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N-acetylcysteine and *S*-methylcysteine inhibit MeIQx rat hepatocarcinogenesis in the post-initiation stage

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***N*-acetylcysteine (NAC) and *S*-methylcysteine (SMC), water soluble organosulfur compounds contained in garlic, were evaluated for chemoprevention of hepatocarcinogenesis after 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) initiation in rats. Intergastric treatment with NAC or SMC five times a week resulted in decreased numbers and areas of preneoplastic, glutathione *S*-transferase placental form (GST-P) positive foci of the liver in a dose-dependent manner. Moreover, cell proliferation was reduced in GST-P positive foci by NAC and SMC. Insulin-like growth factor I (IGF-I) and inducible nitric oxide synthase (iNOS) mRNA expressions were found downregulated in the liver by NAC. The studies indicate that NAC can serve as a chemopreventive agent for rat hepatocarcinogenesis induced by MeIQx by reducing cell proliferation, which may involve IGF-I and iNOS downregulation.**

Introduction

An understanding of how cancer may be prevented is one key objective of research. This can be achieved to some extent by avoiding exposure to carcinogens, increasing host defenses through immunologic responses, modifying lifestyle or using chemopreventive agents. The latter is a promising strategy for cancer control, but depends on the discovery of naturally occurring or synthetic compounds that can suppress or prevent the processes leading to tumor development (1–7). For implementation of chemoprevention, it is essential not only to identify good candidate agents but also to evaluate their safety and efficacy using a variety of methodologies and to elucidate their mechanisms of action.

Abbreviations: NAC, *N*-acetylcysteine; SMC, *S*-methylcysteine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; GST-P, glutathione *S*-transferase placental form; IGF-I, insulin-like growth factor I; iNOS, inducible nitric oxide synthase; OSC, organosulfur compound; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; AgNORs, silver-stained for nucleolar organizer regions; IGFBP-3, insulin-like growth factor binding protein 3; GAPDH, glyceraldehyde-3-*P* dehydrogenase; GH, growth hormone.

N-acetylcysteine (NAC), an organosulfur compound (OSC) in garlic, is considered to be one of the most promising cancer chemopreventive agents. Efficacy of NAC has been demonstrated in various experiments *in vitro* and *in vivo* (8), the compound significantly decreased the incidences of neoplastic and preneoplastic lesions in the lung, trachea, colon, liver, mammary gland, Zymbal gland, bladder and skin induced by a variety of chemical carcinogens in rodents (mice, rats, hamsters) (9). It has been reported that the protective mechanism of NAC against chemical carcinogenesis is due to its dual role as a nucleophile and as a -SH residue donor. In addition, NAC can prevent *in vivo* formation of carcinogen–DNA adducts and reactive oxygen species, and inhibit cell proliferation, prostaglandin synthesis and ornithine decarboxylase (ODC) activity. NAC exerts antioxidant, antimutagenesis and anticarcinogenesis activities, and has been clinically tested as a chemopreventive agent in USA and Europe (8–10).

In our previous study, *S*-methylcysteine (SMC), another OSC in garlic, was examined for its modifying effects on diethylnitrosamine-induced liver neoplasia in rats using a medium-term bioassay (Ito test) based on the two-step model of hepatocarcinogenesis (11,12). We have found that SMC significantly reduced diethylnitrosamine-induced putative preneoplastic lesions, glutathione *S*-transferase placental form (GST-P) positive foci and cell proliferation in rat liver.

Various cooked foods contain a variety of mutagenic and carcinogenic compounds, including heterocyclic amines such as 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx). Humans are exposed to MeIQx in daily life, and daily exposure is estimated to be in the range of 0.2–2.6 μg/person (13). Experimentally MeIQx induces mainly liver tumors in mice and rats (14), with formation of DNA adducts (15,16).

In the present study, we investigated the modifying effects of NAC and SMC on rat hepatocarcinogenesis after initation with MeIQx, which is categorized as a possible human carcinogen (13), and then focused on possible mechanisms underlying the observed inhibition.

Materials and methods

Chemicals

NAC (purity min. 99%) was purchased from Sigma Chemical Co. (St Louis, MO), and SMC (purity min. 99%) was kindly provided by Wakunaga Pharmaceutical Co., Ltd. (Osaka, Japan). The chemical structures are shown in Figure 1. MeIQx (purity min. 99.8%) was purchased from Nard Institute (Osaka, Japan).

