Protective effects of fermented rice vinegar sediment (Kurozu moromimatsu) in a diethylnitrosamine-induced hepatocellular carcinoma animal model

Toru Shizuma,1,* Kazuo Ishiwata,1 Masanobu Nagano,2 Hidezo Mori1 and Naoto Fukuyama1

1Department of Physiology, School of Medicine, Tokai University, 143, Shimokasuya, Isehara, Kanagawa 259-1193, Japan
2Sakamoto Kurozu Inc., 21-15, Uenosonochou, Kagoshimashi, Kagoshima 890-0052, Japan

(Received 14 September, 2010; Accepted 25 October, 2010; Published online 17 June, 2011)

Kurozu moromimatsu is the sediment of Kurozu, a jar-fermented Japanese black vinegar produced from unpolished rice. Here, we examined the protective effects of Kurozu moromimatsu in a diethylnitrosamine-induced model of hepatocellular carcinoma. Thirty-two F344 rats were divided into two groups; the control group received basal CE-2 diet, and the Kurozu moromimatsu group received CE-2 diet containing Kurozu moromimatsu. At 16 weeks after initial intraperitoneal administration of diethylnitrosamine (150 mg/kg/week), serum was collected from half the rats. These rats were sacrificed and the liver was resected for histological examination of hematoxylin-eosin-stained sections and assay of matrix metalloproteinase-2 and matrix metalloproteinase-9 levels in tumor tissues. Glutathione S-transferase placental form-positive foci were evaluated by immunostaining for glutathione S-transferase placental form-positive areas. The remaining rats were maintained for evaluation of survival. There were no significant differences of serum transaminases, tumor necrosis factor-alpha, and also no marked hepatic histological differences, between the two groups. However, the size of hepatocellular carcinomas was greatly decreased and the levels of activated matrix metalloproteinase-2 and -9 were significantly reduced in the Kurozu moromimatsu group. Further, survival was significantly prolonged in the Kurozu moromimatsu group compared with the control. These results indicate that Kurozu moromimatsu inhibited the growth of hepatocellular carcinoma.

Key Words: Kurozu moromimatsu, hepatocellular carcinoma

Kurozu is a traditional black vinegar produced from unpolished rice and has been widely used in Japan as seasoning for food or as a health supplement. Kurozu is produced mainly in Kagoshima, Japan, by fermentation of vinegar with several bacilli for more than one year in earthenware jars. The supernatant is Kurozu-M (Tokyo, Japan) and bred under specific pathogen-free conditions at a room temperature of 24–25°C, under constant humidity, with a 12-h light/dark cycle. The rats were randomized into two groups; the control group received basal CE-2 diet (CLEA Japan Inc., Tokyo, Japan) (n = 16) and the Kurozu-M group received CE-2 diet including 2% Kurozu-M (Sakamoto Kurozu Inc., Kagoshima, Japan) (n = 16). The volume of Kurozu-M added to the diet of rats was chosen based on the typical volume of ingestion by humans, adjusted for body weight. Basal CE-2 diet and special diet

*To whom correspondence should be addressed.
E-mail: shizuma@is.isc.u-tokai.ac.jp

Materials and Methods

Experimental animal model. The experimental procedures were approved by the Animal Experimentation Committee, School of Medicine, Tokai University, Japan. Thirty-two F344 male rats (8 weeks of age) were obtained from CLEA Japan Inc. (Tokyo, Japan) and bred under specific pathogen-free conditions at a room temperature of 24–25°C, under constant humidity, with a 12-h light/dark cycle. The rats were randomized into two groups; the control group received basal CE-2 diet (CLEA Japan Inc., Tokyo, Japan) (n = 16) and the Kurozu-M group received CE-2 diet including 2% Kurozu-M (Sakamoto Kurozu Inc., Kagoshima, Japan) (n = 16). The volume of Kurozu-M added to the diet of rats was chosen based on the typical volume of ingestion by humans, adjusted for body weight. Basal CE-2 diet and special diet

doi: 10.3164/jcbn.10-112
©2011 JCBN
including Kurozu-M were started from a week before the initial administration of DEN (Sigma, St. Louis). Administrations of DEN (150 mg/kg/week) were performed by intraperitoneal injection 3 times during 3 weeks. The concentration of iron in basal CE-2 diet was 32.2 mg/100 g (data from manufacturer) and that of CE-2 diet containing 2% Kurozu-M, used as the experimental diet in this study, was calculated to be 31.7 mg/100 g (based on iron concentration of 4.8 mg/100 g in Kurozu-M).

