Cytotoxicity of WBC or spleen cells treated with PA2/4

WBC treated with PA2/4 exhibited markedly enhanced cytotoxicity against Co26Lu cells; the effect of PA2/4 treatment on the cytotoxicity of spleen cells against Co26Lu cells was not significant (Figure 10).

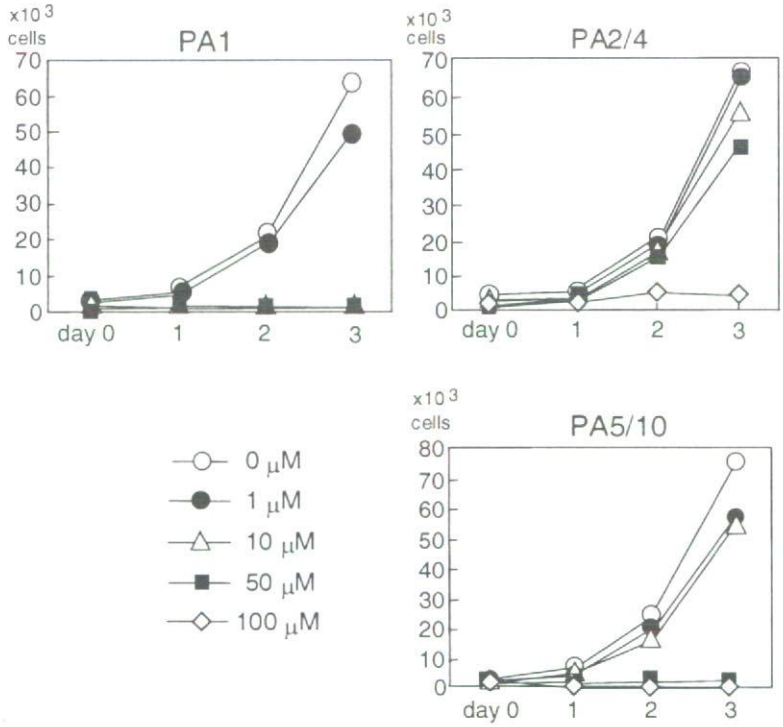


Figure 9. Cytotoxicity of PA1, PA2/4, and PA5/10 against Co26Lu cells.

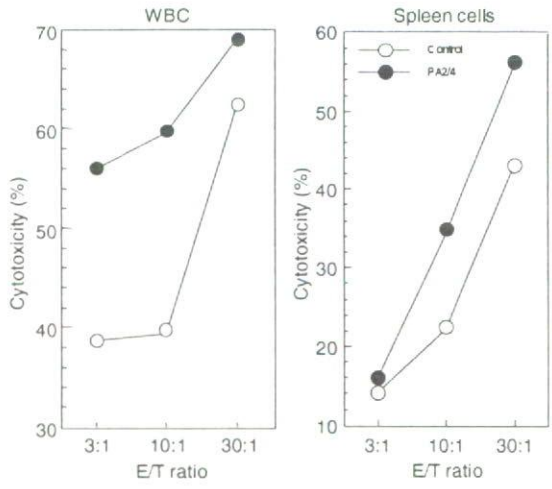


Figure 10. Effect of PA2/4 on the cytotoxicity of white blood cells (A) and spleen cells (B) against Co26Lu cells. PA2/4 was administered daily for 5 days by oral administration at 300 mg/kg/day. At 3 h after the last PA2/4 treatment, white blood cells were collected and used in the cytotoxicity assay.

Population of $DX5^+$, $CD3^+$ and $CD11b^+$ cells in white blood cells (WBC) following oral administration of PAs

A study was carried out to determine whether subpopulations of WBC were changed by treatment with PAs. Pan-NK ($DX5^+$) cells in animals treated with PA2/4 at 300 mg/kg for 5 days were significantly increased as compared with the control (12.3±1.1% vs 18.9 ± 2.1%, $p<0.05$), but $CD3^+$ and $CD11b^+$ cells were not affected (Figure 11). PA1 and PA5/10 had no effect on WBC subpopulations.

Effects of orally administered PAs on T, B and NK cells in the small intestines of mice

Oral administration of PA2/4 significantly increased the number of $DX5^+$(31.4%, $p<0.05$, Figure 12B), but not $CD4^+$ (Figure 12A) or $CD11b^+$ (Figure 12C) cells in the lamina propria of the small intestine.

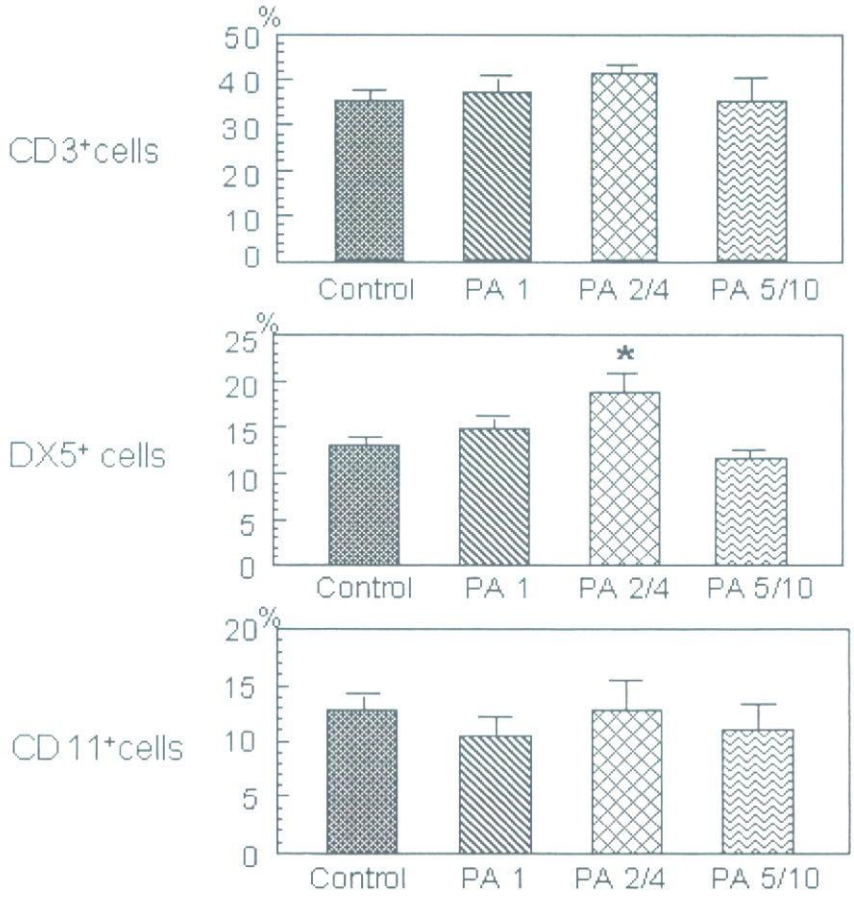


Figure 11. Effects of PA1, PA2/4, and PA5/10 on the proportion of $CD3^+$, $DX5^+$ and $CD11b^+$ cells in the white blood cell population. PA1, PA2/4, or PA5/10 was administered daily for 5 days by oral administration at 300 mg/kg/day. At 3 h after the last PA treatment, WBC were collected and analyzed by FACS.

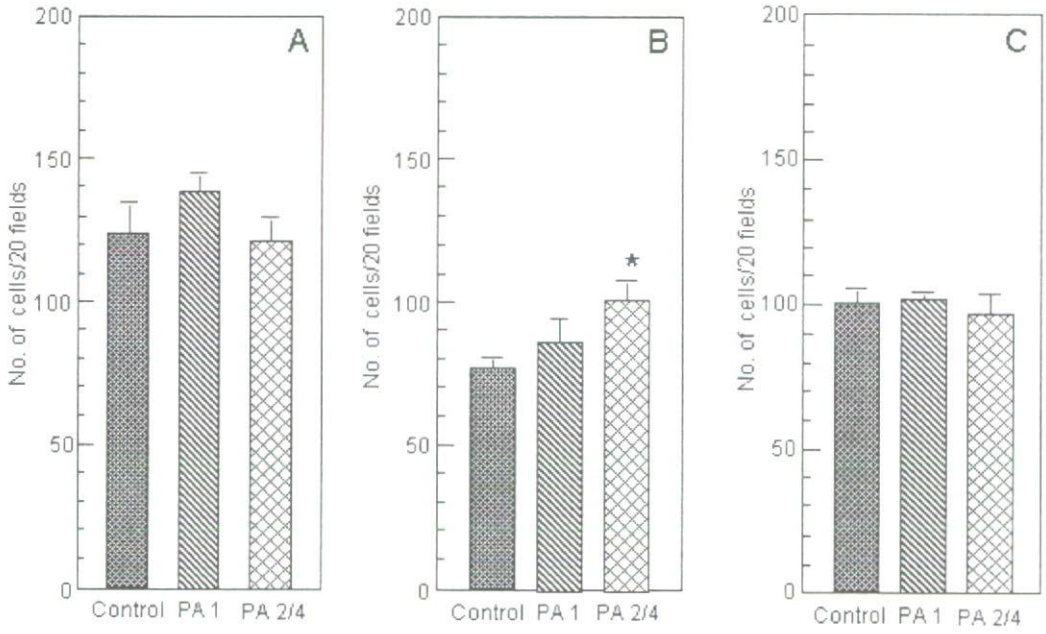


Figure 12. Effects of PA1 and PA2/4 on the number of $CD4^+$(A), $DX5^+$(B), and $CD11b^+$(C) cells in the lamina propria of the small intestine. PA1 or PA2/4 was administered daily for 5 days by oral administration at 300 mg/kg/day. At 3 h after the last PA treatment, the small intestines were excised and fixed in acetone and the $CD4^+$, $DX5^+$, and $CD11b^+$ cells identified by immunohistochemistry and counted.