Animals

For all experiments, 20-day-old male F344 rats were purchased from Charles River Japan, Inc. (Atsugi, Kanagawa, Japan). They were housed in an air-conditioned room at a targeted temperature of 23 ± 1°C and relative humidity

of 36 ± 6%, and with a 12 h light and 12 h dark cycle. MeIQx was added to MF basal pellet diet (Oriental Yeast Co., Tokyo, Japan) at a concentration of 100 p.p.m. Rats were given tap water and the pelleted diet *ad libitum*. The animals were handled in accordance with the Guide for the Care and Use of Animals (Osaka City University Medical School).

Treatments

Experiment 1. The experimental design is shown in Figure 2A. In this experiment, we studied the modifying effects of post-initiation treatment of NAC and SMC on rat MeIQx-induced hepatocarcinogenesis. Forty-five rats (21 day old) were randomly divided into three groups (15 rats in each group). They received MeIQx at a dose of 100 p.p.m. in the diet for the first nine weeks, and then were administered basal diet. Rats were given NAC (Groups 1 and 4) or SMC (Groups 2 and 5) at a dose of 100 mg/kg body wt dissolved in saline (4 ml/kg body wt) by intragastric administration, five times/week from weeks 10 to 24. Animals in Group 3 received only saline (4 ml/kg body wt) with the same schedule as the controls. All rats were killed under ether anesthesia at the end of week 24. Their livers were examined immunohistochemically for GST-P positive foci as endpoint marker lesions of hepatocarcinogenesis and silver-stained for nucleolar organizer regions (AgNORs) to examine characters of cell proliferation. Because AgNOR staining has been suggested to be a useful marker of cell proliferation (17) and AgNOR analysis bears characterization of cell kinetic and metabolic activity of cells (18), the significant increase of AgNORs in the promotion period of carcinogenesis reveals the potential value of cellular alteration (19).

Experiment 2. To examine whether inhibitory effects on GST-P positive foci development depend upon the dose of NAC or SMC, 125 rats, 21 day old, were randomly divided into seven groups (15 rats in each group) (Figure 2A). All rats received MeIQx at a dose of 100 p.p.m. in the diet for the first nine weeks, and then were administered basal diet and NAC (Groups 1–3) or SMC (Groups 4–6) at doses of 100, 30 or 10 mg/kg body wt dissolved in saline (4 ml/kg body wt) by intragastric administration five times/week from weeks 10–24 (Figure 2A). Group 7 received only saline (4 ml/kg body wt), as the control. All rats were killed under ether anesthesia at the end of week 24. Their livers were examined immunohistochemically for GST-P positive foci, and insulin-like growth factor I (IGF-I) levels in blood samples from abdominal aorta.

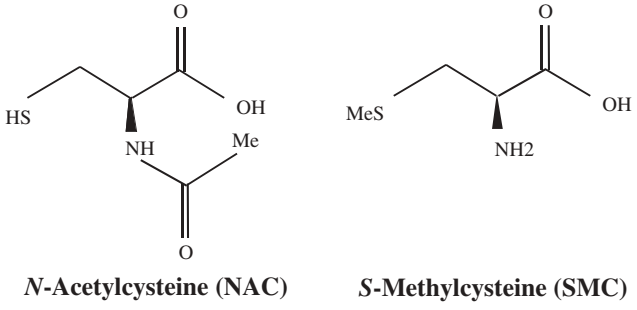

Fig. 1. *N*-acetyl-cysteine (NAC) and *S*-methylcysteine (SMC), water soluble organic sulfur compounds examined in this study.

Experiment 3. To investigate mechanisms underlying the observed inhibitory effects of NAC on rat hepatocarcinogenesis (Figure 2B), 21 day-old rats were divided into three groups of 15 rats each, two of which were given MeIQx, 100 p.p.m. in the diet for five weeks. Then Group 1 received NAC (at dose of 100 mg/kg body wt) dissolved in saline (4 ml/kg body wt) by i.g. five times/week, while Group 2 was given saline (4 ml/kg body wt) by i.g. five times/week during weeks 6–12. Group 3 received only saline (4 ml/kg body wt) by the same schedule without MeIQx, as a control. Five rats in each group were killed under ether anesthesia at weeks 6, 8 and 12 (weeks 0, 2 and 6 after NAC treatment). Blood was sampled from the abdominal aorta of the rats for determination of IGF-I and insulin-like growth factor binding protein 3 (IGFBP-3). Liver samples were frozen in liquid nitrogen and stored at −80°C to determine the expression levels of IGF-I, p53, p21, inducible nitric oxide synthase (iNOS) mRNAs and 8-hydroxy-2′-deoxyguanosine (8-OHdG).

