The Organo- and Cytoprotective Effects of Heat-shock Protein in Response to Injury Due to Radiofrequency Ablation in Rat Liver

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Abstract. *Aim: In treating liver tumors, preserving hepatic reserve and reducing surgical invasiveness are important for minimizing postoperative complications. Geranylgeranylacetone (GGA) is reported to selectively induce heat-shock protein 70 (HSP70), which initiates a powerful cytoprotective effect. We investigated the function of HSP70 under conditions of radiofrequency ablation (RFA) of the liver. Materials and Methods: Male Wistar rats were divided into three groups: a control group, a group administered GGA, and a group administered GGA plus quercetin, an HSP70 synthesis inhibitor. Expression of HSP70 and heat-shock factor-1 (HSF1) in the liver was measured at the protein level, and severity of liver damage was investigated using serum and hepatic tissue. Results: The GGA-treated group had higher expression of HSP70 and HSF1 than the other groups. Peak liver damage in all groups occurred 6 h after RFA. The GGA-treated group also had significantly less liver damage and lower serum level of the inflammatory cytokine tumor necrosis factor-α, and a lower rate of apoptosis in tissue around post-ablation necrosis. Expression of HSP70 and HSF1 was suppressed in the group treated with GGA and quercetin, and this group had severe liver damage. Conclusion: Induction of HSP in the liver by GGA may be applicable in future treatments for hepatocellular carcinoma or liver metastasis. The present findings suggest that if preoperative administration of GGA can offer protective effects in the liver, treatment options could be increased and liver failure and other complications might be avoided.*

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Although treatments for liver cancer have gradually been established in recent years, it is still a disease with a poor prognosis. In Japan, where liver cancer often develops from viral hepatitis or cirrhosis, it is not uncommon for treatment options to be limited by the state of hepatic reserve. Moreover, the 5-year reccurrence rate is a high 70%, even when more radical treatments can be selected (1). Various treatment approaches are being tried to improve the survival rate. Radiofrequency ablation (RFA) has been widely adopted in recent years and has contributed greatly as a local treatment in cases that are unresectable due to cirrhosis, or in which the lesion is in a place that is difficult to reach percutaneously, and in recurrence after hepatecomy (2, 3). These cases often have varying levels of liver damage for the above-mentioned reasons, and reduction of surgical invasiveness is a key issue in avoiding postoperative complications. In general, RFA treatment has been used in patients with relatively well-preserved liver function in cases of tumors of size ≤3 cm and three or fewer lesions (1). However, more radical RFA can be selected even for cases of multiple liver cancer in which hepatic intra-arterial chemotherapy has been used to date. In recent years, RFA has been widely used not only for primary liver cancer,but also for liver metastasis of gastrointestinal cancer (4). De Baere *et al*. performed RFA for metastatic liver cancer and reported that the local control rate 1 year postoperatively was 90% (5).

Heat-shock proteins (HSPs) are proteins that are synthesized rapidly in cells in response to different kinds of stress, acting as molecular chaperones whose actions include intracellular protein repair (6-8). In humans, HSPs are expressed in various organs and have organ-specific protective effects against stress. Heat-shock factor-1 (HSF1), which promotes synthesis of HSPs, is always present in cells. It is maintained as an inactive monomer bound in a complex with HSP90, but when there is an increase in abnormal proteins damaged by stress, HSP90 separates from HSF1 to process those proteins. This HSF1 separated from HSP90 acquires activity, forms trimers and moves inside the

nucleus. If phosphorylation of HSF1 then occurs, large amounts of HSP are synthesized at ribosomes (6, 8).

In addition to different kinds of stress, synthesis of HSP is reported also to be increased by geranylgeranylacetone (GGA) and other pharmaceutical agents through the same mechanism, having a cytoprotective effect (9, 10). GGA is used worldwide as an anti-ulcer drug that repairs the gastric mucosa, but there are also various reports on its cytoprotective effect. It is reported to safely induce stress proteins in cultured gastric mucosa cells (rat) and rat gastric mucosa, selectively and rapidly induce HSP70, and lessen damage and stress ulcers in response to alcohol (9). Other reported effects are, in the lungs, symptom relief and treatment in cases of acute respiratory distress syndrome and inhibition of ischemia perfusion injury in lung following lung grafts (11, 12); in the colon, inhibition of damage to colonic mucosal cells in a mouse model of enteritis (13); and in the heart, post-ischemic myocardial protection (14). In the liver, a hepatoprotective effect has been reported in an ischemia-reperfusion model and in a model of massive hepatectomy (95%) in rats (15, 16).

