Cell Proliferation and Forestomach Carcinogenesis

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To analyze the role of cell proliferation in phenolic compound-induced rat forestomach carcinogenesis, early forestomach histopathological changes as well as oncogene expression and reversibility of early forestomach lesions were examined in F344 male rats. For the analysis of early lesions, five animals each were treated with butylated hydroxyanisole (BHA), caffeic acid, sesamol, or 4-methoxyphenol in the diet, each at a dose of 2%, and killed for histopathological examination after 12 hr, 1, 3, or 7 days. For oncogene analysis, three animals each were treated with BHA for 15, 30 min, 1, 3, 6, or 24 hr and then sacrificed. In the reversibility study, groups of animals were treated with BHA, caffeic acid, sesamol or 4-methoxyphenol for 24 weeks, and basal diet alone was supplied for a further 24-week period. Animals were killed at 24 and 48 weeks and forestomach epithelium was examined histopathologically. DNA synthesis increased within 12 hr to 3 days after commencement of chemical treatment in all cases. Toxicity and cell proliferation became evident subsequent to increase in DNA synthesis in each case. Elevated expression of c-fos and c-myc oncogenes was demonstrated 15 min after beginning treatment with BHA. In the reversibility study, although most of the proliferative lesions induced by these antioxidants regressed after cessation of chemical treatment, some dysplastic lesions were still observed at week 48. The results indicate that these phenolic compounds act primarily as mitogens in rat forestomach epithelium, with regeneration due to toxicity further enhancing cell proliferation. During this continuous elevation of cell turnover, dysplastic lesions appear that persist and presumably play a role in the development of forestomach carcinomas.

Introduction

Recently, phenolic compounds such as butylated hydroxyanisole (BHA), caffeic acid, sesamol, 4-methoxyphenol, and 4-methylcatechol have been shown to induce squamous cell carcinomas in rat forestomach epithelium in 2-year feeding studies (1,2). Such compounds are not mutagenic in the Ames test but induce strong cell proliferation as well as toxicity such as inflammation, erosion, and ulceration during chemical treatment (3–5). Cell proliferation has been suggested to play an important role in the carcinogenicity of nongenotoxic chemicals (6). The present investigation was performed to analyze early histopathological changes induced by these carcinogens in rat forestomach epithelium, as well as oncogene expression and reversibility of such early forestomach lesions to clarify the role of cell proliferation and toxicity in forestomach carcinogenicity induced by phenolic compounds.

Materials and Methods

For the analysis of early forestomach lesions, groups of twenty-five 6-week-old F344 male rats (Charles River Japan Inc., Kanagawa, Japan) were treated with BHA, caffeic acid, sesamol, or 4-methoxyphenol in Oriental MF powdered basal diet (Oriental Yeast Co., Tokyo, Japan) at a dose of 2%, or basal diet alone, and killed for histological examination after 12 hr, 1, 3, or 7 days. A single IP administration of bromodeoxyuridine (BrdU) at a dose of 20 mg per rat was given 1 hr before sacrifice for anti-BrdU immunohistochemical staining. BHA and 4-methoxyphenol were obtained from Wako Pure Chemical Industries (Osaka, Japan), caffeic acid was from Tokyo Kasei Kogyo Co. (Tokyo, Japan), sesamol from Fluka Chemie AG (Buchs, Switzerland), and BrdU from Sigma Chemical Co. (St. Louis, MO).

For oncogene analysis, three animals each were treated with 2% BHA in powdered diet for 15, 30 min, 1, 3, 6, or 24 hr and then sacrificed. The forestomach epithelium was removed using a slide glass, quickly frozen in liquid nitrogen, and total RNA was isolated.

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Oncogene expression was examined by the Northern blotting method. Probes for c-fos and c-myc labeled by \(^{32}P\) were obtained from Takara Shuzo Co., Ltd. (Kyoto, Japan).

In the reversibility study, groups of F344 male rats were treated with BHA, caffeic acid, sesamol, or 4-methoxyphenol at a dose of 2% in powdered basal diet for 24 weeks, and, after cessation of chemical treatment, basal diet alone was supplied for a further 24 weeks. Another group of rats was treated with basal diet alone during the experiment. Ten animals were killed at weeks 24 and 48. The forestomach epithelium was examined histopathologically for lesion development.

**Results**

DNA synthesis in the mid-region of the forestomach epithelium, expressed as the number of labeled cells per 100 basal cells (labeling index), increased 12 hr after treatment with caffeic acid, sesamol, or 4-methoxyphenol. In the case of BHA, an increase in labeling index was apparent 3 days after treatment. After 7 days of continuous antioxidant administration, labeling indexes increased or continued to be high, especially in the groups treated with sesamol or 4-methoxyphenol followed by caffeic acid and BHA (Fig. 1).