Immunohistochemical staining for GST-P and AgNORs in the liver

The livers of the rats ($n = 15$) in experiments 1 and 2 were fixed in 10% phosphate-buffered formalin and embedded in paraffin wax. GST-P immunohistochemistry was performed using the avidin–biotin–peroxidase complex method as described by Takada *et al*. (12). The numbers of GST-P positive foci (≤2 cell) were counted microscopically, their areas and the total areas of liver sections were measured using a color video image processor (IPAP; Sumika Technos, Osaka, Japan).

AgNORs staining was performed as double stainings with GST-P. To allow comparison, GST-P was visualized with alkaline phosphatase-labeled avidin–biotin complex and the new fuchsin substrate system (Dako Japan, Kyoto, Japan) (20). For the AgNORs staining, sections were treated with an acetic acid–ethanol (1:3) mixture for 5 min and rinsed with absolute ethanol. Thereafter, sections were pretreated with absolute alcohol then 70% ethanol and rinsed in distilled water. Silver colloid solution was prepared by dissolving 2 g/dl gelatine in 1 g/dl aqueous formic acid and mixing this with 50% (w/w) aqueous silver nitrate solution. Specimens were allowed to react with silver colloid for 30–40 min in a darkroom and finally washed with distilled water. Then they were fixed with 5% sodium thiosulfate for 5 min. For all specimens, the number of AgNORs dots/cells was randomly counted for 500 hepatic cells in GST-P positive cell foci.

Determination of 8-OHdG formation in liver DNA

The 8-OHdG formation levels in DNA were measured using frozen liver samples of rats ($n = 5$) at weeks 6 and 8 (before and 2 weeks after starting NAC administration, respectively) following MeIQx initiation in experiment 3. Rat liver DNA 8-OHdG levels were determined using a HPLC-ECD method (21).

Measurement of IGF-I and IGFBP-3 in plasma

To measure the plasma levels of IGF-I and IGFBP-3 proteins, blood samples in experiments 2 and 3 were centrifuged (5 min at 3000 r.p.m.). Then, skims were separated and stored at −80°C. IGF-I and IGFBP-3 levels were detected with IRMA assay method kits [Somatmedin C·II, Bayer (22,23) and Ab tube IGFBP-3, Diagnostic Systems Laboratories, Inc. Tex, respectively].

Measurement of p53, p21 and IGF-I mRNAs in rat liver by northern blotting

In experiment 3, 20 μg aliquots of total RNA from each liver sample of rats ($n = 5$) that were killed at week 12 (after 6 weeks on NAC), were fractionated

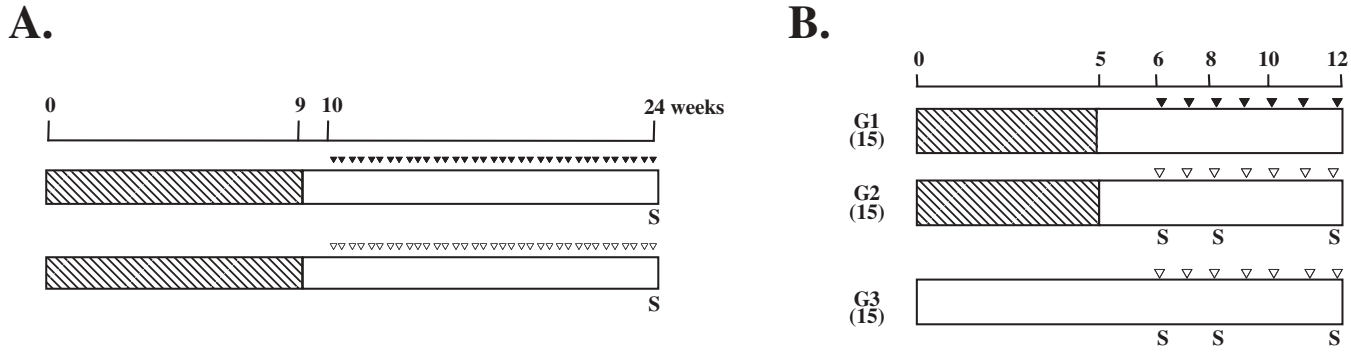

Fig. 2. (**A**) Design in experiments 1 and 2. Striped area, MeIQx, 100 p.p.m in diet; open area, basal diet; closed inverted triangle, NAC or SMC (100 mg/kg body wt in experiment 1, respectively; 100, 30, 10 mg/kg body wt in experiment 2, respectively), i.g., 5 times per week, open inverted triangle, saline, i.g., 5 times per week; S, sacrifice ($n = 15$). (**B**), Design in experiment 3. Striped area, MeIQx, 100 p.p.m om diet; open area, basal diet; closed inverted triangle, G1: NAC, 100 mg/kg body wt, i.g., 5 times per week, open inverted triangle, G2: saline, 4 ml/kg body wt, i.g., 5 times per week; S, sacrifice ($n = 15$).