Preliminary examinations showed that liver tumors more than 10 mm in size at the major axis were present in all rats (n = 5) of the control group at 12 weeks after initial administration of DEN.

**Results**

**Serum examinations.** Levels of serum albumin (g/dl) were 4.3 ± 0.2 in the control group and 4.2 ± 0.2 in the Kurozu-M group. Levels of serum AST/ALT (IU/l) were 236 ± 67/206 ± 51 in the control group and 209 ± 64/183 ± 49 in the Kurozu-M group. There was no significant difference in the levels of albumin or AST/ALT between the two groups. Moreover, the levels of serum hyaluronic acid (ng/ml) were 37.8 ± 3.8 in the control group, and 35.1 ± 2.8 in the Kurozu-M group. There was no significant difference in the level of hyaluronic acid between the two groups.

As regards the levels of cytokines, the levels of TNF-α (pg/ml) were 3.4 ± 1.7 in the control group and 3.1 ± 1.8 in the Kurozu-M group. Levels of IL-2 (U/ml) were 1.6 ± 0.6 in the control group and 1.4 ± 0.4 in the Kurozu-M group. There were no significant differences in the levels of either of the cytokines between the two groups.

**Histology and GST-P staining.** In macroscopic examination of the resected liver, white tumors with a size of more than 10 mm at the major axis were apparent in all rats in the control group (Fig. 1). Such large tumors were not found in the Kurozu-M group. In microscopic examination of HE-stained liver sections, focal necrosis of liver cells and invasion of inflammatory cells in portal areas (corresponding to mild chronic hepatitis in humans) were found in both groups. However, fibrosis or piecemeal necrosis was not obvious. Moreover, there were no marked background changes of liver tissues in the two groups (Fig. 2a and b).

As for GST-P staining, the numbers of GST-P-positive foci were 12.2 ± 6.8 in the control group, and 10.7 ± 5.3 in the Kurozu-M group. There was no significant difference between the two groups. However, large HCC with a diameter of more than 10 mm was noted in liver in all animals of the control group. On the other hand, in the Kurozu-M group, no lesion of more than 3 mm in diameter was found in any liver. The size (mm) of GST-P-positive foci in the Kurozu-M group (0.89 ± 0.46) was significantly (p < 0.001) reduced compared with the control (7.35 ± 5.31) (Fig. 3 and 4).

**MMP-2 and MMP-9 levels in tumor tissues.** Total MMP-2 amounted to 6.7 ± 2.6 ng/mg protein in the control group and 3.6 ± 1.7 ng/mg protein in Kurozu-M group. The corresponding values for activated MMP-2 were 0.28 ± 0.12 ng/mg protein and 0.12 ± 0.06 ng/mg protein, respectively. Total MMP-9 amounted to 20.6 ± 4.6 ng/mg protein in the control group and 12.0 ± 2.7 ng/mg protein in Kurozu-M group. The corresponding values for activated MMP-9 were 1.17 ± 0.38 ng/mg protein and 0.51 ± 0.10 ng/mg protein, respectively.

**Histology and immunostaining.** Immediately after collection of the liver, the same rats (each group; n = 8) were sacrificed under anesthesia induced with isoflurane (Wako Pure Chemicals, Osaka, Japan) and the livers were resected. The presence of liver tumors was confirmed macroscopically. Moreover, microscopic examination of liver tissues by hematoxylin-eosin (HE) staining showed no obvious differences in the levels of either of the cytokines between the two groups.
0.19 ng/mg protein, respectively. Total and activated MMP-2 and MMP-9 levels were all significantly \((p<0.001)\) reduced in the Kurozu-M group compared with the control group (Fig. 5).