In addition, inhibition of inflammatory cytokine expression was previously reported in experimental models (17). Elucidation of the hepatoprotective effect of HSP may contribute to broadening treatment options, preserving hepatic reserve, and reducing postoperative complications in the treatment of patients with liver cancer with severe liver damage, while clarification of the protective effects in damaged liver would be promising for clinical applications. The presence of HSP would also seem to be helpful in the protection of remaining hepatocytes from ischemia when the hepatic blood flow is blocked during liver resection, and in the avoidance of postoperative complications in response to surgical invasiveness. In the treatment of metastatic liver tumors, which often occur in multiple locations, the presence of HSP may also lead to safer treatment by RFA alone or in combination with resection for patients in whom post-resection complications are a concern because of the small capacity of the remaining liver. In this study, we investigated the effect *in vivo* of HSP induced by GGA in response to hepatic injury by RFA.

Materials and Methods

Experimental design. Male Wistar rats were used in this experiment. The rats (n=90) were randomly divided into three groups, a control group, a group that was given oral GGA, and a group that was given oral GGA plus quercetin, which inhibits the synthesis of HSP70 (18).

Animals. Wistar rats, weighing 250-300 g, provided by our laboratory's rat colony, were used. They were housed in Makrolon® cages, two rats per cage, at 20-22˚C room temperature, on a 12 h light:12 h dark cycle. The rats were provided with a commercial pelleted diet and tap water *ad libitum*. The facilities were in accordance with Directive 86/609/EEC. Animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals Prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985) (19).

RFA. GGA was provided by Eisai (Eisai Co., Ltd., Tokyo, Japan). It was adsorbed onto gum arabic and dissolved in distilled water. It was then administered to group A orally under ether inhalation anesthesia twice, 24 and 4 h before ablation, at a dose of 200 mg/kg (6). Group B received quercetin in addition to GGA. Quercetin was administered orally with the same technique 4 h before RFA at a dose of 100 mg/kg. For RFA, laparotomy was performed with the animals under ether inhalation anesthesia. A 1-cm Cool-tip Needle (Radionics Co., Burlington, MA, USA) was used. Ablation was performed at one site in the left lobe of the liver and at one site on the right side of the medial lobe. RFA was started at 5 W and then raised 5 W every minute until 3 min, after which it was continued at 15 W. RFA was performed for 5 minutes (20, 21). The extent of the ablation was about 15-20% of the full liver in the two locations in total.

Sampling. Blood and liver sampling were performed under ether anesthesia. Blood was sampled preoperatively and at 6, 12, 24, and 48 hours postoperatively. Serum chemistry examinations [aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB)] and measurement of the inflammatory cytokine tumor necrosis factor-alpha (TNFα) were obtained. Blood levels of AST, ALT TB, and TNFα were measured by MA Iatron Lab. (Genzyme Corp., Tokyo, Japan).

Expression of HSP70 and HSF1 at the protein level. The excised liver specimens were fixed in 10% formalin and stored at –80˚C after freezing with liquid nitrogen. In the three groups, 50-μg liver specimens excised preoperatively and at 6, 12, 24, and 48 h postoperatively (unablated left side of the medial lobe, right lobe) were used.

The frozen speciments were homogenized in three volumes of lysis buffer (10 mmol/l Tris-HCl, 150 mmol/l NaCl, 5 mmol/l ethylenediamenetetraacetic acid, 1% Triton™ X-100, 2 mmol/l phenylmethylsulfonyl fluoride). The mixture of the cells and the lysis buffer was centrifuged for 15 min. An equal amount of protein in the supernatents (50 μg protein per lane) was separated by sodium dodecylsulfate polyacrylamide gel electrophoresis in a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After nonspecific binding sites were blocked with 4% purified milk casein, the blots were incubated with a monoclonal antibody against HSP70 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Bound antibodies were detected with an enhanced chemiluminescence western blot detection kit (GE Healthcare Japan Corporation, Tokyo, Japan).

The expression of β-actin was measured using the same specimens. The NIH ImageJ software (Image Processing and Analysis in Java) (http://image.nih.gov/ij) was used in the analysis.