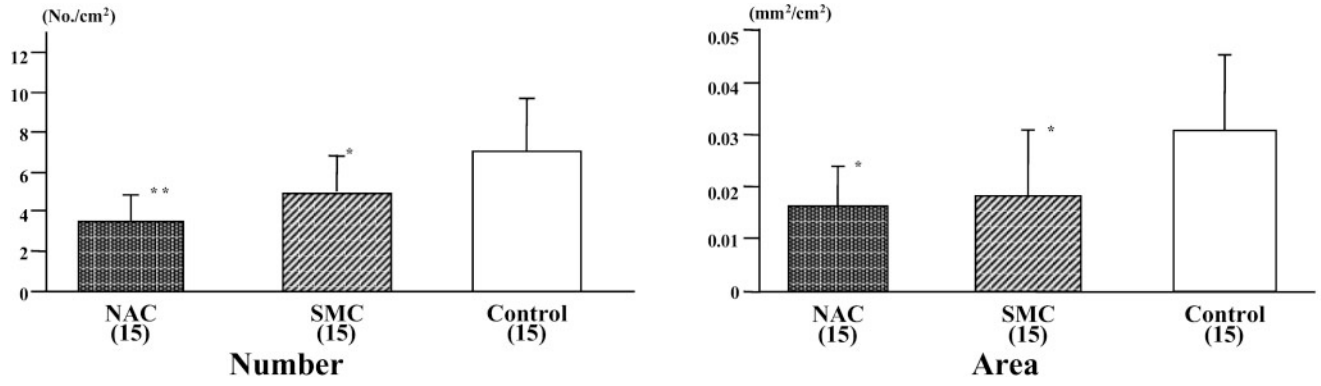

Fig. 3. Numbers and areas of GST-P positive foci in the liver of rats treated with MeIQx followed by NAC, SMC or saline (groups 1, 2 and 3) (experiment 1). *, **Significantly different from the control at $P < 0.01$ and $P < 0.001$.

Table I. Primer nucleotide sequence used for PCR in northern blotting

Target genes	Nucleotide sequence	Orientation
p53	5′-CAGCGACAGGGTCACCATAT-3′	sense
	5′-TTTATGGCGGGACGTAGACT-3′	antisense
p21	5′-CTGGTGATGTCCGACCTGTTC-3′	sense
	5′-CTCCTGACCCACAGCAGAAGA-3′	antisense
IGF-I	5′-GCTGGTGGACGCTCTTCAGTT-3′	sense
	5′-AGCCTCCTCAGATCACAGCTC-3′	antisense

on 1% agarose gel and transferred to Hybond nylon membranes. Primers for PCR analysis were designed as follows (Table I). The design of *IGF-I* primers was as per protocol described in Arkins *et al*. (24). The membranes were hybridized with the [^{32}P] dCTP-labeled probe system (Amersham International plc., Little Chalfont, Buckinghamshire) according to the manufacturer's instructions. After hybridization, the membranes were washed, and signals were detected by autoradiography and quantified using an image analyzer (BAS 2500 II, Fuji, Tokyo, Japan).

Standards for real-time PCR

Using RNAs extracted from the livers of rats without treatment as templates, iNOS (25) and glyceraldehyde-3-*P* dehydrogenase (GAPDH) (25) were amplified by RT–PCR and subcloned in pT-AdV vector plasmid using an AdvanTAge^{TM} PCR cloning Kit (Clontech Laboratories, Inc.). Finally, amplified plasmids were isolated with a QIAGEN Plasmid Mini Kit (Funakoshi Co., Ltd. Tokyo, Japan), measured in a spectrophotometer, and concentrations calculated. Sequence analysis was performed to check the quality of the plasmid obtained. Serial dilutions ranging from 10^9–10^2 molecules were then prepared.