Effects of orally administered PAs on IL-1β, IL-12, IL-13, IL-18 and IFN-γ levels in the small intestine

Oral administration of PA2/4 significantly increased IL-12 levels (15.6 ± 2.4 vs 29.2 ± 4.2 pg/mg protein, $P<0.05$) and tended to increase IL-1β levels (284.5 ± 26.5 vs 392.0 ± 46.6 pg/mg protein) and IL-18 levels (11.2 ± 1.0 vs 14.7 ± 0.8 ng/mg protein,), but had no effect on IL-13 levels, in extracts from the mucosa of the small intestine (Figure 13). IFN-γ levels were significantly increased by both PA2/4 and PA5/10 (Control: 4.7 ± 1.0 pg/mg protein; PA2/4: 7.9 ± 0.9 pg/mg protein, $p<0.05$; PA5/10: 11.3 ± 2.2 pg/mg protein, $P<0.01$). PA1 had no effect on cytokine expression.

Discussion

Recently, the use of naturally-occurring compounds in the development of anti-carcinogenic and anti-tumor agents has become a critical topic. Flavonoids comprise one group of natural products currently receiving a great deal of attention. Treatment with the green tea constituent EGCG resulted in inhibition of cell proliferation, with induction of apoptosis (11-14) being one mechanism. Treatment with green tea also inhibits metastasis of tumor cells to lungs and increases the survival period of test animals (27). Several studies have reported

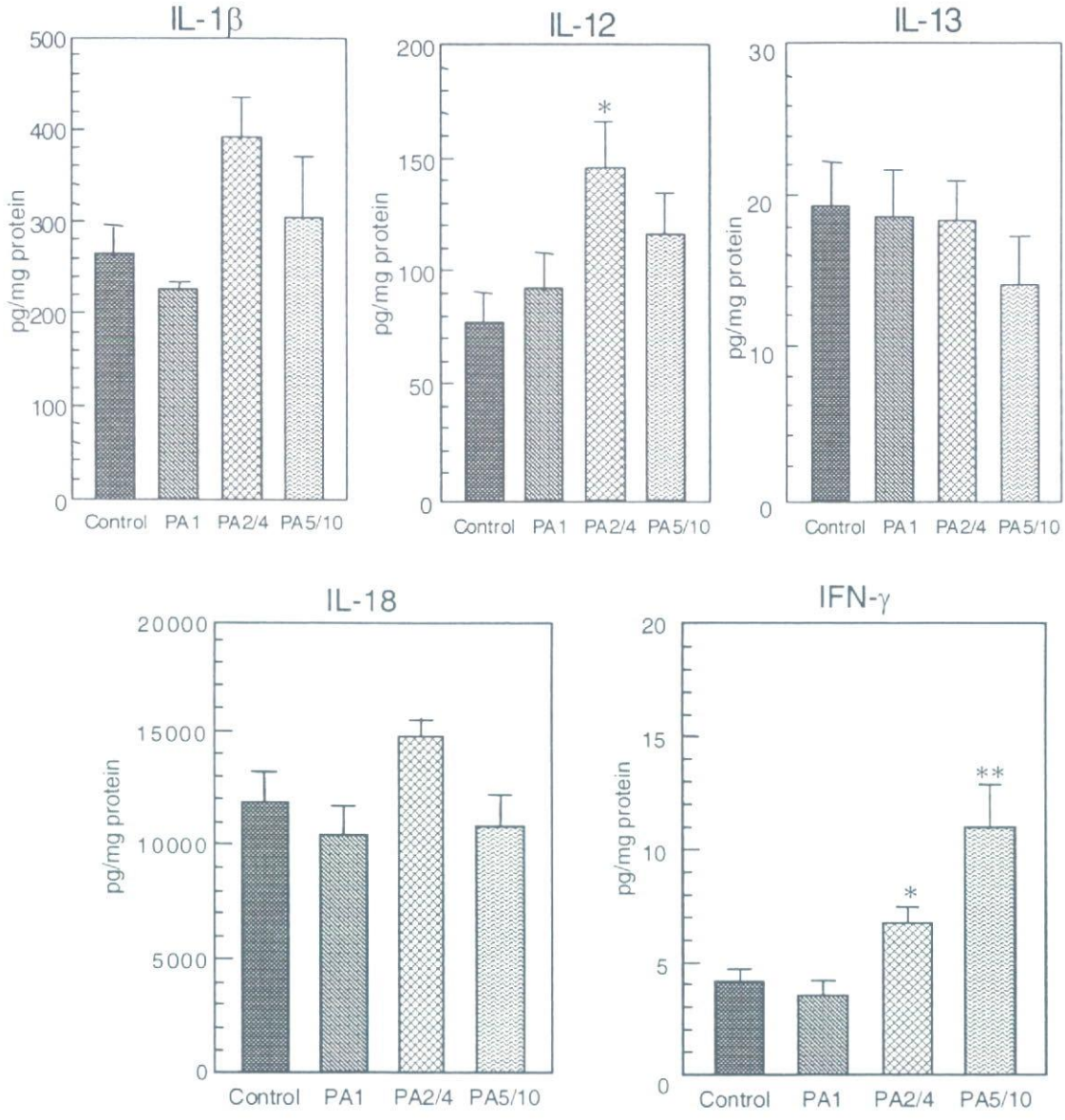


Figure 13. Effects of PA1, PA2/4, and PA5/10 on cytokine levels in the mucosa of the small intestine. PA1, PA2/4, or PA5/10 was administered daily for 5 days by oral administration at 300 mg/kg/day. At 3 h after the last PA treatment, the small intestines were excised and extracted using lysis buffer. IL-1β, IL-12, IL-13, IL-18, and IFN-γ levels were measured by ELISA.

that other flavonoids, including kaempferol and quercetin, exert anti-oxidant effects and can also inhibit carcinogenesis (28-32). PAs, another kind of flavonoid, are the most abundant polyphenolic compounds in plants and are common constituents of various fruits and seeds. The daily intake of PAs has been estimated at 0.1 – 0.5 g (33). Various epidemiologic data on PA-rich wine and tea have suggested that they prevent cancers and cardiovascular diseases in

humans (34). Nomoto et al. (25) reported that PAs enhanced apoptosis against colon carcinoma cell lines and also enhanced NK activity in rats; NK cells may be involved in anti-carcinogenesis and prevention of metastasis (35). The polymeric nature and the complexity of the chemical structure of PAs make the pharmacological properties of individual PAs uncertain. We purified polyphenolic compounds from grape seed extracts by HPLC and obtained PA1, PA2, PA3, and fractions of PA2/4, PA5/10, and >PA11. Among these PAs, PA5/10 induced the strongest apoptotic response in colon carcinoma cell lines. Moreover, PA5/10 showed strong anti-angiogenesis in the chick embryo CAM assay. Thus, PA5/10 may have a direct effect on colon carcinogenesis.

On the other hand, PA2/4 has strong anti-tumor activity in Co26Lu-bearing mice in *in vivo*. Following oral administration of PA2/4, high levels of PAs were found in the intestinal mucosa compared to control or PA5/10 treatment. Possibly, PA2/4 may be more easily absorbed into the epithelial cells of the small intestine compared to PA5/10. Highly polymerized PAs may not be easily absorbed if they are not degraded by the colonic microflora into low-molecular weight aromatic acids (36). Importantly, after oral administration of PA2/4, it was undetectable in the serum by Vanillin's method (threshold detection of about 10 μM). These results indicate that serum PA2/4 levels were far too low to effectively kill tumor cells: the concentration of PA2/4 needed to kill tumor cells is more than 100 μM (Table 1). This suggests that other mechanisms, such as stimulation of immune cells, may account for the observed *in vivo* anti-tumor activity of PA2/4.

The importance of NK cells as effector cells for the anti-tumor/anti-metastatic activity effected by PA2/4 treatment is strongly supported by the findings reported here. Following oral administration of PA2/4, WBC killing of tumor cells was markedly enhanced. Moreover, the population of NK ($DX5^+$) cells was significantly increased by PA2/4 treatment in both the blood and the mucosa of the small intestine. PA2/4 treatment also induced significant increases in IL-12 expression in the mucosa of the small intestine ($P<0.05$). In another experiment, using more than 20 mice each, similar results were obtained (Control group: 87.7 ± 6.4 pg/mg protein, n=24; PA2/4 treatment group: 134.5 ± 18.6 pg/mg protein, n=22, $p<0.05$). IFN-γ levels in the intestinal mucosa were also markedly increased after treatment with PAs, especially PA5/10 (2.4-fold) ($p<0.01$) and PA2/4 (1.7-fold)($p<0.05$). Finally, IL-18 and IL-1β levels also increased in the intestinal mucosa after treatment with PA2/4. The high levels of these cytokines in the mucosa of the small intestine could activate immune cells, especially NK cells, which would then serve as an effective means for preventing metastasis and carcinogenesis.

Taken altogether, our results indicate that PA mixtures, such as those found in grape seed extracts, exert more than a single type of effect on cancers. PA5/10 is able to directly induce apoptosis of abnormal cells and may also

possibly be able to affect neovascularization of the tumor. PA2/4 exerts anti-metastasis and anti-carcinogenesis effects indirectly via enhancement of immune function.

Acknowledgements

We thank Hiroshi Nomoto and Tomoko Izaki for assistance with flow cytometry detection. This work was supported in part by a Grant-in-Aid for the Second Term Comprehensive 10-Year Strategy for Cancer Control and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare Japan.