Hyperplasia was also observed 3 days after treatment with caffeic acid at an incidence of 80%, but this change first became evident only later in the cases of BHA, sesamol, and 4-methoxyphenol (Fig. 2). Toxic changes such as erosion or ulceration developed in 60–100% of the animals treated with caffeic acid, sesamol, or 4-methoxyphenol, but were not found in those treated with BHA (Fig. 3).

Strongly elevated expression of the c-fos oncogene in forestomach epithelium was demonstrated 15 min after beginning treatment with BHA but rapidly decreased thereafter. c-myc expression was similarly observed after 15 min of treatment, then decreased slowly.

In the reversibility study, moderate hyperplasia (thickness between 0.1 and 0.5 mm) was found in all animals treated with BHA, caffeic acid, sesamol, and 4-methoxyphenol for 24 weeks. However, incidences of moderate hyperplasia had decreased to 40% in the BHA case, 20% with caffeic acid, 40% with sesamol and 10% with 4-methoxyphenol 24 weeks after cessation of chemical treatment. On the other hand, atypical hyperplasia, which was not observed at 24 weeks, was found 24 weeks later in 10% of the animals receiving caffeic acid, sesamol, or 4-methoxyphenol.

**Discussion**

The present experiments clearly show that all four phenolic compounds induce increases in DNA synthesis within 3 days after treatment followed by hyperplasia and evidence of epithelial damage. Expression of the protooncogenes c-fos and c-myc was clearly found after 15 min of treatment with BHA. These results indicate that almost immediately after commencement of treatment with phenolic compounds, factor(s) stimulating or responsible for cell proliferation are generated, eventually resulting in hyperplasia. This might be further enhanced by regenerative changes subsequent to erosion or ulceration. In the liver, cell proliferation can be divided into two categories: compensatory cell
proliferation and direct cell proliferation. Partial hepa-
tectomy and CCl_{4} intoxication induce compensatory
cell proliferation and \(c-fos\) and \(c-myc\) expression with a
maximum increase between 0.5 and 2 hr for \(c-fos\) and
2–3 hr for \(c-myc\). On the other hand, ethylene dibro-
mide, cyproterone acetate, lead nitrate, and nafenopin
induce direct cell proliferation without any associated
increase in the expression of \(c-fos\) during the first 24 hr
(7–9). In this sense, phenolic compounds seem to
induce a different type of direct hyperplasia with pro-
toecogene expression in the early stage, which later
is supplemented by compensatory hyperplasia.

It is of interest that although hyperplasia regressed
24 weeks after cessation of chemical treatment, some
focal dysplastic lesions still remained in the forestom-
ach epithelium. These lesions are histopathologically
similar to those found in animals treated with the
genotoxic carcinogens \(N\)-methyl-\(N^{\prime}\)-nitro-\(N\)-nitroso-
guanidine or \(N\)-methyl-N-nitrosourea and are also
found in hamster forestomach epithelium after cess-
ation of BHA treatment (10). Therefore, it is conceiv-
able that during continuous, strong cell proliferation,
genetic alterations in DNA may take place, resulting
in focal populations responsible for the development of
carcinomas. However, the genotoxic effect of BHA
may be very weak because continuous oral treatment
with 2% BHA for 24 weeks was insufficient for initia-
tion of two-stage rat forestomach carcinogenesis (11).

Recently, we established that small amounts of DNA
adducts were indeed formed in the forestomach ep-
thelium but not in the glandular stomach epithelium
of rats treated with BHA for 2 weeks as evaluated by the
enzymatic 32P-postlabeling method (12). \textit{In vitro}, DNA
adducts were not formed by BHA alone, requiring the
addition of S-9 mixture to the medium and acidic condi-
tions. The adduct spots coincided with those formed by
t-butylquinone, a metabolite of BHA (12); oral admin-
istration of this compound has, in fact, been shown to
induce DNA damage in rat forestomach epithelium
(13). Therefore, quinone metabolites may be responsi-
bile for the induction of cytotoxicity or genotoxicity. On
the other hand, formation of 8-hydroxydeoxyguano-
sine, a DNA-damage product associated with active
oxygen species, and/or elevation of lipid peroxidation,
was not demonstrated in rat forestomach epithelium
treated with these phenolic compounds for 2–48 weeks
(14). Therefore, active oxygen species may not be
involved in the cytotoxicity or probable genotoxic
events caused by these chemicals, at least later than 2
weeks after treatment.

