Protective effect of Sanguisorbae Radix against apoptosis and function of renal tissues subjected to ischemia-reperfusion

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Abstract

DNA ladders were detected by gel electrophoresis of DNA obtained from rat kidney subjected to 60 min of ischemia followed by 24 h of reperfusion, indicating the involvement of apoptosis in ischemia-reperfusion injury. This ladder formation was significantly inhibited by oral administration of Sanguisorbae Radix extract to rats for 30 days prior to ischemia-reperfusion. In addition, blood levels of urea nitrogen and creatinine, two parameters of renal function, were markedly lower in the Sanguisorbae Radix-treated animals than in the untreated controls. These results suggest that Sanguisorbae Radix has potential for attenuating renal injury and accelerating the recovery of renal function after ischemia-reperfusion injury, which might involve inhibition of apoptosis.

Key words Sanguisorbae Radix, ischemia-reperfusion, apoptosis, DNA fragmentation, renal function, kidney.

Introduction

Renal ischemia-reperfusion injury is a frequently encountered phenomenon in renal transplantation, and certain disease states.[1,2] A major consequence of ischemic damage to the kidney is lethal or sublethal injury to tubular epithelial cells, causing renal dysfunction. In earlier studies, such tubule injury was characterized as "acute tubule necrosis". However, in recent years, a large number of investigations have provided strong evidence that apoptosis, a programmed form of cell death, occurs in renal tissue subjected to brief periods of ischemia followed by reperfusion, which may contribute to subsequent renal tubule cell death.[3-5]

Extensive studies on the mechanism of ischemia-reperfusion injury have revealed that reactive free radicals play a major role.[6,7] On the other hand, these free radicals and oxidative stress have also been implicated in the induction of apoptosis.[8,9] Interventions aimed at attenuating cell and renal damage through manipulation of free radical production have been shown to have therapeutic potential.[10,11]

Traditional crude drugs and prescriptions have been shown to protect renal cells and tissues, and inhibit the progression of renal failure efficiently *in vivo* and *in vitro*. This protective effect is also involved in the mechanism of apoptosis suppression.[12,13] Investigations of the active principles have frequently focused on tannin, a species of constituent that extensively scavenges free radicals and suppresses lipid peroxidation. In the present study, using a model of renal ischemia-reperfusion injury, we examined the effect of Sanguisorbae Radix, a hemostasis drug used in traditional Chinese medicine, which contains a large amount of tannin as its major constituent, and shows strong radical-scavenging activity in experiments using sodium nitropresside as a nitric oxide (NO) donor *in vitro*.[14]

Materials and Methods

Preparation of extract from Sanguisorbae Radix:

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The roots of Sanguisorbae Radix (*Sanguisorba officinalis* L.), grown in China and supplied by Uchida Wakan-yaku Co., Ltd., Tokyo, Japan, were finely powdered and extracted with distilled water at 100°C for 1 h (roots : water=1 : 10, w/v). After removal of the insolubles by filtration, the filtrate was concentrated under reduced pressure and then lyophilized to yield a brown residue. The yield of the extract was 17.4 % by weight of the original material.

Animals and treatments: Male LWH: Wistar rats with a body weight of 125-130 g were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan). They were kept in wire-bottomed cages under a conventional lighting regimen with a dark night. The room temperature (about 25°C) and humidity (about 60 %) were controlled automatically. Laboratory pellet chow (CLEA Japan Inc., Tokyo, Japan; comprising 24.0 % protein, 3.5 % lipid and 60.5 % carbohydrate) and water were given *ad libitum*. Following several days of adaptation, the animals were divided into 4 groups-a normal, a control, and two treatment groups-avoiding any intergroup difference in body weight. The normal and control groups were given water, while the others were given Sanguisorbae Radix extract orally at a dose of 100 or 200 mg/kg body weight/day for 30 consecutive days. After induction of anesthesia by intraperitoneal administration of sodium pentobarbital 50 mg/kg body weight, bilateral flank incisions were made and the renal arteries were exposed. Bilateral renal artery occlusion was then carried out for 60 min using a nontraumatic vascular clamp. Following release of the occlusion, the abdomen was sutured, and the animal was returned to the cage. At 24 h after reperfusion, blood samples were obtained by cardiac puncture under anesthesia, and the serum was separated immediately by centrifugation. The kidneys were subsequently extirpated from each rat following renal perfusion through the renal artery with ice-cold physiological saline. The tissues were quickly frozen and kept at -80°C until analysis. Five rats were used for each experimental group.