References

1. Block, G., Patterson, B., and Subar, A. 1992, Nutr. Cancer, 18, 1.
2. Witte, J.S., Longnecker, M.P., Bird, C.L., Lee, E.R., Frankl, H.D., and Haile, R.W. 1996, Am. J. Epidemiol., 144, 1015.
3. Neuhouser, M.L. 2004, Nutr. Cancer, 50, 1.
4. Knekt, P., Jarvinen, R., Seppanen, R., Hellovaara, M., Teppo, L., Pukkala, E., and Aromaa, A. 1997, Am. J. Epidemiol., 146, 223.
5. Deschner, E.E., Ruperto, J., Wong, G., and Newmark, H.L. 1991, Carcinogenesis, 12, 1193.
6. Singh, A.K., Seth, P., Anthony, P., Husain, M.M., Madhavan, S., Mukhtar, H., and Maheshwari, R.K., 2002, Arch. Biochem. Biophys., 401, 29.
7. Yang, C.S., Chung, J.Y., Yang, G., Chhabra, S.K., and Lee, M.J. 2000 J. Nurt., 130, 472S.
8. Fujiki, H., Suganuma, M., Okabe, S., Sueoka, N., Komori, A., Sueoka, E., Kozu, T., Tada, Y., Suga, K., Imai, K., and Nakachi, K. 1998, Mutat. Res., 402, 307.
9. Wang, Z.Y., Huang, M.T., Ho, C.T., Chang, R., Ma, W., Ferraro, T., Reuhl, K.R., Yang, C.S., and Conney, A.H. 1992, Cancer Res., 52, 6657.
10. Lu, Y.P., Lou, Y.R., Xie, J.G., Peng, Q.Y., Liao, J, Yang, C.S., Huang, M.T., and Conney, A.H. 2002, Proc. Natl. Acad. Sci. USA, 99, 12455.
11. Roy, A.M., Baliga, M.S., and Katiyar, S.K., 2005, Mol. Cancer Ther., 4, 81.
12. Gupta, S., Hastak, K., Afaq, F., Ahmad, N., and Mukhtar, H. 2004, Oncogene 23, 2507.
13. Brusselmans, K., Vrolix, R., Verhoeven, G., and Swinnen, J.V. 2005, J. Biol. Chem., 280, 5636.
14. Gupta, S., Hussain, T., and Mukhtar, H. 2003, Arch. Biochem. Biophys., 410, 177.
15. Koga, T., Moro, K., Nakamori, K., Yamakoshi, J., Hosoyama, H., Kataoka, S., and Ariga, T. 1999, J. Agric. Food Chem., 47, 1892.
16. Busserolles, J., Gueux, E., Balasinska, B., Piriou, Y., Rock, E., Rayssiguier, Y., and Mazur, A. 2006, Int. J. Vitam. Nutr. Res., 76, 22.
17. Miller, K.B., Stuart, D.A., Smith, N.L., Lee, C.Y., McHale, N.L., Flanagan, J.A., Ou, B., and Hurst, W.J. 2006, J. Agric. Food Chem., 54, 4062.
18. Zhao, J., Wang, J., Chen, Y., and Agarwal, R. 1999, Carcinogenesis, 20, 1737.
19. Waffo-Teguo, P., Hawthorne, M.E., Cuendet, M., Merillon, J.M., Kinghorn, A.D., Pezzuto, J.M., and Mehta, R.G. 2001, Nutr. Cancer, 40, 173.

20. Ye, X., Krohn, R.L., Liu, W., Joshi, S.S., Kuszynski, C.A., McGinn, T.R., Bagchi, M., Preuss, H.G., Stohs, S.J., and Bagchi, D. 1999, Mol. Cell Biochem., 196, 99.
21. Vayalil, P.K., Mittal, A., and Katiyar, S.K. 2004, Carcinogenesis, 25, 987.
22. Agarwal, C., Sharma, Y., Zhao, J., and Agarwal, R. 2000, Clin. Cancer Res., 6, 2921.
23. Tyagi, A., Agarwal, R., and Agarwal, C. 2003, Oncogene, 22, 1302.
24. Agarwal, C., Singh, R.P., and Agarwal, R. 2002, Carcinogenesis, 23, 1869.
25. Nomoto, H., Iigo, M., Hamada, H., Kojima, S., and Tsuda, H. 2004, Nutr. Cancer, 49, 81.
26. Butler, L.G., Price, M.L., and Brotherton, J.E. 1982, J. Agric. Food Chem., 30, 1087.
27. Liu, J.D., Chen, S.H., Lin, C.L., Tsai, S.H., and Liang, Y,C. 2001, J. Cell Biochem., 83, 631.
28. Nguyen, T.T., Tran, E., Ong, C.K., Lee, S.K., Do, P.T., Huynh, T.T., Nguyen, T.H., Lee, J.J., Tan, Y., Ong, C.S., and Huynh, H. 2003, J. Cell Physiol., 197, 110.
29. Murtaza, I., Marra, G., Schlapbach, R., Patrignani, A., Kunzli, M., Wagner, U., Sabates, J., and Dutt, A. 2006, Biotechnol. Appl. Biochem., 45, 29.
30. Park, C.H., Chang, J.Y., Hahm, E.R., Park, S., Kim, H.K., and Yang, C.H. 2005, Biochem. Biophys. Res. Commun., 328, 227.
31. Nguyen, T.T., Tran, E., Nguyen, T.H., Do, P.T., Huynh, T.H., and Huynh, H. 2004, Carcinogenesis, 25, 647.
32. Yang, K., Lamprecht, S.A., Liu, Y., Shinozaki, H., Fan, K., Leung, D., Newmark. H., Steele, V.E., Kelloff, G.J., and Lipkin, M. 2000, Carcinogenesis. 21, 1655.
33. Scalbert, A., and Williamson, G. 2000, J. Nutr., 130, 2073S.
34. Kris-Etherton, P.M., and Keen, C.L. 2002, Curr. Opin. Lipidol, 13, 41.
35. Racz, T., Sacks, P.G., Taylor, D.L., and Schantz, S.P. 1989, Arch. Otolaryngol Head Neck Surg., 115, 1322.
36. Deprez, S., Brezillon, C., Rabot, S., Philippe, C., Mila, I., Lapierre, C., and Scalbert, A. 2000, J. Nutr., 130, 2733.

Gpx2 Is an Overexpressed Gene in Rat Breast Cancers Induced by Three Different Chemical Carcinogens

Aya Naiki-Ito,[1] Makoto Asamoto,[1] Naomi Hokaiwado,[1] Satoru Takahashi,[1] Hiroko Yamashita,[2] Hiroyuki Tsuda,[3] Kumiko Ogawa,[1] and Tomoyuki Shirai[1]

[1]Departments of Experimental Pathology and Tumor Biology, [2]Oncology and Endocrinology, and [3]Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, Mizuho-cho, Mizuho-ku, Nagoya, Japan

Abstract

Gene expression alterations are essential for the process of carcinogenesis. A carcinogen may have specific mechanisms for inducing tumors, which may involve inducing characteristic gene expression alterations. In this study, we attempted to identify genes crucial for mammary carcinogenesis. For this purpose, we used human *c-Ha-ras* proto-oncogene transgenic rats (Hras128), which are highly sensitive to mammary carcinogens including *N*-methyl-*N*-nitrosourea, 7,12-dimethyl benz[*a*]anthracene, and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. DNA microarray analysis revealed that *glutathione peroxidase 2* (*Gpx2*) was commonly up-regulated in the mammary carcinomas induced by the three different carcinogens, and its up-regulation was confirmed by quantitative reverse transcriptase-PCR and Western blotting analysis. In addition, expression of GPX2 was recognized in all 41 immunohistochemically examined cases of human breast cancer. Forced suppression of *GPX2* expression by siRNA resulted in significant growth inhibition in both rat and human mammary carcinoma cell lines with wild-type *p53* cells. Thus, these data suggested that GPX2 may be involved in mammary carcinogenesis and cell proliferation in both rats and humans, indicating that GPX2 may be a novel target for the prevention and therapy of breast cancer. [Cancer Res 2007;67(23):11353–8]

Introduction

Breast cancer is the most frequent type of cancer in women and, after lung cancer, represents the second leading cause of cancer death (1). Therefore, to reduce the incidence of death from this cancer among women, it is important to establish new approaches to its prevention and treatment.

Many genes have been reported to be involved in the mammary carcinogenic process. For instance, the receptor tyrosine kinase HER2 (Neu/ErbB2) is overexpressed in ~20% to 30% of primary breast cancers, and these patients have a poor prognosis (2, 3). The identification of overexpressed genes in the majority of breast cancer cases might lead to good candidates as target molecules for the prevention and therapy of the disease.

We have hypothesized that common gene expression changes in mammary cancers, induced by different carcinogens, are critical for rat mammary carcinogenesis. Moreover, such common genes might also be involved in the development of human breast cancer. *N*-Methyl-*N*-nitrosourea (MNU), 7,12-dimethyl benz[*a*]anthracene (DMBA), and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) are well-known rat mammary carcinogens. We have previously established a strain of human *c-Ha-ras* proto-oncogene transgenic rats (Hras128), which are highly susceptible to these mammary carcinogens, resulting in the development of a high incidence of mammary carcinomas over a relatively short period of time (4–6). This rat model is a valuable one for investigating mammary carcinogenesis *in vivo*.

In the present study, using DNA microarray analysis, we found that, compared with normal mammary gland tissues, the expression of *glutathione peroxidase 2* (*Gpx2*) was commonly elevated in the mammary carcinomas of Hras128 rats, which had been induced by three different mammary carcinogens: MNU, DMBA, and PhIP. *Gpx2* overexpression was confirmed by quantitative reverse transcriptase-PCR (RT-PCR), Western blotting, and immunochemistry. Furthermore, to clarify the function of this overexpression in the carcinogenetic process, we examined the down-regulation effect of *Gpx2* expression using siRNA.

Materials and Methods

Production of transgenic rats. Female Hras128 rats were produced by mating transgenic male with nontransgenic female Sprague-Dawley animals (Clea Japan, Inc.; ref. 4). Rats were maintained in plastic cages on hardwood chips in an air-conditioned, specific pathogen-free animal room at 22 ± 2°C and 50% humidity with 12:12 h light/dark cycle. All animal experiments were done under protocols approved by the Institutional Animal Care and Use Committee of Nagoya City University School of Medical Sciences.

Experimental protocols. The 7-week-old Hras128 transgenic rats were treated with MNU (a single i.p. injection of 50 mg/kg, body weight), DMBA (a single intragastric administration of 50 mg/kg of body weight), and PhIP (intragastric administration of 100 mg/kg of body weight, twice a week for 4 weeks). These chemicals were obtained from WAKO. Each carcinogen was given to four transgenic rats. Four nontreated transgenic rats and four littermate nontransgenic rats served as the controls. Rats were sacrificed at 14 weeks for sampling of the mammary cancers of MNU-treated transgenic rats and the normal mammary tissues of nontreated animals, and at 23 weeks for the transgenic rats treated with DMBA and PhIP. Mammary cancers were induced in Hras128 as described in Table 1.