**Survival.** In the control group, all 8 rats died within 36 weeks after initial DEN administration. On the other hand, the survival rate in the Kurozu-M group was 37.5% (3/8) at 48 weeks. The survival rate in the Kurozu-M group was significantly higher than that in the control group \((p<0.001)\) (Fig. 6).

**Discussion**

Kurozu-M treatment markedly inhibited growth of HCC and improved survival in the DEN-induced HCC animal model. One of the mechanisms of its anti-cancer effects was considered to be suppression of activated MMP-2 and MMP-9, which degrade the extracellular matrix, thereby promoting the growth and metastasis of various solid cancers.

Occurrence of HCC is generally associated with liver fibrosis or inflammatory changes. Clinically, chronic hepatitis or liver cirrhosis (LC) with HCV infection often leads to HCC. For example, the occurrence rate of HCC in LC patients with HCV infection is 6–8%/year in Japan.\(^{12}\) In patients with hepatitis, inflammation and regeneration in the liver are associated with...
development of HCC,(13) though exceptionally, HCC rarely develops in patients with autoimmune hepatitis. However, our study revealed that the extents of histological liver fibrosis or inflammatory change, as well as the serum levels of transaminases or hyaluronic acid as a marker of liver fibrosis, were not significantly different between the Kurozu-M group and the control. Therefore, inhibition of HCC growth in the Kurozu-M group in this study was not associated with differences in background factors. It has been reported that iron overload, which induces oxidative stress, aggravates liver diseases(14) and increases the risk of malignancy.(15) However, the levels of iron in the diets of the control and Kurozu-M groups were essentially the same in this study.

Increased levels of the gelatinases; MMP-2 and MMP-9 are considered prognostic for solid cancers, including HCC.(16) In fact, it has been suggested that inhibition of gelatinases can suppress development of HCC in an animal model.(17) Since activated MMP-2 promotes angiogenesis,(18) suppression of MMP-2 and MMP-9 by Kurozu-M may have led to inhibition of HCC growth in our model. We found that the number of GST-P-positive foci (which were estimated foci corresponding to low-grade dysplastic nodules) in the Kurozu-M group showed no difference from the control, but the size of developed GST-P-positive foci was greatly decreased. Thus, a possible explanation is that Kurozu-M inhibited angiogenesis, which may not greatly influence the initial progression from premalignant tumor, but may be an important promoting factor at the stage of high-grade dysplastic nodules or early HCC. This would be consistent with our findings.

Activation of MMPs is important for carcinogenesis. MMP-2 is activated by membrane type (MT)-1-MMP(19) It is also activated by peroxylnitrite(20) which exhibits cytotoxicity via direct oxidation of sulphydryl groups, leading to severe tissue damage. We have already reported that Kurozu-M inhibits nitrosyrgene generation in colon cancer cells.(13) Nitrosyrgene is produced in vivo via two pathways, i.e., from the reaction of tyrosine with peroxylnitrite, which is generated from O$_2^-$ and nitric oxide (NO),(21) and from the reaction of tyrosine with nitrite, catalyzed by myeloperoxidase (MPO).(22) Therefore, Kurozu-M might reduce MMP-2 activation by inhibiting the generation of peroxylnitrite. In addition, MMP-9 is activated by MMP-2,(23) as well as by several cytokines.(24) However, in our study, serum levels of TNF-α and IL-2 were not increased.

The active component of Kurozu-M remains to be identified. Acetic acid, which is the main component of Kurozu, is not included in Kurozu-M. However, Kurozu-M contains amino acids, oligopeptide, organic materials including metabolites of lactic acid or Koji molds, minerals, saccharides, lipids, etc. Candidate active components include branched chain amino acid (BCAA), which are widely used for the treatment of protein malnutrition in LC patients. It was reported that BCAA suppressed insulin-resistance-based carcinogenesis in diabetic rats,(7) and suppressed angiogenesis in a chemical carcinogenesis model.(7) Furthermore, various other amino acids have anti-oxidative activity, acting as radical scavengers.(25) Therefore, various amino acids may suppress the generation of O$_2^-$ or peroxylnitrite, thereby suppressing activation of MMP-2. On the other hand, oligosaccharides are also candidate active components, because oligosaccharides from agar suppressed NO generation in vitro.(26) Oligosaccharides also reduced the activity of inducible NO synthase (iNOS), which might lead to a decrease of peroxylnitrite generation. Organic materials, including metabolites of lactic acid or Koji formed in the earthenware jars are also potential anti-oxidants.(26) and anti-cancer agents such as bleomycin or mitomycin C or their derivatives are known to be produced as bacterial metabolites.
In conclusion, Kurozu-M inhibited the growth of HCC in a DEN-induced HCC animal model and prolonged survival. The active component(s) has not yet been identified, but our results suggest that Kurozu-M may be beneficial as a dietary supplement in humans.