Analysis of DNA fragmentation: According to the method of Katoh,[15] the kidney was homogenized and lysed in a cold lysis buffer (10mM Tris-HCl, 5 mM disodium EDTA, and 0.5 % Triton X-100, pH 8.0) for 10 min at 4°C. The DNA was sequentially extracted two times with half volumes of phenol/chloroform and incubated at 55°C for 10 min. After centrifugation at 3,000 rpm for 20 min, the upper layer was incubated with 2 μl proteinase K (20 mg/ml) at 37°C for 60 min followed by incubation with 2 μl ribonuclease (20 mg/ml) at 37°C for 60 min. The DNA was precipitated by adding 0.1 volume of 10 M ammonium acetate and 2.5 volumes of 100 % ethanol and maintained at -20°C overnight. DNA was collected by centrifugation at 15,000 x g for 20 min, air-dried, and resuspended in TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 7.4). The resulting DNA preparations were electrophoresed through a 2 % agarose gel containing ethidium bromide. Equal quantities of DNA (according to determination of the optical density at 260 nm) were loaded in each lane, and 1-kb and 50-bp multimers were employed as molecular weight standards. DNA fragmentation was visualized and photographed under ultraviolet illumination. Semiquantitative densitometric analysis of DNA fragmentation was conducted using a BIO-RAD Model GS-670 Imaging Densitometer with Molecular Analyst/PC Software.

Determination of blood constituents : Urea nitrogen and creatinine (Cr) were determined using the commercial reagents BUN Kainos and CRE-EN Kainos (Kainos Laboratories, Tokyo, Japan).

Statistics : Statistical analysis was performed by Dunnett's test.

Results

DNA fragmentation

As shown in Fig. 1, kidney DNA was fragmented into lower-molecular-weight molecules after ischemia-reperfusion. Analysis of the agarose gel electrophoresis pattern revealed a ladder, which was absent in kidney not subjected to ischemia-reperfusion, indicating that oxidative stress induced apoptosis. However, it was confirmed in terms of the electrophoresis pattern and semiquantitative densitometry that oral administration of 100 mg of Sanguisorbae Radix extract decreased the DNA fragmentation significantly compared with that in ischemia-reperfused control kidney. A further increase in the dose to 200 mg produced a further decrease in the

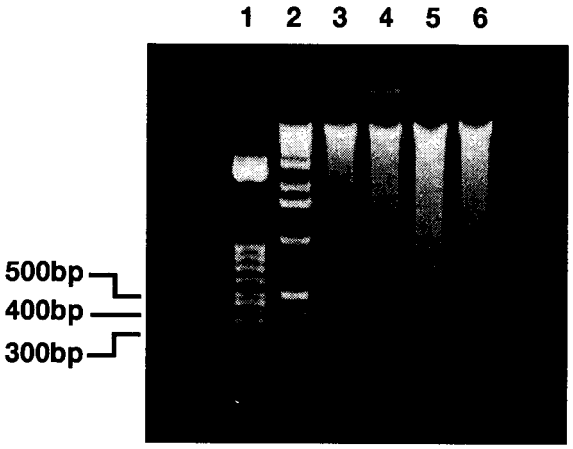

Fig. 1 Agarose gel electrophoresis of DNA. lane 1 : 50-bp marker DNA ; lane 2 : 1-kb marker DNA ; lane 3 : normal ; lane 4 : ischemic and reperfused control ; lane 5 : ischemic and reperfused Sanguisorbae Radix extract-treated (100 mg/kg B.W./day) ; lane 6 : ischemic and reperfused Sanguisorbae Radix extract-treated (200 mg/kg B.W./day).

Table I Effect of Sanguisorbae Radix extract on DNA fragmentation.

Group	Dose (mg/kg B.W./day)	Fragmentation rate (%)
Normal	–	–
Ischemic and reperfused		
Control	–	14.6±2.7
Sanguisorbae Radix extract	100	7.1±1.4[a]
Sanguisorbae Radix extract	200	6.5±1.3[a]

Statistical significance: [a]$p<0.001$ *vs.* control values with ischemia-reperfusion.

DNA fragmentation rate (Table I).

Blood urea nitrogen and Cr

Table II shows the effect of Sanguisorbae Radix extract on parameters of blood constituents after administration of an oral dose. The blood urea nitrogen and Cr levels in ischemia-reperfused control rats were increased significantly in comparison with normal rats. In contrast, the blood urea nitrogen level in rats given Sanguisorbae Radix extract decreased from 138.4 to 82.1 mg/dl at the 100-mg level (a 41 % change, $p<0.001$) and from 138.4 to 54.9 mg/dl at the 200-mg level (a 60 % change, $p<0.001$). Similarly, the Cr level in rats given Sanguisorbae Radix extract showed a significant decrease at both the 100- and 200-mg dosage levels as compared with that in the control rats, as shown in Table II.