Human breast cancer samples. From 2002 to 2003, breast cancer samples were collected from 41 female patients (32–79 years old) undergoing surgery for their disease at Nagoya City University Hospital; all patients had given their prior informed consent. Histologic grade of the breast cancer was assessed according to Elston and Ellis method (7).

Immunohistochemistry. Deparaffinized slide sections of rat and human mammary tissues were fixed with acetone (rat) or 10% formalin (human) and then incubated with 1:100 diluted GPX2 (IMGENEX) antibody. Antibody binding was visualized by a conventional immunostaining method using an autoimmunostaining apparatus (Ventana HX System, Ventana Japan). For intensity of GPX2 cytoplasmic immunoreactivity, clinical breast cancers were classified as weak (1+), moderate (2+), or strong (3+) in each histologic grade.

Requests for reprints: Makoto Asamoto, Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, 1-Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. Phone: 81-52-853-8156; Fax: 81-52-842-0817; E-mail: masamoto@med.nagoya-cu.ac.jp.

doi:10.1158/0008-5472.CAN-07-2226

Table 1. Final body weight, number, and incidence rates of mammary tumor in Hras128

	No. rats	Body weight (g)	Mammary tumor		
			Total no.	Incidence (%)	Multiplicity*
At 7 wk (14 wk old)					
Nontreated nontransgenic	4	256.2 ± 16.47	0	0	0
Nontreated transgenic	4	273.5 ± 21.64	2	50	0.5
MNU-treated transgenic	4	198.5 ± 17.78	102	100	25.5
At 14 wk (23 wk old)					
DMBA-treated transgenic	4	253.9 ± 46.69	48	100	12
PhIP-treated transgenic	4	264.5 ± 22.46	65	100	16.25

*Multiplicity = tumor number / animal number.

Quantitative RT-PCR. One microgram of RNA was converted to cDNA with avian myoblastosis virus reverse transcriptase (Takara) in a 20-μL reaction mixture. Aliquots of 2 μL of cDNA samples were subjected to quantitative PCR in 20 μL using SYBR Premix ExTaq (Takara) in a LightCycler apparatus (Roche Diagnostics). The primers used were 5′-GACACGAGGAAACCGAAGCA-3′and 5′-GGCCCTTCACAACGTCT-3′ for *Gpx2* (rat), 5′-CCAGGACCTTGAGATTGAAT-3′ and 5′-GTGTCAGCACG-CACGTTA-3′ for cytokeratin 19, 5′-GCCTCCTTAAAGTTGCCATA-3′ and 5′-GCCCAGAGCTTACCCA-3′ for *GPX2* (human), and 5′-GCATCCTGCAC-CACCAACTG-3′ and 5′-GCCTGCTTCACCACCTTCTT-3′ for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Initial denaturation was at 95°C for 60 s, followed by 40 cycles with denaturation at 95°C for 5 s, annealing at 55°C for 15 s, and elongation at 72°C for 30 s. Cytokeratin-19 mRNA levels were used to normalize for the sample cDNA content of rat tissues and cell lines and *GAPDH* mRNA levels for the human cell line samples.

Western blotting. A total of 20 μg protein per lane were separated on 12% acrylamide gels and electroblotted onto nitrocellulose membranes (Hybond-ECL, GE Healthcare UK Ltd.). Gpx2 expression levels were assessed with the same antibody used for immunohistochemical staining. β-Actin expression was evaluated to confirm equal amount of protein loadings by monoclonal anti-β-actin, AC-74 (Sigma-Aldrich Corp.).

Cell culture. The rat mammary carcinoma cell lines (C1, C2, C3, C6, C11, and C17), established from a DMBA-induced mammary carcinoma in Hras128 rats (8), and the human mammary carcinoma cell lines MCF-7, T47D, and MDA-MB-231 were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS. The human mammary epithelial cell line (HMEC) was obtained from Lonza Walkersville, Inc. All cell cultures were maintained under 5% CO_2/95% air at 37°C in a humidified incubator.

RNA interference and cell counts. Stealth Select RNAi targeting rat *Gpx2*, human *GPX2*, and control sequences were obtained from Invitrogen. Cells (5×10^4) from each of the two rat breast cancer cell lines, C2 and C11, were seeded in six-well plates and cultured for 24 h. They were then transfected with 100 pmol/well of siRNA using LipofectAMINE RNAiMAX (Invitrogen) at 10% cell confluence. Cell numbers were counted after 2 and 4 days. For the two human mammary carcinoma cell lines (MCF-7 and T47D), after preparing 100 pmol/well of siRNA samples, 3×10^5 MCF-7 cells and 2.5×10^5 T47D cells were seeded in six-well plates, and the cell numbers counted after 4 days. These experiments were done thrice.

Results

Mammary carcinomas induced by carcinogens. Mammary tumors were palpable in most of the Hras128 rats at the end of week 5 following treatment with the carcinogen. Faster growth and greater numbers of tumors (25.5 per rat) were noted in rats treated

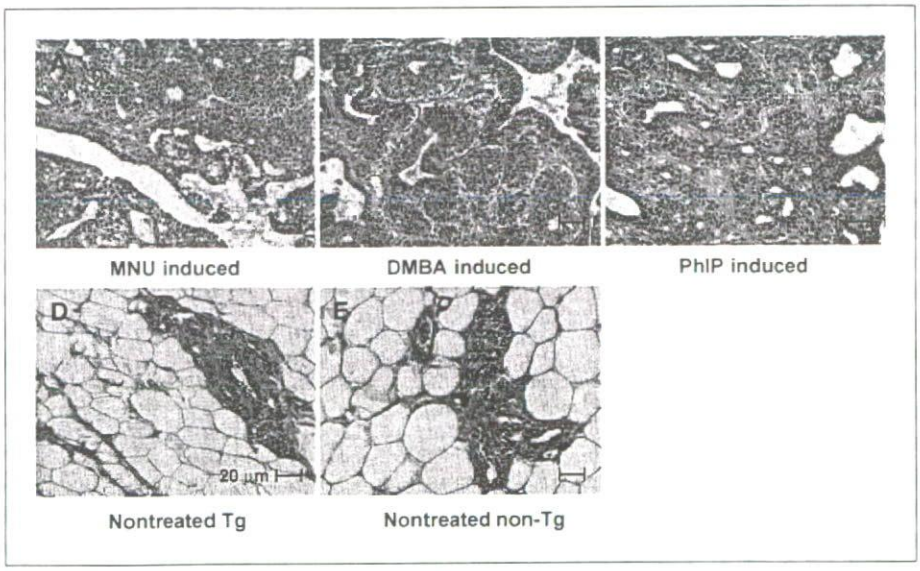


Figure 1. Histologic appearance of mammary carcinomas induced in Hras128 rat (transgenic). *A* to *C*, mammary cancers induced by MNU, DMBA, and PhIP, respectively. All tumors were adenocarcinomas. *D* and *E*, mammary glands of nontreated transgenic (*Tg*) and littermate nontransgenic (*non-Tg*) rats.