Acknowledgments

This work was supported by grants in 2009 Tokai University School of Medicine Research Aid, 2009 and 2010 Grant-in-Aid for Scientific Research in Japan Society for the Promotion of Science (No. 21659295 and No. 22659106) and 2009 Grant-in-Aid for Japanese Society for Parenteral and Enteral Nutrition.

References

1. Nanda K, Miyoshi N, Nakamura Y, et al. Extract of vinegar “Kurosu” from unpolished rice inhibits the proliferation of human cancer cells. J Exp Clin Cancer Res 2004; 23: 69–75.

2. Shimoji Y, Kohno H, Nanda K, et al. Extract of Kurosu, a vinegar from unpolished rice, inhibits azoxymethane-induced colon carcinogenesis in male F344 rats. Nutr Cancer 2004; 49: 170–173.

3. Fukuyama N, Jujo S, Ito I, and et al. Kurozu moromimatsu inhibits tumor growth of Lovo cells in a mouse model in vivo. Nutrition 2007; 23: 81–86.

4. Masterton GS, Hayes PC. Coffee and the liver: a potential treatment for liver disease? Eur J Gastroenterol Hepatol 2010; 22: 1277–1283.

5. Bravi F, Bosetti C, Tavani A, La Vecchia C. Coffee drinking and hepatocellular carcinoma: an update. Hepatology 2009; 50: 1317–1318.

6. Weng CJ, Chau CF, Yen GC, Liao JW, Chen DH, Chen KD. A traditional Japanese medicine Kurozu moromimatsu inhibits tumor growth of HCC in a rat hepatic carcinogenesis model. Anticancer Res 2009; 29: 2301–2305.

7. Ohta M, Konno H, Tanaka T, and et al. Effect of combination therapy with matrix metalloproteinase inhibitor MMI-166 and mitomycin C on the growth and liver metastasis of human colon cancer. Jpn J Cancer Res 2001; 92: 688–695.

8. Muñoz-Nájara UM, Neurath KM, Vumbaca F, Claffey KP. Hypoxia stimulates breast carcinoma cell invasion through MT1-MMP and MMP-2 activation. Oncogene 2006; 25: 2379–2392.

9. Murugan RS, Vinothini G, Hara Y, Nagai S. Black tea polyphenols target like growth factor binding protein-5 is a target of matrix metalloproteinase-7: implications for epithelial-mesenchymal signaling. Hum Pathol 2005; 36: 245–253.

10. Hemers E, Duval C, McCaig C, Handley M, Dockray GJ, Varro A. Insulin-like growth factor binding protein-5 is a target of matrix metalloproteinase-7: implications for epithelial-mesenchymal signaling. Cancer Res 2005; 65: 7363–7369.

11. Ignarro LJ, Buga GM, Wood KS, and Chaudhuri G. Role of endogenous nitric oxide in the relaxing response to increased luminal flow of canine coronary arteries. Circ Res 1988; 63: 54–61.

12. Hemers E, Duval C, McCaig C, Handley M, Dockray GJ, Varro A. Insulin-like growth factor binding protein-5 is a target of matrix metalloproteinase-7: implications for epithelial-mesenchymal signaling. Cancer Res 2005; 65: 7363–7369.

13. Tsuchikami H, Ishida K, and et al. Comparison of outcomes between patients with alcoholic cirrhosis and those with hepatitis C virus-related cirrhosis. J Gastroenterol 2004; 39: 676–685.