Histological investigation of the ablated liver. The ablated liver was fixed in 10% formalin for 24 h and embedded in paraffin, after which thinly sliced specimens were prepared and stained with hematoxylin and eosin (HE). Sections with the largest amount of ablation were prepared and the area of ablation was measured. Specimens were examined using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate–biotin nick end

labeling (TUNEL) to assess hepatocytes outside the ablation area that had become apoptotic. An ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (S7101, EMD Millipore Corporation, Billerica, MA, USA) was used in staining.

Statistical analysis. Comparison of estimated tumor volume was performed using two-factor factorial analysis of variance (ANOVA) and Student's *t*-test. Other data comparisons were performed using Student's *t*-test. Statistical significance was set at $p<0.05$.

Results

Higher expression of HSP70 was seen in the group treated with GGA alone than in the other groups from before RFA until 48 h after. The expression of HSP70 was found to be inhibited with the oral administration of quercetin (Figure 1A). Quercetin is a natural flavonoid, also known as vitamin P, which has long attracted attention due to its antioxidant and anticancer activity, but it was later reported to also inhibit the expression of *HSP70* mRNA (19). HSP expression is inhibited by inhibiting the expression of HSF1, a transcription factor for HSP expression (18). Therefore the expression of HSF1 was then investigated. Since the expression was confirmed to be uniform from the measurement of monomers, dimers, and trimers, the amount of expression was determined with monomers. The group treated with GGA alone was found to have mild expression even with preoperative administration, with a peak at 6 h postoperatively. In the group treated with GGA-plus-quercetin, expression of HSF1 was found to be inhibited, similarly to the expression of HSP (Figure 1B). The experiment was repeated at least three times to confirm reproducibility of data, and almost identical results were obtained.

Next, serum chemistry reflecting liver damage from RFA were compared. The peak liver damage as shown by AST, ALT, and TB occurred at 6 h after RFA in all groups. The level of liver damage was significantly lower in the group treated with GGA alone than in the other groups, with the value at the time of peak damage limited to approximately a surprising one third of that in the other groups. Serum TNFα was also significantly inhibited in the this group compared with the control group at 6 and 24 h after RFA (Figure 2).

The severity of histological damage due to RFA was investigated. HE staining of the liver parenchyma at the ablation margin showed numerous apoptotic cells presenting chromatin condensation, nuclear rupture, and condensation of the cytoplasm. Similarly, TUNEL staining showed numerous TUNEL-positive cells (Figure 3A and B). Using these results, the number of apoptotic cells in the liver parenchyma was compared, excluding the margin within 5 mm of the area of ablation. The number of TUNEL-positive cells was counted in 10 areas of 0.25-mm^2. The results are given as average±SE in Figure 3C. The appearance of apoptotic cells was significantly less frequent in the group treated with GGA alone than in the other groups. In each group, the greatest number of apoptotic cells appeared at 12 h after RFA. The range of ablation with a 1-cm Cool-tip needle has been shown histologically to be about 1.2×1.2 cm=1.44 cm^2 (18); no significant difference from this value was seen in the present study. In the three groups, the mean area of post-ablation coagulation necrosis was 1.58 cm^2 in the control, 1.37 cm^2 in the GGA-treated group, and 1.47 cm^2 in the group treated with GGA and quercetin, showing no clear, statistically significant difference.

Discussion

In the treatment of hepatocellular carcinoma, preservation of hepatic reserve and reduction of surgical invasiveness are important in terms of reducing postoperative complications. RFA and other local treatments, with which preservation of hepatic reserve can be expected, have a large role in the treatment of HCC, which often develops from chronic viral hepatitis or cirrhosis. Even with RFA, however, when hepatic reserve is low, the occurrence of postoperative complications remains sufficiently likely (2).

GGA is a gastric mucosal protectant that binds with mucous cells in the gastric mucosa and is already in use as an anti-ulcer drug for repair of the gastric mucosa. GGA is reported to have diverse effects, including actions to increase gastric mucosa, phospholipids, bicarbonate ion secretion, and gastric mucosal blood flow (7). Rokutan *et al*. first reported that GGA induces expression of HSP (7). Regarding the liver, there are reports of an organoprotective effect in a liver ischemia-reperfusion model and a model of massive hepatectomy (95%) in rats (15, 16), and of suppression of expression of inflammatory cytokines accompanying invasion in an experimental model (16, 17).