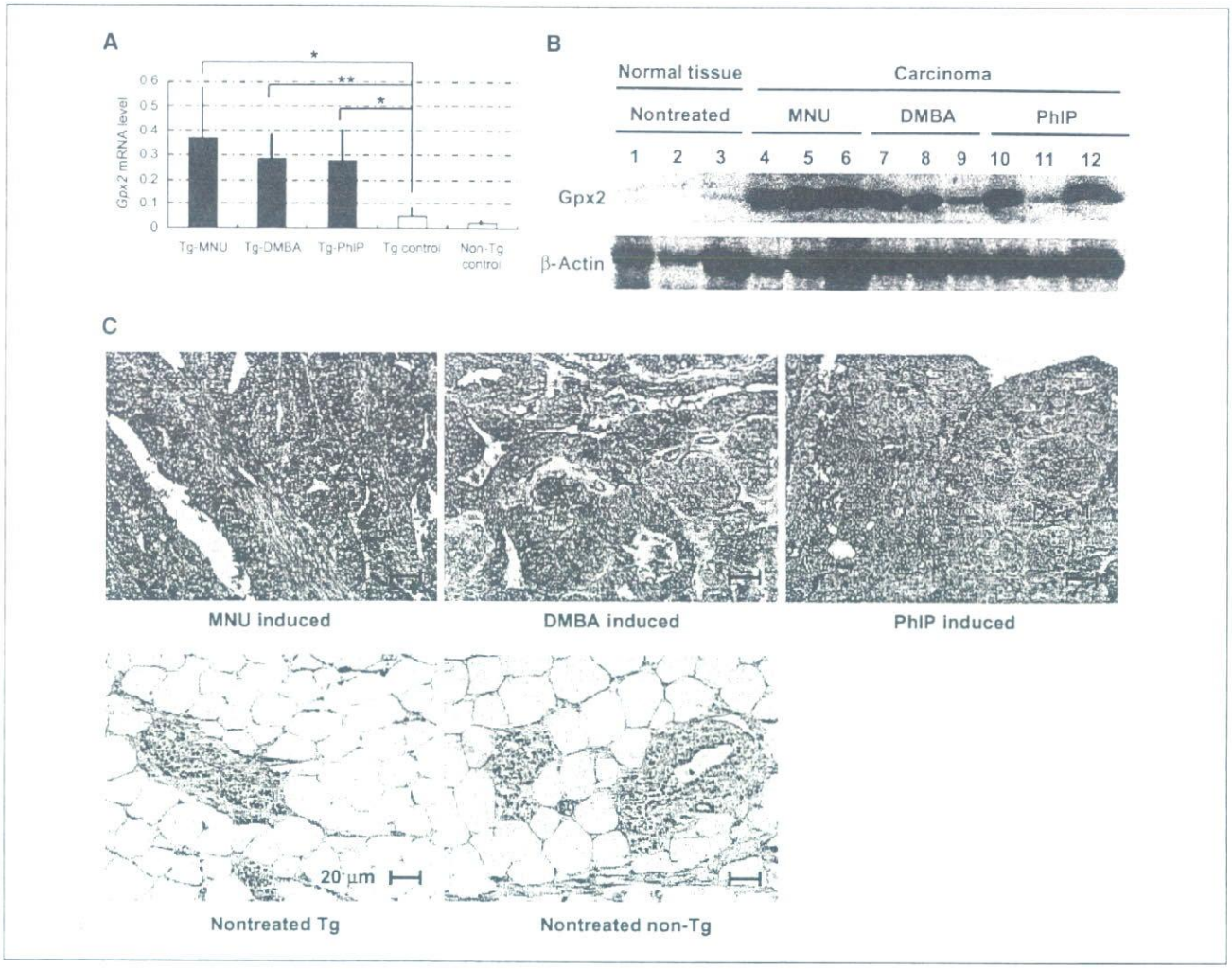


Figure 2. *A*, relative Gpx2 expression levels in carcinogen-induced mammary cancers. The data were obtained by quantitative RT-PCR as described in Materials and Methods. *Gpx2* mRNA expression level was adjusted by cytokeratin 19. *, $P = 0.01$; **, $P = 0.001$, compared with control value (Student's *t* test). *B*, Western blot of normal mammary tissue and carcinoma in Hras128 rats. *Lanes 1* to *3*, normal mammary tissue. *Lanes 4* to *12*, carcinogen-induced mammary cancer: *lanes 4* to *6*, MNU; *lanes 7* to *9*, DMBA; *lanes 10* to *12*, PhIP-induced. *C*, immunohistochemical findings of Gpx2 in rat mammary carcinomas induced by MNU, DMBA, and PhIP. Gpx2 was strongly positive in the cytoplasm of tumor cells. Nontumorous mammary glands of transgenic and nontransgenic rats were completely negative.

with MNU than in those treated with DMBA (16.25 per rat) or PhIP (12 per rat; Table 1). All mammary tumors were adenocarcinomas as shown in Fig. 1; there were no clear differences in tumor types among the three groups.

***GPX2* overexpressed in rat and human mammary carcinoma cell lines.** According to gene expression analysis for the rat mammary cancers using DNA microarray analysis, we found *Gpx2* as an up-regulated gene in the mammary cancer induced by the three different mammary carcinogens, compared with the nontreated normal mammary glands in the Hras128 rats. *Gpx2* mRNA expression was elevated >5-fold over the control value by quantitative RT-PCR (Fig. 2*A*), and Western blot analysis showed that Gpx2 protein levels were obviously high in all carcinogen-induced mammary cancers compared with normal mammary tissues of nontreated Hras128 (Fig. 2*B*).

GPX2 expression in rat and human mammary carcinoma cell lines was examined by quantitative RT-PCR. Gpx2 was highly expressed in all rat mammary carcinoma cell lines established from a DMBA-induced mammary carcinoma in Hras128 rats, C1, C2, C3, C6, C11, and C17 (8). In contrast, normal rat mammary tissues from nontreated transgenic rats exhibited very low levels of *Gpx2* (Table 2). Among these cancer cell lines, we selected C2 and C11 for further experiments *in vitro* because of their higher mRNA expression levels of *Gpx2*. Gpx2 protein expressions were confirmed by Western blot analysis. These rat breast cancer cell lines expressed Gpx2 protein. However, there is no clear difference among protein levels of the cell lines (data not shown). Interestingly, the human mammary carcinoma cell lines MCF-7, T47D, and MDA-MB-231 also showed overexpression of *GPX2* compared with the HMEC. Furthermore, *GPX2* expression in the estrogen-dependent cell lines MCF-7 and T47D tended to be higher than the estrogen-independent MDA-MB-231 (Table 2).

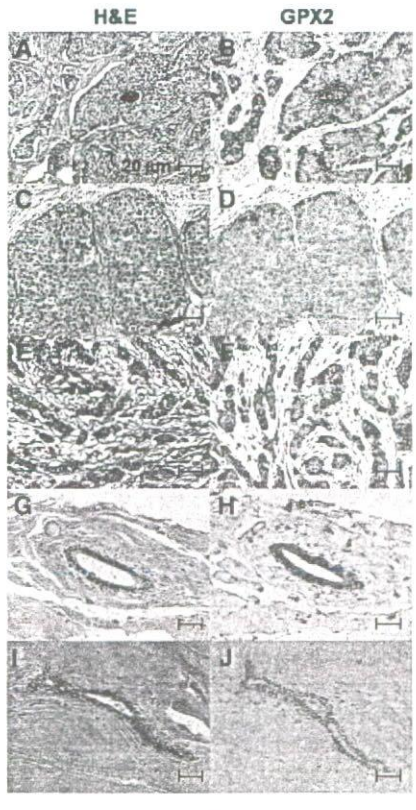
Immunohistochemical analysis of GPX2 in rat and human mammary carcinomas. Gpx2 protein was clearly detected in the cytoplasm of rat mammary cancers. Stromal tissue also exhibited partially positive staining. All 18 tumors were positive for Gpx2.

Table 2. GPX2 mRNA levels in rat and human mammary carcinoma cell lines

Cell lines (rat)	p53	*Gpx2*	Cell lines (human)	p53	*GPX2*
C1	Wild	134.8 ± 6.8	MCF-7	Wild	5.72 ± 0.77
C2	Wild	472.9 ± 143.2	T47D	Mutant	7.64 ± 0.65
C3	Wild	9.1 ± 0.9	MDA-MB-231	Mutant	1.89 ± 0.32
C6	Wild	367.3 ± 68.4	HMEC		1.00
C11	Mutant	519.0 ± 118.6			
C17	Wild	86.4 ± 5.2			
Control mammary tissue of transgenic		0.2 ± 0.1			

NOTE: GPX2 expression of three human mammary carcinoma cell lines was relative to its expression of HMEC.

In contrast, Gpx2 was not immunohistochemically detected in normal mammary tissues of the nontreated transgenic and nontransgenic rats (Fig. 2*C*). These results suggest that Gpx2 may play a critical role in the proliferation of mammary cancer cells. To clarify the relevance of this finding in human cases, expression levels of GPX2 were assessed in 41 human breast cancers (all cases were invasive ductal carcinoma; 18 papillotubular carcinomas, 10 solid-tubular carcinomas, and 13 scirrhous carcinomas) by immunohistochemistry. Cytoplasmic positive immunostaining was clearly detected in all the human tumor tissues (Fig. 3*B, D, F*). Fourteen (32%) cases of low histologic grade (grade 1) invasive ductal carcinomas were strongly positive for GPX2 (average



K

Case no.	Histology	Grade	Gpx2 intensity	Case no.	Histology	Grade	Gpx2 intensity
1	PTC	1	2	21	PTC	2	3
2	PTC	1	2	22	SC	2	2
3	PTC	1	2	23	SC	2	2
4	SC	1	2	24	SC	2	3
5	SC	1	2	25	STC	2	3
6	PTC	1	3	26	PTC	2	1
7	PTC	1	3	27	STC	2	1
8	PTC	1	3	28	PTC	2	2
9	SC	1	3	29	PTC	2	2
10	SC	1	3	30	PTC	2	2
11	SC	1	3	31	SC	2	2
12	SC	1	3	32	PTC	2	3
13	PTC	1	3	33	PTC	3	1
14	SC	2	2	34	PTC	3	1
15	SC	2	2	35	STC	3	1
16	STC	2	2	36	STC	3	1
17	STC	2	2	37	STC	3	1
18	PTC	2	1	38	STC	3	1
19	STC	2	1	39	PTC	3	2
20	SC	2	2	40	PTC	3	2
				41	STC	3	2

Figure 3. Histologic appearance of human breast cancers and GPX2 immunohistochemical staining. *A, C, E, G,* and *I,* H&E; *B, D, F, H,* and *J,* immunohistochemistry. GPX2 staining was positive in the cytoplasm of human breast cancer cells regardless of tumor type. *A* to *F,* invasive ductal carcinoma: *A* and *B,* papillotubular carcinoma; *C* and *D,* solid-tubular carcinoma; *E* and *F,* scirrhous carcinoma. *G* and *H,* a representative normal mammary gland with GPX2-positive surrounding breast cancer lesion. *I* and *J,* negative for GPX2 in noncancerous mammary lesions. *K,* inverse correlation between the low and high histologic grade samples for GPX2 intensity in clinical breast cancer.