14. Miyakawa K, Tarao K, Ohshige K, and et al. High serum alanine aminotransferase levels for the first three successive years can predict very high incidence of hepatocellular carcinoma in patients with Child Stage A HCV-associated liver cirrhosis. Scand J Gastroenterol 2009; 44: 1340–1348.

15. Asare GA, Kew MC, Mossanda KS, Paterson AC, Siziba K, Kahler-Venter CP. Effects of exogenous antioxidants on dietary iron overload. J Clin Biochem Nutr 2009; 44: 85–94.

16. Tsurupeniemi-Hujanen T. Gelatinases (MMP-2 and -9) and their natural inhibitors as prognostic indicators in solid cancers. Biochimie 2005; 87: 287–297.

17. Murugan RS, Vinothini G, Hara Y, Nagai S. Black tea polyphenols target matrix metalloproteinases, RECK, proangiogenic molecules and histone deacetylase in a rat hepatocarcinogenesis model. Anticancer Res 2009; 29: 2301–2305.

18. Ohta M, Konno H, Tanaka T, and et al. Effect of combination therapy with matrix metalloproteinase inhibitor MMI-166 and mitomycin C on the growth and liver metastasis of human colon cancer. Jpn J Cancer Res 2001; 92: 688–695.

19. Muñoz-Nájara UM, Neurath KM, Vumbaca F, Claffey KP. Hypoxia stimulates breast carcinoma cell invasion through MT1-MMP and MMP-2 activation. Oncogene 2006; 25: 2379–2392.

20. Migita K, Maeda Y, Aibara S, and et al. Peroxynitrite-mediated matrix metalloproteinase-2 activation in human hepatic stellate cells. FEBBS Lett 2005; 579: 3119–3125.

21. Ahmad R, Rasheed Z, Ahsan H. Biochemical and cellular toxicity of peroxynitrite: implications in cell death and autoimmune phenomenon. ImmunopharmacolImmunotoxicol 2009; 31: 388–396.

22. Eisier JP, Hristova M, Cross CE, and et al. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. Nature 1998; 391: 393–397.

23. Wittrant Y, Theoleyre S, Couillaud S, Dunstan C, Heymann D, Redini F. Relevance of an in vitro osteoclastogenesis system to study receptor activator of NF-κB ligand and osteoprotegerin biological activities. Exp Cell Res 2004; 293: 292–301.

24. Steinbrenner H, Ramos MC, Stuhlmann D, Mitic D, Sies H, Bremesien P. Tumor promoter TPA stimulates MMP-9 secretion from human keratinocytes by activation of superoxide-producing NADPH oxidase. Free Radic Res 2005; 39: 245–253.

25. Wu G. Amino acids: metabolism, functions, and nutrition. Amino Acids 2009; 37: 1–17.

26. Enoki T, Okuda S, Kudo Y, Takashima F, Sagawa H, Kato I. Oligosaccharides from agar inhibit pro-inflammatory mediator release by inducing heme oxygenase 1. Biosci Biotechnol Biochem 2010; 74: 766–770.

27. Daddaoua A, Puerta V, Requena P, and et al. Goat milk oligosaccharides are anti-inflammatory in rats with hapten-induced colitis. J Nutr 2006; 136: 672–676.

28. Fukuda Y, Tao Y, Tomita T, and et al. A traditional Japanese medicine mitigates TNBS-induced colitis in rats. Scand J Gastroenterol 2006; 41: 1183–1189.

Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| Kurozu-M | Kurozu moromimatsu |
| HCC | hepatocellular carcinoma |
| HBV | hepatitis B virus |
| MMP | matrix metalloproteinase |
| DEN | diethylaminoethylamine |
| TNF | tumor necrosis factor |
| IL | interleukin |
| HE | hematoxylin-eosin |
| GST-P | glutathione S-transferase placental form |
| APMA | aminophenylmercuric acetate |
| LC | liver cirrhosis |
| MT | membrane type |
| NO | nitric oxide |
| MPO | myeloperoxidase |
| BCAA | branched chain amino acid |
| iNOS | inducible nitric oxide synthase |

©2011 JCBN