Discussion

Cells die by either apoptosis or necrosis. Cell death by necrosis occurs when cells are exposed to severe injurious conditions. In contrast, apoptosis occurs in a number of physiological processes such as embryogenesis, metamorphosis,[16] cytotoxic T cell-mediated killing of target cells,[17] and death of autoreactive thymocytes,[18] which play an important role in maintaining a normal balance in cell growth, development and elimination. However, recent studies have shown that multiple cytotoxic stimuli causing necrosis can also initiate apoptosis when cells are exposed to the same noxious agents at lower concentration. Apoptosis has been shown to contribute to extensive cell loss in many pathological states including ischemic renal failure.[19] Indeed, the involvement of

Table II Effect of Sanguisorbae Radix extract on blood urea nitrogen and creatinine.

Group	Dose (mg/kg B.W./day)	Urea nitrogen (mg/dl)	Cr (mg/dl)
Normal	–	20.9± 1.0	0.36±0.03
Ischemic and reperfused			
Control	–	138.4±13.1[a]	2.81±0.47[a]
Sanguisorbae Radix extract	100	82.1± 9.9[a,b]	1.65±0.20[a,b]
Sanguisorbae Radix extract	200	54.9± 5.5[a,b]	1.05±0.16[a,b]

Statistical significance : [a]$p<0.001$ *vs.* normal values, [b]$p<0.001$ *vs.* control values with ischemia-reperfusion.

apoptosis in ischemia-reperfusion injury was also clearly demonstrated in the present study. When rats were subjected to a 60-min period of complete ischemia, followed by 24 h of reperfusion, a ladder pattern of low-molecular-weight DNA was detected by agarose gel electrophoresis. These fragments displayed a typical DNA ladder pattern indicative of apoptosis at intervals of about 180 bp, a feature that was absent in tissues not subjected to ischemia-reperfusion, indicating that oxidation stress induces apoptosis. In contrast, rats given Sanguisorbae Radix extract orally at a dose of 100 or 200 mg/kg body weight/day for 30 consecutive days prior to ischemia and reperfusion demonstrated sufficient inhibition of apoptosis. Although precise quantitative analysis of the extent of DNA fragmentation was not conducted, semiquantitative detection showed a significant difference between the controls and the groups treated with Sanguisorbae Radix extract. The percentage of DNA fragmentation was lower in the treated rats than in the controls. This result indicated that Sanguisorbae Radix was able to inhibit apoptotic cell death.

Gobe *et al.*[20] obtained pathologic evidence for both necrosis and apoptosis of renal epithelial cells during the first 2 to 8 days after occlusion of the renal artery, whereas from 10 to 28 days when the renal mass was markedly reduced, cell death continued, but only apoptosis was observed. Other experiments have also yielded similar results.[3 5] These results demonstrate that apoptosis plays an important role in ischemic cell injury and is responsible for renal dysfunction in ischemic acute renal failure. On the other hand, Hellberg and Kallskog[21] have reported that ischemia-reperfusion causes aggregation of polymorphonuclear cells in the glomeruli and stimulates them to release chemical mediators including free radicals, which then undergo ultrafiltration and subsequently injure the tubule cells from the luminal side. They explained that the initial change therefore occurs at the brush border, followed by deciduation of tubule cells into the lumen, forming casts in the distal part, resulting in tubule occlusion and hence decreased renal function. In this regard, we observed reversal of the decrease in renal function (in terms of increased levels of blood urea nitrogen and Cr) after administration of Sanguisorbae Radix extract.

The mechanism by which Sanguisorbae Radix inhibits apoptosis and protects against renal failure is not clear. However, reactive free radicals and oxidative stress have been extensively implicated in ischemia-reperfusion injury. As Sanguisorbae Radix contains a large amount of tannin as its major constituent, a species shown to have marked antioxidant and radical-scavenging activity,[22,23] and furthermore in a preliminary study we have verified that Sanguisorbae Radix extract significantly scavenges superoxide and hydroxyl radical (data not shown), we speculate that these properties of Sanguisorbae Radix might contribute to its protective effect on renal function and inhibition of apoptosis.

In summary, although the complete mechanism and therapeutic indications of Sanguisorbae Radix extract remain to be elucidated and verified, the present data provide evidence that this agent attenuates renal damage and accelerates renal recovery from ischemia-reperfusion injury. Furthermore, we suggest that the protective effect of Sanguisorbae Radix extract on renal function may involve inhibition of apoptosis.

和文抄録

60分間虚血後，24時間再灌流したラット腎のDNAにラダーが出現し，腎虚血－再灌流障害にアポトーシスが関与していることが示唆された。このようなDNAの断片化は，地楡エキスを前もって30日間経口投与した場合，有意に抑制され，腎機能の指標の血中尿素窒素とクレアチニンレベルも地楡エキス処理群で著しく低下していた。このことから，地楡はアポトーシスが関与した腎虚血－再灌流障害を軽減して，腎機能の回復をはかっているものと推測された。

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