Light Cycler real-time quantitative (LC-RT) PCR

PCR for iNOS and the internal control, GAPDH, was performed using single pairs of primers and fluorescent probes and a light Cycler-Fast Start DNA Master HybProbe Kit (Roche Moleculer Biochemicals). Probes were designed to hybridize to the antisense strands of transcripts and labeled with 6-carboxy-fluorescein phosphoramidite at the 5′ end, with 5-carboxy-tetramethyl-rhodamine (Nihon Gene Research Labs Inc., Japan) as a quencher. The 20 μl PCR reaction mix contained 2 μl 10× PCR buffer, 25 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM of each primer and 0.2 μM probe, 2 U of FastStart Taq DNA polymerase and 100 ng of sample cDNA. PCR amplification began with a 10 min preincubation step at 95°C, followed by 45 cycles of denaturation at 95°C for 40 s, annealing at 54.2°C (iNOS) for 20 s and elongation at 78°C for 40 s (iNOS). The relative concentration of the PCR product derived from the target gene was calculated using software of the Light Cycler System. A standard curve for each run was constructed by plotting the crossover point against the log (number of starting molecules). The number of target molecules in each sample was then calculated automatically with reference to this curve. Results were expressed relative to the number of GAPDH transcripts used as an internal control. Some amplification products in the Light Cycler were checked by electrophoresis on 3% ethidium bromide-stained agarose gels. All experiments were performed in quintuplicate.

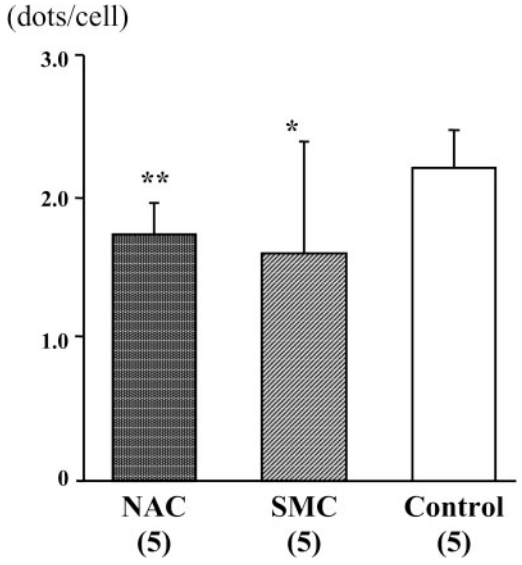

Fig. 4. Silver-stained nucleolar organizer regions (AgNORs) in the liver of rats treated with MeIQx followed by cysteine or saline (experiment 1). Figure shows number of AgNORs. Number of rats examined are shown in parentheses. *, **Significantly different from the control at $P < 0.05$ and $P < 0.01$.

Statistical analysis

Statistical analysis of our data was conducted with the Stat View-J 4.02 program using the Fisher's method of Abacus Concepts (Abacus Concepts, Inc., Berkeley, CA).

Results

All rats survived in the three experiments. In all experiments, final body weights and relative liver weights did not differ between groups, and no significant intergroup variations were found regarding water intake and food consumption. In experiment 1, total intakes of MeIQx for 9 weeks were 79.5 mg/rat (NAC 100 mg/kg body wt), 80.8 mg/rat (SMC 100 mg/kg body wt) and 83.1 mg/rat (saline 4 ml/kg body wt). In experiment 2, intakes of MeIQx were 82.2 mg/rat (NAC 100 mg/kg body wt), 80.3 mg/rat (NAC 30 mg/kg body wt), 81.9 mg/rat (NAC 10 mg/kg body wt), 82.7 mg/rat (SMC 100 mg/kg body wt), 83.1 mg/rat (SMC 30 mg/kg body wt), 81.3 mg/rat (SMC 10 mg/kg body wt) and 84.6 mg/rat (saline 4 ml/kg body wt). The intakes of MeIQx did not differ between experiments 1 and 2.