However, few studies have been conducted on HSP in relation to RFA. In the present experiment of liver RFA using rats, we found high levels of HSF1 and HSP70 expression from before RFA in the livers of the rats given GGA orally. Similarly to previously reported results on suppression of HSF1 and HSP70 in the liver with oral administration of quercetin, strong HSP expression inhibiting activity was shown. In addition, hepatic dysfunction was more strongly inhibited in the GGA-treated group than in the other groups, and TNFα in the blood was also significantly suppressed. TNFα expression was found to be biphasic, at 6 and 24 h postoperatively. While biphasic expression of TNFα in response to liver invasion has been seen in various reports to date, the possibility has been pointed out that is mainly involved in liver regeneration (22). In cases of RFA, as with hepatic resection, TNFα may promote liver regeneration.

In addition to regeneration and processing of denatured proteins, HSP70 blocks the apoptotic pathway and maintains the membrane potential and shape of mitochondria during stress. It is reported that HSP70 inhibits the release of cytochrome *c* and

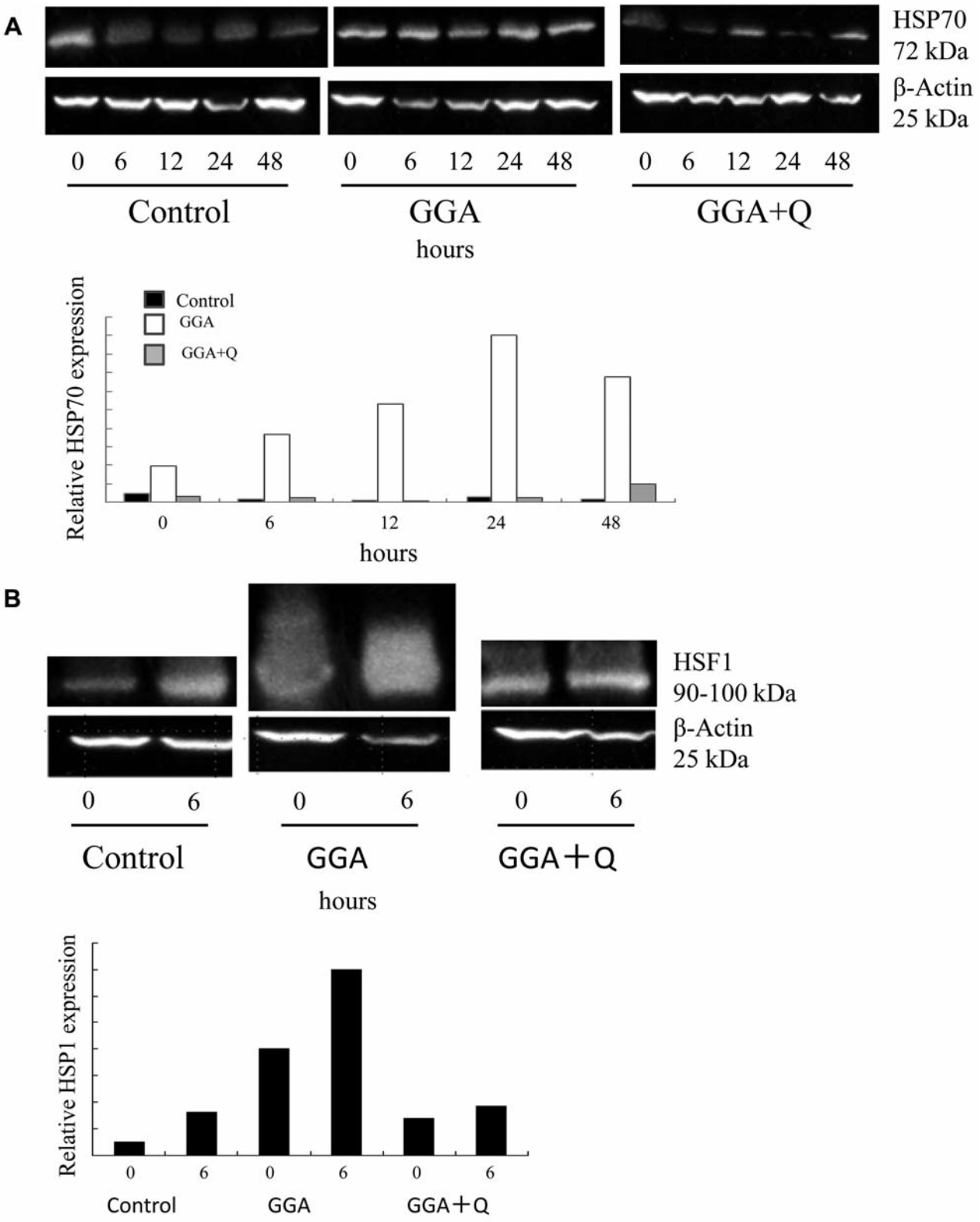

Figure 1. *Expression of heat-shock protein 70 (HSP70) (A) and heat-shock factor-1 (HSF1) (B) relative to that of β-actin in rats treated with radiofrequency ablation (RFA) and geranylgeranylacetone (GGA), alone and in combination with quercetin (GGA+Q), and controls. The group treated with GGA only had higher expression of HSP70 than the other groups. In the GGA+Q group,expression of HSP was inhibited. The group treated with GGA alone also had higher expression of HSF1, both pre- and postoperatively, compared with the other groups.*

