13–15].

There are several systems involved in producing oxygen free radicals in the mammalian body. The site of production may be divided into two compartments, intra- and extracellular. In the intracellular compartment, xanthine oxidase (XOD) is considered to produce the greater part of superoxide ion (O_2^-) by the transmission of one electron to groud state of oxygen. Parks et al.[16] reported that the excessive permeability was inhibited by prior treatment of allopurinol, an inhibitor of XOD, in an animal model of bowel ischemia and postulated the elevation of XOD activity. In the ischemic cells, xanthine gehydrogenase has been asertained to be hydrolyzed to XOD by a protein kinase activated by Ca ion excessively fluxed into the cell[17]. SOD, catalase and glutathine peroxidase are enzymatic scavengers of radicals, whereas vitamin E, β-carotin, and reduced glutathion act as chemical quenchers.

In the extracellular compartment, phagocytes such as leucocytes, macrophages and monocytes continuously produce O_2^- by membrane NAD(P)H oxidase, reducing ground-state oxygen. There are some quenchers of radicals, such as ceruloplasmin, systeine, ascorbic acid, and transferin, but few enzymatic scavengers. Thus the total scavenging capacity is thought to be weak.

The cellular injury may be prevented, if the accumulation of oxygen free radicals is blocked by effective measures. The measures now proposed are: 1) inhibition of XOD by allopurinol, 2) exogenous supply of enzymatic scavengers, and 3) supplementation of chemical quenchers. The most promising measure is reported to be exogenous supply of XOD, because the enzyme can catalyze the O_2^- to H_2O_2. The molecular weight of SOD is relatively small as 33,000 to 80,000, permitting the rapid elimination through the kidney. Kunimoto et al.[8] reported that a large amount of bovine SOD exceeding 1 mg/100 g was required to prevent the accumulation of LPO in rats with lethal endotoxemia. So, the dose of 2 mg/100 g was adopted in the present study. Single injection of large amount of SOD produced significant improvements of survival rate in the present study.

The human SOD does not penetrate the cell membrane because of high molecular weight. The present study indicates one possibility that the human SOD scavenges the oxygen free radicals produced in the extracellular space, suggesting the principal role of phagocytes in the pathogenesis of excessive permeability of microcirculatory system after temporary occlusion of splanchnic vessels.

The results obtained in the present study

suggest the effectiveness of human SOD as a scavenger of oxygen free radicals. The massive production of human SOD is established using microorganisms as clone cell recently. The human SOD used in the present study was reported to be extremely purified and stable[18]. The clinical introduction of human SOD is expected because of no hypersensitive reactions.

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