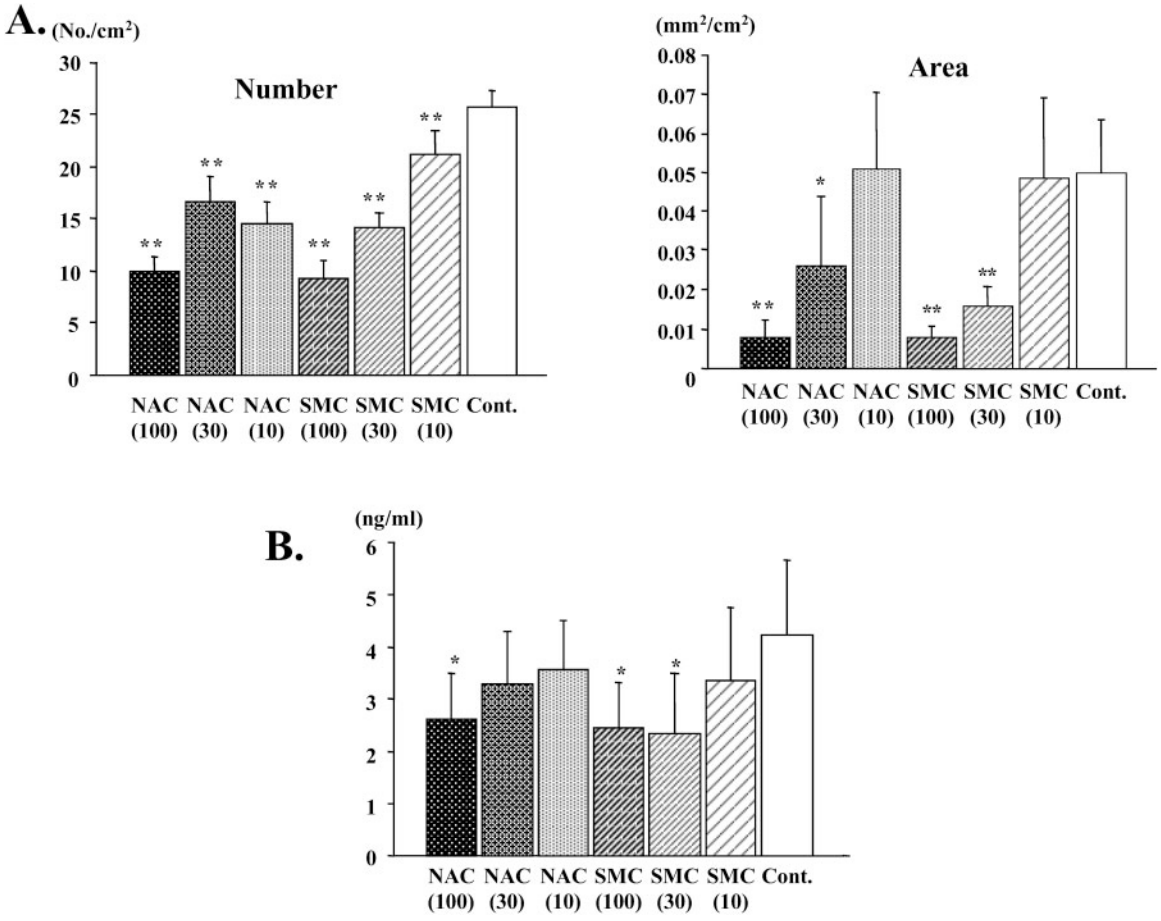

Fig. 5. (**A**) Numbers and areas of GST-P positive foci in the liver of rats (experiment 2) ($n = 15$). *, **Significantly different from the control at $P < 0.01$ and $P < 0.001$; (**B**) Level of insulin-like growth factor-I (IGF-I) in plasma due to NAC or SMC treatment (experiment 2) ($n = 5$). *Significantly different from the control at $P < 0.05$.

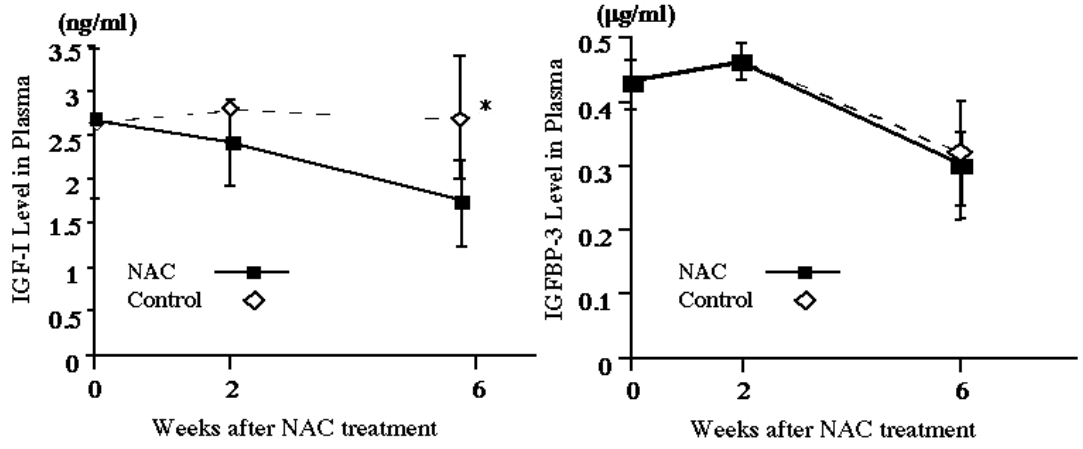

Fig. 6. Level of insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein (IGFBP)-3 in plasma due to NAC at 0, 2 and 6 weeks after starting treatment of NAC in experiment 3. Closed squares, NAC (100 mg/body wt); open diamond, saline (4 ml/body wt) ($n = 5$). *Significantly different from the control at $P < 0.05$.