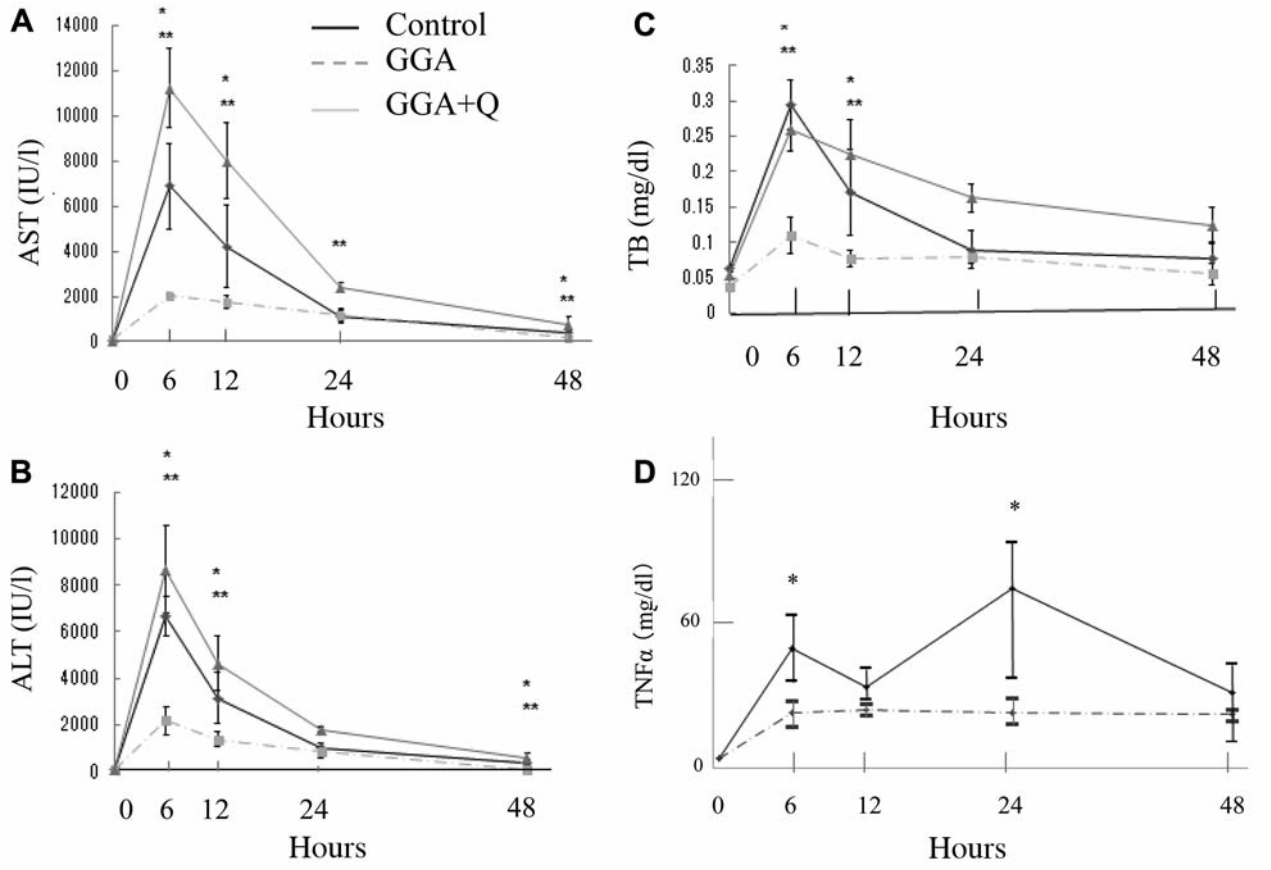

Figure 2. *Trends in serum values of aspartate aminotransferase (AST; A), alanine aminotransferase (ALT; B), total bilirubin (TB; C) and of the inflammatory cytokine tumor necrosis factor-alpha (TNFα; D) in rats with livers damaged by radiofrequency ablation (RFA). In all groups, peak liver damage was seen at 6 h after RFA. In the group treated with geranylgeranylacetone (GGA) alone, the level of liver damage was significantly inhibited compared with the other groups. Blood TNFα was significantly inhibited at 6 and 24 h postoperatively in this group compared with the controls. GGA+Q: GGA in combination with quercetin. Differences significant at p<0.05 for *GGA vs. control and **GGA vs. GGA+Q.*

apoptosis-inducing factor, inhibits the activation of c-JUN *N*-terminal kinases, binds to apoptotic protease-activating factor 1 and blocks the formation of its complexes, and inhibits the activation of procaspase 9 (23, 24). HSP70 is also reported to bind directly to apoptosis-inducing factor and inhibit its activation (25). In the present study, organoprotection of the liver or the cytoprotective effect may also have been obtained through the contribution of such a mechanism.