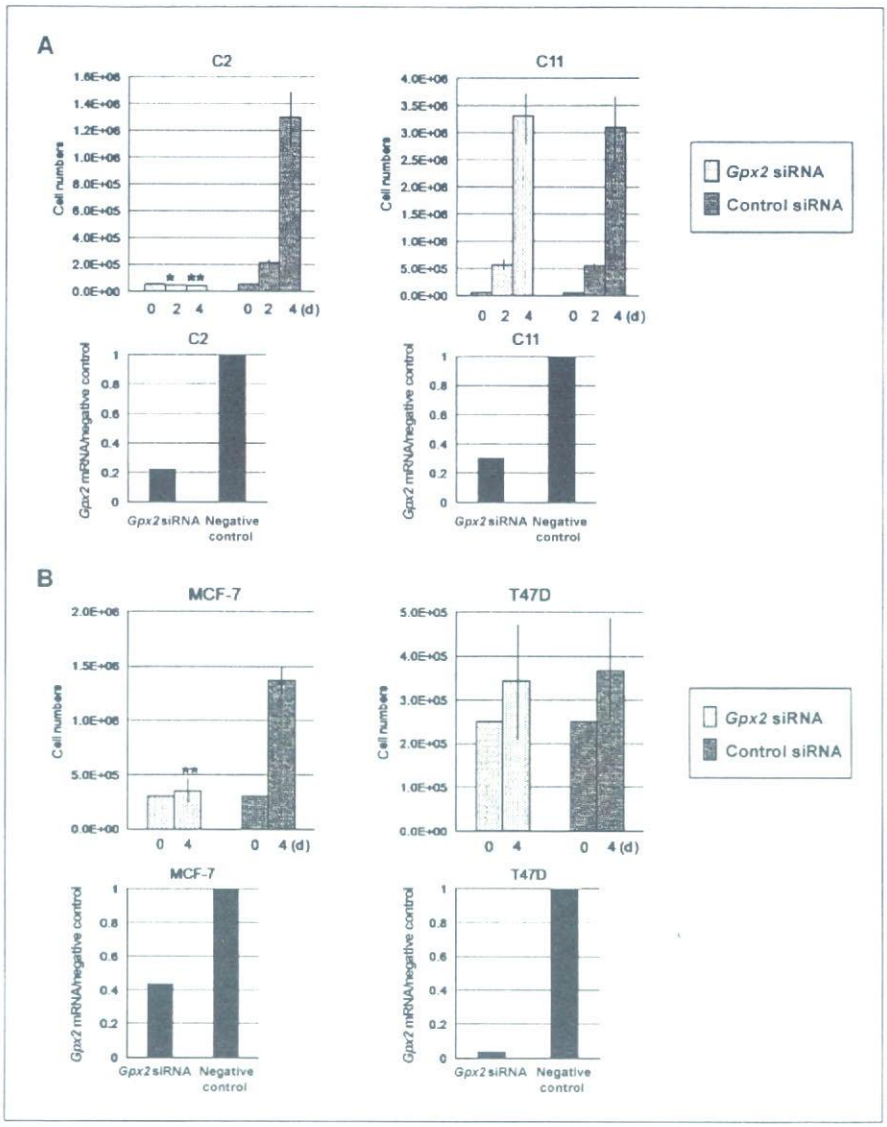


Figure 4. *GPX2* affects p53-dependent cell proliferation in both rat and human mammary cancer cells. *A*, rat mammary cancer cell line C2 with wild-type p53 showed clear inhibition of cell proliferation by *Gpx2* silencing whereas the C11 with mutant p53 did not. *, $P = 0.02$; **, $P = 0.008$ (Student's *t* test). *Bottom*, knockdown efficiency by quantitative RT-PCR. *B*, human mammary carcinoma cell line, MCF-7 with wild-type p53, showed significant inhibition of cell proliferation by *GPX2* suppression, whereas another human breast cancer cell line, T47D with mutant *p53*, showed no apparent change (**, $P = 0.001$, Student's *t* test). *Bottom*, knockdown efficiency by quantitative RT-PCR.

intensity, 2.6 ± 0.5) and the other high-grade (grade 2 and 3) tumors (27 cases; 68%) had weaker GPX2 expression (average score, 1.8 ± 0.7; Fig. 3*K*). These data showed inverse correlation between the low-grade and the high-grade samples for GPX2 intensity (Student's *t* test, $P = 0.00014$). Interestingly, normal-appearing breast epithelial cells surrounding the cancer often gave positive GPX2 staining (Fig. 3*G* and *H*); however, samples of fibrocystic change derived from noncancer patients did not show positive staining of GPX2 (Fig. 3*I* and *J*).

GPX2 silencing effects in rat and human mammary carcinoma cell lines. Next, we investigated the role of *GPX2* by using siRNA to knockdown its expression in the rat mammary carcinoma cell lines C2 and C11. Down-regulation of *GPX2* expression resulted in the dramatic inhibition of cell proliferation in C2 with wild-type *p53* but not in C11 with mutated *p53* (Fig. 4*A*). We also examined the effects of silencing *GPX2* in the human mammary carcinoma cell lines MCF-7 and T47D. Interestingly, MCF-7 with wild-type *p53* showed a large number of dead cells following *GPX2* knockdown, which resulted in a decrease of cell numbers, whereas T47D with mutant *p53* did not have any significant effect on cell growth (Fig. 4*B*).

Discussion

In the present study, we have attempted to identify genes that are important for mammary carcinogenesis. Chemical carcinogens induce mammary cancer by means of their own characteristic mechanisms, including changes in gene expression. However, we believe that common important mechanisms for mammary carcinogenesis probably exist, and that such mechanisms are also likely to be involved in human cases. Therefore, we used DNA microarray analysis to identify gene expression changes in mammary cancers, which had been induced in Hras128 transgenic rats by three different carcinogens; this strain is very sensitive to the chemical induction of mammary carcinomas (Table 1).

Among the genes that showed statistically significant expression changes, we focused particularly on *Gpx2*, the expression of which was elevated in all of the induced mammary carcinomas but was

very reduced in normal mammary glands (Fig. 2). GPX2 is a selenium-dependent glutathione peroxidase belonging to the glutathione peroxidase (GPX) family; it reduces H_2O_2 and alkyl hydroperoxides and possesses anti-inflammatory activity (9, 10). Because GPX2 has been reported to be expressed in selected tissues including mammary glands and the mucosal epithelium of the gastrointestinal tract (GI), it is also known as GPX-GI (11, 12).

There have already been a few reports showing an involvement of Gpx2 in cancer: mice with target disruption of *Gpx2* together with *Gpx1* have been associated with inflammation-induced cancer formation; the expression of *Gpx2* was shown to be reduced in the epithelium of prostatic intraepithelial neoplasia in Nkx3.1 mutant mice; and overexpression of *GPX2* has been found in human colorectal adenomas, Barrett's mucosa of the esophagus, and rat hepatocarcinogenesis (13–18). In addition, inactivation of *GPX2* has been associated with UV-induced squamous cell carcinoma of skin (19), and in the public data base Gene Expressing Omnibus (GEO), a similar result to our present data, which shows that *Gpx2* is overexpressed in MNU-induced breast cancers of Wistar-Furth female rats, is deposited (GDS1363).

Immunohistochemically, Gpx2 was clearly detected in the mammary cancers of Hras128 rats (Fig. 2*C*), and expression of this protein was located in the cytoplasm of cancer cells in all the human breast cancer tissues examined (Fig. 3*A–F*). These positive findings indicate that GPX2 could play an important role in not only rat but also human breast cancer development. In human cases, normal-appearing mammary tissues surrounding the breast cancer often show GPX2 positivity (Fig. 3*G* and *H*). On the other hand, negative staining for GPX2 was shown in noncancerous mammary glands of a patient without cancer (Fig. 3*I* and *J*). These results may suggest that similar alteration of GPX2 expression has already occurred in normal-appearing tissues adjacent the cancer lesion.

To clarify how GPX2 might function in the proliferation of mammary cancer cells, we carried out *GPX2* silencing by using siRNA in both rat and human mammary carcinoma cell lines. Yan and Chen (20) have recently reported that suppression by *GPX2* of oxidative stress–induced apoptosis in MCF-7, a human breast cancer cell line, was wild-type *p53* dependent. Therefore, in the present study, mammary cancer cell lines with both wild-type p53 [C2 (rat) and MCF-7 (human)] and mutant *p53* [C11 (rat) and T47D (human)] were analyzed to investigate any association between *GPX2* and *p53* (8, 21). Interestingly, knockdown of *GPX2* expression caused the inhibition of cell proliferation in both rat and human mammary carcinoma cell lines with wild-type *p53* but not with mutation of *p53* (Fig. 4). These results were compatible with the report by Yan and Chen.

According to a review article by Lacroix M, et al. (21), 20% to 35% of breast cancers express a mutant *p53*; breast cancer patients with *p53* mutations have been associated with poor prognosis (22). Therefore, *Gpx2* could be a target gene for therapy of breast cancer with wild-type *p53*, especially in the early stages of mammary carcinogenesis including precancerous condition.

In conclusion, the present data show that GPX2 may be involved in breast cancers with wild-type *p53* and indicate that GPX2 may be a novel target for the prevention and therapy of breast cancer.

Acknowledgments

Received 6/18/2007; revised 8/27/2007; accepted 10/3/2007.

Grant support: Long-range Research Initiative grant 2005CC05 by Japan Chemical Industry Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Yoichiro Matsuoka (Second Department of Pathology, Kansai Medical University, Osaka, Japan) for the generous gift of rat mammary carcinoma cell lines.

References

1. Dumitrescu RG, Cotarla I. Understanding breast cancer risk—where do we stand in 2005? J Cell Mol Med 2005;9:208–21.
2. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177–82.
3. Sjogren S, Inganas M, Lindgren A, Holmberg L, Bergh J. Prognostic and predictive value of c-erbB-2 overexpression in primary breast cancer, alone and in combination with other prognostic markers. J Clin Oncol 1998;16:462–9.
4. Asamoto M, Ochiya T, Toriyama-Baba H, et al. Transgenic rats carrying human c-Ha-ras proto-oncogenes are highly susceptible to *N*-methyl-*N*-nitrosourea mammary carcinogenesis. Carcinogenesis 2000;21:243–9.
5. Tsuda H, Asamoto M, Ochiya T, et al. High susceptibility of transgenic rats carrying the human c-Ha-ras proto-oncogene to chemically induced mammary carcinogenesis. Mutat Res 2001;477:173–82.
6. Naito A, Suzuki A, Ueda S, et al. Preferential mammary carcinogenic effects of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in human c-Ha-ras proto-oncogene transgenic rats. Cancer Sci 2004;95:399–403.
7. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403–10.
8. Hamaguchi T, Matsuoka Y, Bechberger J, et al. Establishment of an apoptosis-sensitive rat mammary carcinoma cell line with a mutation in the DNA-binding region of p53. Cancer Lett 2006;232:279–88.
9. Chu FF, Esworthy RS, Doroshow JH. Role of Se-dependent glutathione peroxidases in gastrointestinal inflammation and cancer. Free Radic Biol Med 2004;36:1481–95.
10. Chu FF, Doroshow JH, Esworthy RS. Expression, characterization, and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. J Biol Chem 1993;268:2571–6.
11. Chu FF, Esworthy RS, Lee L, Wilczynski S. Retinoic acid induces Gpx2 gene expression in MCF-7 human breast cancer cells. J Nutr 1999;129:1846–54.
12. Esworthy RS, Swiderek KM, Ho YS, Chu FF. Selenium-dependent glutathione peroxidase-GI is a major glutathione peroxidase activity in the mucosal epithelium of rodent intestine. Biochim Biophys Acta 1998;1381:213–26.
13. Chu FF, Esworthy RS, Chu PG, et al. Bacteria-induced intestinal cancer in mice with disrupted Gpx1 and Gpx2 genes. Cancer Res 2004;64:962–8.
14. Esworthy RS, Aranda R, Martin MG, Doroshow JH, Binder SW, Chu FF. Mice with combined disruption of Gpx1 and Gpx2 genes have colitis. Am J Physiol Gastrointest Liver Physiol 2001;281:G848–55.
15. Ouyang X, DeWeese TL, Nelson WG, Abate-Shen C. Loss-of-function of Nkx3.1 promotes increased oxidative damage in prostate carcinogenesis. Cancer Res 2005;65:6773–9.
16. Mork H, Scheurlen M, Al-Taie O, et al. Glutathione peroxidase isoforms as part of the local antioxidative defense system in normal and Barrett's esophagus. Int J Cancer 2003;105:300–4.
17. Mork H, al-Taie OH, Bahr K, et al. Inverse mRNA expression of the selenocysteine-containing proteins GI-GPx and SeP in colorectal adenomas compared with adjacent normal mucosa. Nutr Cancer 2000;37:108–16.
18. Dewa Y, Nishimura J, Muguruma M, et al. Gene expression analyses of the liver in rats treated with oxfendazole. Arch Toxicol 2007;81:647–54.
19. Walshe J, Serewko-Auret MM, Teakle N, et al. Inactivation of glutathione peroxidase activity contributes to UV-induced squamous cell carcinoma formation. Cancer Res 2007;67:4751–8.
20. Yan W, Chen X. GPX2, a direct target of p63, inhibits oxidative stress-induced apoptosis in a p53-dependent manner. J Biol Chem 2006;281:7856–62.
21. Lacroix M, Toillon RA, Leclercq G. p53 and breast cancer, an update. Endocr Relat Cancer 2006;13:293–325.
22. Bull SB, Ozcelik H, Pinnaduwage D, et al. The combination of p53 mutation and neu/erbB-2 amplification is associated with poor survival in node-negative breast cancer. J Clin Oncol 2004;22:86–96.