In experiment 1, significantly decreased numbers and areas of GST-P positive foci per unit area of liver section in the groups treated with MeIQx followed by both OSCs were found compared with controls (Figure 3). These inhibition effects of NAC and SMC did not differ. The results of AgNORs examination, used for evaluation of cellular proliferation in Groups 1–3, are shown in Figure 4. The numbers of AgNORs in the GST-P positive foci were decreased in the groups treated with NAC and SMC in comparison with controls. However, the shape of AgNORs were not different among the groups.

Figure 5A represents numbers and areas of GST-P positive foci in the liver in experiment 2. In comparison with the control (Group 7), both numbers and areas of GST-P positive foci with NAC (Groups 1–3) and SMC (Groups 4–6) treatments were decreased in a dose-dependent manner. Moreover,

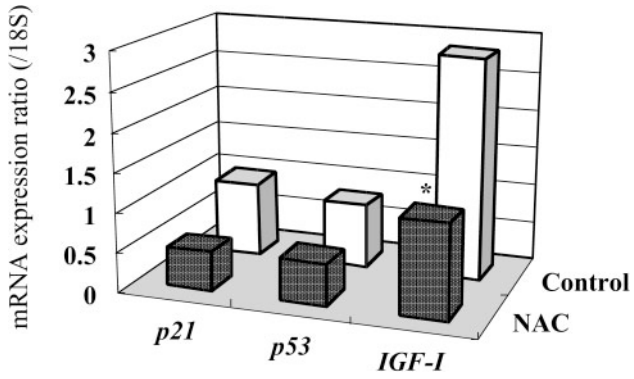

Fig. 7. *IGF-1*, *p53*, *p21* mRNA expression in the liver of rats sacrificed at week 12 (6 weeks after starting NAC administration) by northern blotting ($n = 5$) (experiment 3). *Significantly different from the control at $P < 0.001$.

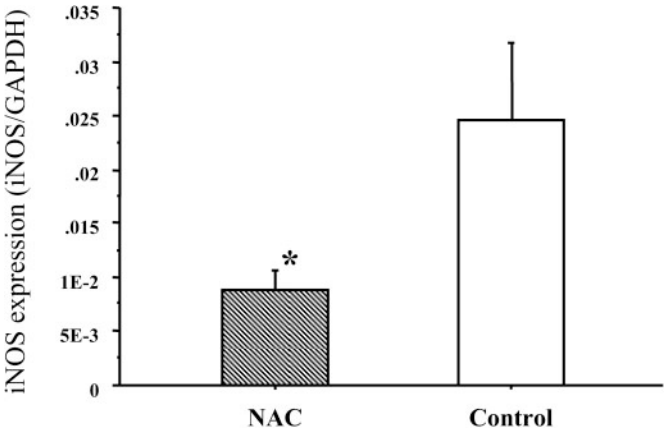

Fig. 8. iNOS mRNA expression in the liver of rats sacrificed at week 12 (6 weeks after starting NAC treatment) detected by LC-PCR (experiment 3). Striped area, NAC (100 mg/body wt); open area, saline (4 mg/body wt.) ($n = 5$). *Significantly different from the control at $P < 0.01$.

the levels of IGF-I in plasma were decreased with similar dose-dependence (Figure 5B).

In experiment 3, plasma levels of IGF-I were significantly decreased after 6 weeks of NAC treatment (Figure 6). However, NAC did not influence plasma IGFBP-3 levels. In addition, *IGF-I* mRNA expression in the liver of the group treated with NAC was significantly decreased compared to the control group (Figure 7). In contrast, *p53* mRNA and *p21* mRNA expression in NAC-treated group did not differ from the control.

The level of 8-OHdG, a marker of oxidative DNA damage, in the liver was $1.17 \pm 0.16/10^6$ dG, at one week after cessation of MeIQx treatment (before organic chemical treatment). After 2 weeks on NAC, the level of 8-OHdG in the group treated with NAC ($0.37 \pm 0.02/10^6$ dG) was significantly lower than in the control group ($0.42 \pm 0.05/10^6$ dG). Moreover, iNOS mRNA expression in liver was significantly decreased in the group administered NAC with respect to the control (Figure 8).