In the histological investigation, apoptosis of hepatocytes in the margin of the post-ablation necrosis resulting from RFA was assessed morphologically with HE stain and evaluated with TUNEL. Apoptosis of hepatocytes around the ablated liver parenchyma that was undergoing post-ablation coagulation necrosis was seen to be at the maximum at 12 h postoperatively in all three groups. In comparing the groups, apoptosis was found to be inhibited significantly in the group treated with GGA alone compared with the other groups. The group that also received oral quercetin, which inhibits the expression of HSP70, showed increased apoptosis compared with the group treated with GGA alone. The expression level in the combination-treated group was similar to that in the control group. Thus, it is possible that preconditioning with GGA before RFA may have caused HSP70 to be expressed within the liver and inhibited apoptosis of the liver parenchyma around the area of RFA. The size of the area ablated with RFA did not differ significantly between the three groups, and post-ablation necrosis was certain to occur even in livers with HSP induced by GGA.

In the present study, HSP was thought to give almost no protective effect against ablation. In fact, the temperature immediately after ablation reached 80˚C, a condition in which HSP is inactivated. Clinically, therefore, no remaining ablation area will likely exist if a needle of appropriate size is selected that matches the lesion-area to be ablated. The present findings suggest that, in addition to the conventional role of GGA as an anti-ulcer drug that repairs the gastric mucosa, GGA may