In conclusion, the process of forestomach carcino-
genesis by nongenotoxic phenolic compounds is complex,
and several steps should be considered as indicated in
Figure 4: a) initiation of DNA synthesis, b) stimulation
of continuous cell proliferation, and c) DNA alteration
(adduct formation). Further studies are required to
clarify the factors that cause these changes.

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\textbf{REFERENCES}

1. Hirose, M., Fukushima, S., Shirai, T., Hasegawa, R., Kato, T.,
Tanaka, H., Asakawa, E., and Ito, N. Stomach carcinogenicity of
caffeic acid, sesamol and catechol in rats and mice. Jpn. J.
Cancer Res. 81: 207–212 (1990).

2. Hagiwara, A., Hirose, M., Takahashi, S., Ogawa, K., Shirai, T.,
and Ito, N. Forestomach and kidney carcinogenicity of caffeic
acid in F344 rats and C57BL/6N X C3H/HeN F; mice. Cancer
Res. 51: 5655–5660 (1991).

3. Hirose, M., Masuda, A., Imaida, K., Kagawa, M., Tsuda, H., and
Ito, N. Induction of forestomach lesions in rats by oral adminis-
trations of naturally occurring antioxidants for 4 weeks. Jpn. J.
Cancer Res. 78: 317–321 (1987).

4. Altman, H. J., and Gronow, W. Effects of BHA and related
phenols on the forestomach of rats. Food Chem. Toxicol. 24:
1188–1188 (1986).

5. Rodrigues, C., Lok, E., Nera, E., Iverson, F., Page, D.,
Karpinski, K., and Clayson, D. B. Short-term effects of various
phenols and acids on the Fisher 344 male rat forestomach
epithelium. Toxicology 38: 103–117 (1986).

6. Cohen, S. M., and Ellwein, L. B. Cell proliferation in carcino-
genesis. Science 249: 1007–1011 (1990).

7. Herbst, H., Milani, S., Schuppan, D., and Stein, H. Temporal and
spatial patterns of proto-oncogene expression at early stages of
toxic liver injury in the rat. Lab. Invest. 65: 324–333 (1991).

8. Coni, P., Pichiri-Coni, G., Ledda-Columbano, G. M., Rao, P. M.,
Rajalakshmi, S., Sarma, D. S. R., and Columbano, A. Liver
hyperplasia is not necessarily associated with increased expres-
sion of \(c-fos\) and \(c-myc\) mRNA. Carcinogenesis 11: 885–889
(1990).

9. Ledda-Columbano, G. M., Columbano, A., Curto, M., Ennas,
M. G., Coni, P., Sarma, D. S. R., and Pani, P. Further evidence that
mitogen-induced cell proliferation does not support the forma-
tion of enzyme-altered islands in rat liver by carcinogens.
Carcinogenesis 11: 847–850 (1990).

10. Hirose, M., Masuda, A., Hasegawa, R., Wada, S., and Ito, N.
Regression of butylated hydroxyanisole (BHA)-induced hyper-
plasia but not dysplasia in the forestomach of hamsters.
Carcinogenesis 11: 239–244 (1990).

11. Hirose, M., Uwagawa, S., Ozaki, K., Takaba, K., and Ito, N.
Effects of butylated hydroxyanisole pretreatment on low dose
\(N\)-methyl-\(N^{\prime}\)-nitro-\(N\)-nitrosoguanidine- or \(N, N\)-dibutylnitro-
samine-induced rat forestomach or esophageal carcinogenesis.
Carcinogenesis 12: 1773–1776 (1991).

12. Nakagawa, S., Kogiso, S., Yoshitake, A., Hirose, M., and Ito, N.
32P-postlabeling analysis of DNA adducts in the forestomach and

\begin{figure}
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\includegraphics[width=\textwidth]{cell proliferation and forestomach carcinogenesis}
\caption{Putative pathway of rat forestomach carcinogenesis
induced by phenolic compounds.}
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glandular stomach of rats treated with catechol or related phenolic compounds. Proc. Jpn. Cancer Assoc. 48: 70 (1991).

13. Morimoto, K., Tsuji, T., Iio, T., Miyata, N., Uchida, A., Osawa, R., Kitsutaka, H., and Takahashi, A. DNA damage in forestomach epithelium from male F344 rats following oral administration of tert-butylquinone, one of the forestomach metabolites of 3-BHA. Carcinogenesis 12: 703-708 (1991).

14. Ito, N., Hirose, M., and Takahashi, S. Cellular proliferation and stomach carcinogenesis induced by antioxidants. Prog. Clin. Biol. Res. 369: 43-52 (1991).