Med Mol Morphol (2007) 40:185–190
DOI 10.1007/s00795-007-0369-4

SPECIAL REVIEW SERIES: Carcinogenesis of breast carcinoma and its development

**Yoichiro Matsuoka · Tetsuya Hamaguchi
Katsumi Fukamachi · Midori Yoshida · Gen Watanabe
Kazuyoshi Taya · Hiroyuki Tsuda · Airo Tsubura**

Molecular analysis of rat mammary carcinogenesis: an approach from carcinogenesis research to cancer prevention

Received: March 28, 2007 / Accepted: April 23, 2007

Abstract A rat strain carrying the human c-Ha-*ras* proto-oncogene is highly susceptible to chemically induced mammary carcinogenesis. All the transgenic rats develop preneoplastic mammary lesions within 20 days of an injection of *N*-methyl-*N*-nitrosourea, and mammary carcinomas appear within 8 weeks of treatment with a variety of chemical carcinogens. In this review, we summarize molecular aspects of mammary carcinogenesis in transgenic rats and the potential application of this model for studies of breast cancer prevention.

Key words Mammary carcinoma · Preneoplasia · Prevention · *ras* · Transgenic rat

Introduction

The rat mammary gland treated with carcinogens is one of the most widely studied and useful models of mammary carcinogenesis. A rat line carrying the human c-Ha-*ras* proto-oncogene shows an increased susceptibility to chemical carcinogens targeting the mammary glands.[1,2] All the transgenic (Tg) rats developed preneoplastic mammary lesions within 20 days of an injection of *N*-methyl-*N*-nitrosourea (MNU),[3] and mammary carcinomas appeared within 8 weeks of treatment with a variety of chemical carcinogens.[4,5] Interestingly, these carcinomas harbor activating mutations in the human transgene.[1,6] Tg rats also spontaneously develop alveolar hyperplasia and adenocarcinomas. Elevated expression, rather than mutations in the c-Ha-*ras* proto-oncogene, seems to be sufficient to cause a highly proliferative phenotype of mammary alveoli.[7]

Strong epidemiological evidence indicates that women who have a full-term pregnancy early in their lives have a significantly reduced risk of developing breast cancer.[8,9] In both rat and mouse models, full-term pregnancy confers resistance to chemical carcinogen-induced mammary tumorigenesis;[10–14] this effect can be mimicked by treatment with estrogen and progesterone[13,15] or human chorionic gonadotropin.[16] Hormone-induced protection is manifested at a cellular level as a block in carcinogen-induced proliferation shortly after carcinogen treatment. In addition, the cellular content and nuclear localization of p53 tumor suppressor protein are increased in mammary glands involuted with hormone treatment, compared with nontreated virgin glands before and after carcinogen treatment.[12,17] Recently, Medina and Kittrell[18] demonstrated that the absence of the p53 gene abrogates the protective effect of hormones against carcinogen-induced mammary carcinogenesis in mice.

Because the Ras pathway negatively regulates p53 function,[19,20] we examined whether parity-induced protection against mammary tumorigenesis and mammary involution would normally occur in c-Ha-*ras* proto-oncogene Tg rats. We found that parity protected the Tg mammary glands against carcinogen-induced tumorigenesis and that involution occurred normally in the glands. Interestingly, early malignant lesions and *ras* mutations occurred in the parous glands at incidences similar to those incidences in the virgin glands. Our data suggest that pregnancy protects Tg rats from mammary carcinogenesis mainly during the promotion phase.

Y. Matsuoka (✉) · A. Tsubura
Second Department of Pathology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Japan
Tel. +81-6-6993-9432; Fax +81-6-6992-5023
e-mail: matsuoyo@takii.kmu.ac.jp

T. Hamaguchi
Second Department of Surgery, Mie University School of Medicine, Mie, Japan

K. Fukamachi · H. Tsuda
Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

M. Yoshida
Experimental Pathology Team, Research Center for Radiation Protection, National Institute of Radiological Sciences, Chiba, Japan

G. Watanabe · K. Taya
Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan

Chemically induced mammary preneoplastic and neoplastic lesions in rats

After exposure to chemical carcinogens, the earliest visible histological changes in the rat mammary glands are focal or multifocal hyperplasias primarily within the terminal ductule or alveolar bud or both.[21,22] The terminal end buds (TEBs) and terminal ducts are the sites of origin of malignancies, whereas benign lesions, such as cysts, adenomas, alveolar hyperplasias, and fibroadenomas, originate from the alveolar buds in the rat mammary glands.[7,21–24]

A common type of chemically induced preneoplastic lesions found in rats is ductal hyperplasia, lesions characterized by intraluminal proliferation of epithelial cells (i.e., an increase in the number of epithelial cell layers within a duct). Ductal hyperplasia, which exhibits intraductal epithelial proliferation, progresses through a phenotype very similar to human ductal carcinoma in situ (DCIS). In histological sections, intraductal proliferation increases in size and leads to the formation of microadenocarcinomas. When young virgin rats (21–50 days old) are inoculated with 7,12-dimethylbenz[a]anthracene (DMBA) or MNU, ductal hyperplasias are initially detected within 14 days, and intraductal carcinomas are detected 20 days after carcinogen administration. Eventually, locally invasive carcinomas develop from these intraductal lesions, and they become palpable tumors by approximately 13 weeks after the injection.[22,25]

Studies of the pathogenesis of rat mammary carcinomas have revealed that carcinogens act on TEBs and terminal ducts mainly when these structures are differentiating into alveolar buds. Transformed TEBs and terminal ducts evolve into ductal hyperplasias, carcinomas in situ, and invasive carcinomas.[21,24]

Morphological similarities and dissimilarities of mammary lesions in rats and humans

Rat mammary tumors have been classified by several authors.[24–26] Benign tumors, such as intraductal papilloma, papillary cystoadenoma, and adenoma, and malignant tumors, such as papillary carcinoma, cribriform carcinoma, comedo carcinoma, and tubular carcinoma, have been recognized. Most neoplastic lesions found in the rat mammary glands have counterparts in human pathology; the exceptions are human-specific lesions, such as lobular carcinoma and Paget's disease, which have no counterpart in rats. Although lobular carcinoma, in situ or invasive, has not been described in conventional strains of rats, the alveolar epithelia can transform into carcinomas in rats with certain genetic backgrounds, such as human c-Ha-*ras* Tg rats.[7]

Molecular biology of mammary carcinogenesis in human c-Ha-*ras* proto-oncogene transgenic (Tg) rats

In contrast to conventional strains, a rat strain carrying the human c-Ha-*ras* proto-oncogene is highly susceptible to mammary chemical carcinogens.[1,2] When the Tg rats are intravenously injected with 50 mg/kg body weight of MNU at 50 days of age, atypical ductal hyperplasias develop in 44% of the animals by day 15, and small invasive carcinomas develop in almost all animals by day 20. Adenocarcinomas become palpable in all animals by day 56.[3] This rat model can be used for short-term screening of chemopreventive agents as well as midterm screening of promoting agents for mammary carcinogenesis.

The Tg rats exhibit increased numbers of TEBs compared to non-Tg littermates. Confocal microscopy has revealed the level of active mitogen-activated protein kinase to be elevated in these TEBs, and a close correlation between the number of TEBs and the tumorigenic response initiated by DMBA was confirmed.[7] Single injections of MNU into the Tg rats cause mutations in codon 12 of human c-Ha-*ras* transgene in TEBs before tumor development, supporting the conclusion that these structures are the major targets of chemical carcinogens. In contrast, with spontaneous development of lesions, alveolar hyperplasia with elevated expression levels of rat and human c-Ha-*ras* proto-oncogenes is the first morphological alteration that becomes apparent. Some, but not all, hyperplastic alveolar nodules harbor mutations in the transgene.[7] These results indicate that elevated expression of the c-Ha-*ras* proto-oncogene is sufficient in itself to cause a highly proliferative phenotype of mammary alveoli. Our data suggest that TEBs and acini are the major targets for chemical and sporadic carcinogenesis, respectively, in the mammary glands of Tg rats.