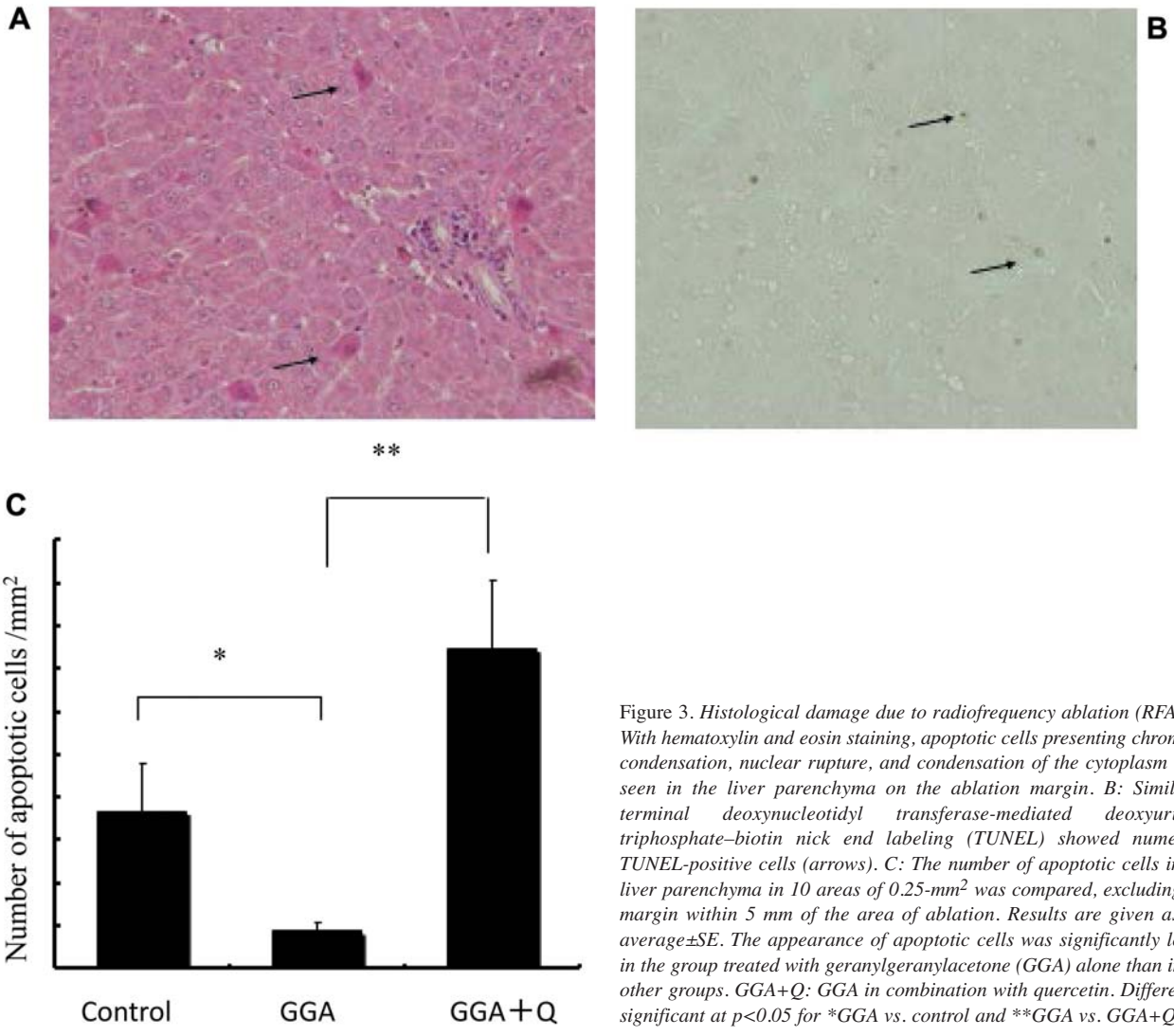

Figure 3. *Histological damage due to radiofrequency ablation (RFA). A: With hematoxylin and eosin staining, apoptotic cells presenting chromatin condensation, nuclear rupture, and condensation of the cytoplasm were seen in the liver parenchyma on the ablation margin. B: Similarly, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate–biotin nick end labeling (TUNEL) showed numerous TUNEL-positive cells (arrows). C: The number of apoptotic cells in the liver parenchyma in 10 areas of 0.25-mm^2 was compared, excluding the margin within 5 mm of the area of ablation. Results are given as the average±SE. The appearance of apoptotic cells was significantly lower in the group treated with geranylgeranylacetone (GGA) alone than in the other groups. GGA+Q: GGA in combination with quercetin. Differences significant at p<0.05 for *GGA vs. control and **GGA vs. GGA+Q.*

also be expected to induce HSP in the liver and exert a strong protective effect against liver damage. However, there is a problem with regard to the appropriate dosage of GGA. In a rat experimental model, a dose of 100-200 mg/kg was used; this value converted to a human dose would be a massive dose of 6-12 g. However, there is one report that lower doses are sufficient for humans (26). Another report noted a powerful effect with continuous administration of low doses (26). There would thus seem to be a strong possibility that GGA can be applied clinically. There is an ongoing search for non-toxic molecular chaperone inducers other than GGA, such as zinc L-carnosine, polaprezinc (27) or rebamipide (28). The development of new drugs that induce stronger organo- and cytoprotective effects is anticipated.

Conclusion

We have shown in an experimental rat model that expression of HSP70 is promoted in the liver with administration of GGA, reducing damage to hepatocytes. Induction of HSP in the liver by GGA may be clinically applicable. The present findings suggest that it may be possible to broaden treatment options. Liver failure, as well as various other complications, could be avoided if the protective effect on the liver could be optimized. This might be accomplished through preoperative administration of GGA in the treatment of HCC with severe liver damage or liver metastasis of colonic cancer, which often occurs at multiple sites within the liver.

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