Discussion

In the present study, NAC treatment in the post-initiation stage dose-dependently inhibited the development of GST-P positive foci in the livers of rats treated with MeIQx. Moreover, SMC showed the same inhibitory effect as described previously (11). Numbers of AgNOR positive cells (a marker of cell proliferation) in GST-P positive foci of the liver were decreased by NAC and SMC. Previously, Takada *et al*. (11) similarly reported that numbers of proliferating cell nuclear antigen-positive cells in the liver were diminished by SMC treatment. The results indicate that NAC and SMC inhibit hepatocellular proliferation *in vivo*, resulting in post-initiation inhibition of hepatocarcinogenesis, and that there is little difference in effective intensity between NAC and SMC.

In this study, the numbers and areas of GST-P positive foci differed between experiments 1 and 2. The reason is unclear, but the experimental data showed the same tendency, so that the variation did not affect interpretation of the effects of NAC and SMC.

The mRNA and protein levels of IGF-I, an important cell growth factor, were also found to be reduced in blood and/or liver by NAC treatment. IGF-I, a 70 amino acid peptide with sequence and structural homology to insulin, is produced in a variety of organs and tissues, including liver (26). Recently, IGF-I has attracted particular attention with regard to its involvement in carcinogenesis. For example, disregulation of the IGF axis, including the autocrine production of IGFs, IGF binding proteins (IGFBPs), IGFBP proteases and expression of the IGF receptors has been identified in the development of hepatocellular carcinoma and hepatoma cell lines (27). Another report suggested that IGF-I and IGFBP were increased in the serum of colorectal cancer patients (28). In our experiments, the level of IGF-I was reduced in the group treated with NAC, while the level of IGFBP-3 did not change. These results indicated that the synthesis of IGF-I in the liver was suppressed by NAC treatment, and this may have contributed to the reduced liver cell proliferation. Regulators of the bioactivity or synthesis of IGF-I include growth hormone (GH) and insulin, GH providing the key stimulus for synthesis of IGF-I and IGFBP-3. Insulin enhances the GH-stimulated synthesis of IGF-I and IGFBP-3 by increasing the levels of GH receptors and by stimulating cellular uptake of amino acids for protein synthesis. How NAC influences cell proliferation needs to be examined in more detail with further studies.

It is known that some compounds in garlic can induce apoptosis in cancer cells *in vitro* (29). For example, a phenethyl isothiocyanate NAC diet upregulated inhibitors of cyclin-dependent kinases p21 (WAF-1/Cip-1) and p27 (Kip1) in xenogeneic human prostate cancer cells (30). However, in the present study *p53* and *p21* mRNA expression in the liver was not changed by the NAC treatment. Therefore, the p53 and p21 pathway may not contribute to NAC-chemopreventive effects.

It is well known that oxidative stress has an important role in chemical carcinogenesis (18). 8-OHdG, produced by the oxidation of deoxyguanosine, is considered as the most sensitive and useful maker of DNA adducts. It has been shown that 8-OHdG is closely associated with aging and cancer (31). Its formation is reported to be increased in rat hepatocarcinogenesis induced by MeIQx (32). The reactive oxygen species are involved in activation of NF-κB. NF-κB is a redox-sensitive transcription factor, and nuclear translocation of NF-κB is observed in the suffered nucleus by oxidative stress. A recent report has suggested that NAC suppressed the translocation of NF-κB (33). It has been stated that redox status is changed by oxidative stress, NF-κB is involved in the cellular response to

oxidative stress, and redox modulation by NAC, the exogenous thiol antioxidant affects the transcription level of intracellular stress signaling. iNOS is one of NOS, enzymes which carry out NO production by endotoxin or carcinogen. NO is considered to damage DNA and bring out carcinogenesis. It is reported that NAC inhibits iNOS, induced by endotoxin from *Escherichia coli*, *in vivo* (34). In the present study, the levels of 8-OHdG formation and iNOS mRNA expression were decreased by NAC treatment, in line with strong scavenging of superoxide radicals.

NAC is readily deacetylated inside cells to yield cysteine, a rate-limiting amino acid in the synthesis of glutathione through an ATP-dependent two-step pathway catalysed by γ-glutamylcysteine synthetase and glutathione synthetase (10). NAC is thus related to glutathione conjugation, which is part of the detoxifying system. The protective effect of NAC against the toxicity of chemicals may be due to its dual role as a nucleophile and as a SH donor. Cysteine also has demonstrated inhibitory effects in the post-initiation stage of MeIQx hepatocarcinogenesis in rats (35).

In conclusion, our present studies indicated that NAC and SMC can serve as chemopreventive agents for hepatocarcinogenesis, reducing cellular proliferation by a mechanism which may involve suppression of IGF-I and oxidative stress.

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Conflict of Interest Statement: None declared.

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