Biological comparisons between non-Tg and Tg rats

Normal levels of gonadal steroids, except during the last term of pregnancy, in Tg rats

In non-Tg rats, the plasma 17β-estradiol (E_2) level reached a maximum value on day 15 of gestation and then remained high until before delivery, falling to prepregnancy levels on day 5 of lactation; then, the E_2 level generally remained low, except for a single major peak 1 day after weaning (Fig. 1A). An elevation in the levels of luteinizing hormones shortly after weaning promotes the growth of follicles, resulting in a transient increase in the E_2 level in rats. No differences between the plasma E_2 levels of non-Tg and Tg rats were observed on any day of the study except for on day 20 of gestation, when the E_2 levels in Tg rats (85.9 ± 40.2 pg/ml) were significantly ($P < 0.01$) higher than the E_2 levels in non-Tg rats (39.3 ± 14.7 pg/ml). The plasma progesterone levels in non-Tg and Tg rats were the same at each time point, except for on day 15 of gestation, when the value in Tg rats (93.5 ± 52.2 ng/ml) was significantly ($P < 0.01$) lower than that in non-Tg rats (225.9 ± 175.8 ng/ml; Fig. 1B).

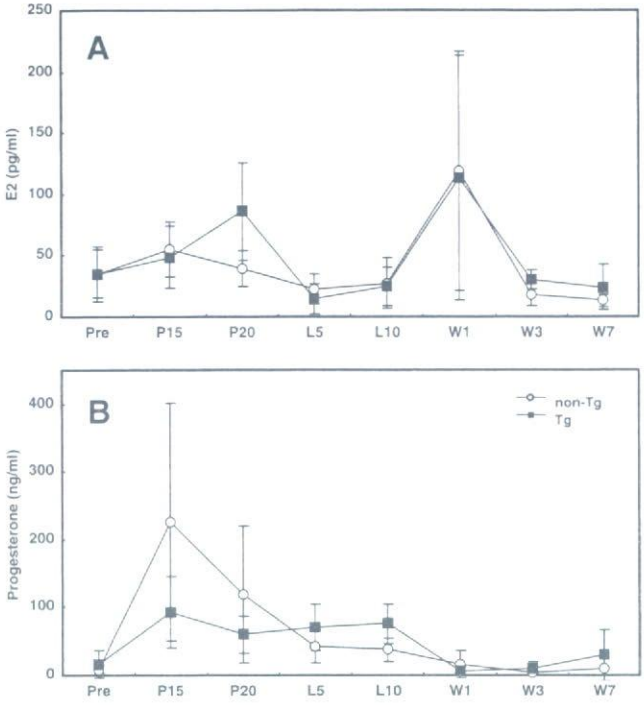


Fig. 1. Serum levels of E_2 and progesterone during pregnancy, lactation, and weaning. **A** Concentrations of serum E_2 and (**B**) serum progesterone in nontransgenic (*non-Tg*) (○) and transgenic (*Tg*) (■) rats. The results are expressed as the mean ± SD. *Pre*, virgin rats; *P15*, day 15 of pregnancy; *P20*, day 20 of pregnancy; *L5*, day 5 of lactation; *L10*, day 10 of lactation; *W1*, *W3*, and *W7*, days 1, 3, and 7 of weaning, respectively

Because E_2 is exclusively supplied by the follicle cells, which convert testosterone released from the placenta into E_2 during pregnancy,[27] larger numbers of follicle cells were expected in the Tg rats. Indeed, a histological examination of ovaries excised on day 15 of pregnancy, day 10 of lactation, and day 1 of involution revealed that the Tg ovaries contain higher numbers and larger sizes of corpora lutea than the non-Tg ovaries (Table 1). However, there is no clear explanation of the underlying mechanism responsible for this phenomenon, and the possibility that the follicle cells in Tg and non-Tg rats may be functionally different cannot be excluded.

Apoptosis occurs normally during involution in Tg mammary glands

p53-dependent apoptosis occurs in the alveolar epithelium during the first phase of involution (1–3 days after weaning), whereas p53-independent apoptosis occurs in the later phase (4 or more days after weaning).[28,29] No differences were found in the morphology or level of apoptosis, as assessed by a single-stranded DNA staining technique, between non-Tg and Tg mammary glands during pregnancy, lactation, and involution (Fig. 2A,B). The levels of apoptosis in the mammary glands of the non-Tg rats during pregnancy and lactation were very low but increased strikingly 24 h after weaning and then gradually decreased at 3 and 7 days

Table 1. Comparison of the numbers of corpora lutea in non-Tg and Tg ovaries

Genotype	Number of corpora lutea/rat[a]			
	P15	L10[b]	W1	Average[b]
non-Tg	15.7 ± 2.1	16.3 ± 3.5	17.3 ± 1.5	16.4 ± 2.3
Tg	19.7 ± 4.5	25.3 ± 2.1	23.0 ± 4.6	22.6 ± 4.2

Tg, transgenic
[a] Only corpora lutea on the surface of the ovaries were counted
[b] Significant ($P < 0.05$) difference between non-Tg and Tg rats

after weaning. The levels and temporal profile of apoptosis in the mammary glands during pregnancy, lactation, and involution in the Tg rats were the same as those in the non-Tg rats (Fig. 2A,B). Similar results were obtained using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) method. We also investigated p53-independent apoptosis in TEBs and found no differences in the levels (15%–32%) or the distribution of apoptotic cells between non-Tg and Tg mammary glands.

The expression of p53 mRNA in non-Tg mammary glands increased during the first 24 h after weaning and was detectable until 7 days after weaning (Fig. 3). The expression level on the first day after weaning was more than 10 fold the level in the lactating glands. Expression of the $p21^{Waf1}$ gene, which is p53 responsive, was very low during lactation but was rapidly induced on weaning. Reduction of $p21^{Waf1}$ to basal level after postweaning day 3 coincided with a decrease in p53 transcripts. The induction of p53 and $p21^{Waf1}$ mRNAs in Tg mammary glands occurred normally during the first 24 h after weaning, and this finding was consistent with the apoptotic profiles. We conclude that the activation of the Ras microtubule-associated protein kinase (Ras-MAPK) cascade in mammary epithelium has little, if any, effect on p53-dependent and p53-independent physiological apoptosis in the mammary glands of Tg rats.

The Ras-MAPK cascade has negative effects on p53-dependent[19] and p53-independent apoptosis under certain conditions.[30,31] However, apoptosis in involuted mammary glands after weaning occurs normally in Tg rats (see Fig. 2). The level of activation of the Ras-MAPK pathway may be sufficiently high to promote the proliferation of Tg mammary epithelium,[7] but not high enough to interfere with apoptosis under physiological conditions. Our results suggest that the promotion of proliferation, rather than the inhibition of apoptosis, by the Ras-MAPK pathway may be primarily responsible for the alveolar hyperplasia and sporadic mammary carcinomas seen in Tg rats.

In the following section, we describe recent progress in the field of breast cancer prevention that is based on results from the rat mammary carcinogenesis model.

Parity-induced protection against mammary tumorigenesis at the promotion phase in rats

In non-Tg rats, parity-induced protection against mammary carcinoma development was observed, as reported

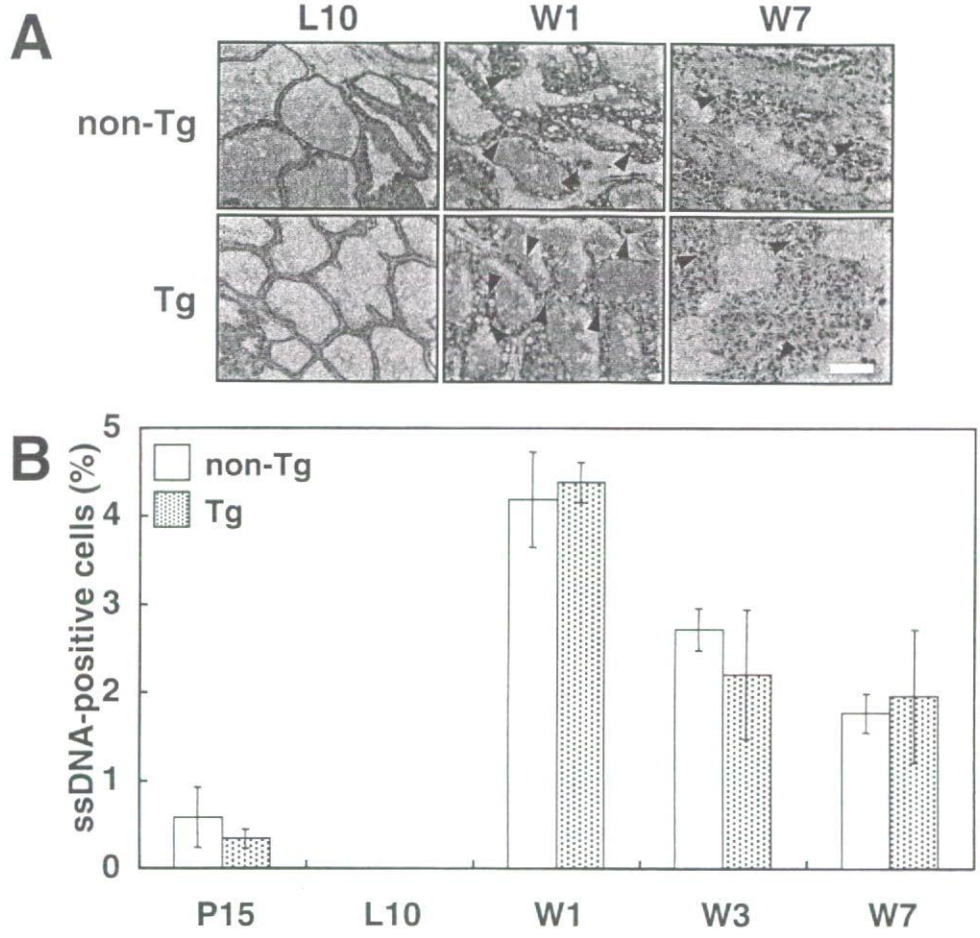


Fig. 2. Apoptosis occurs normally during mammary gland involution in c-Ha-*ras* Tg rats. **A** Single-stranded (ss) DNA staining of mammary glands from non-Tg (*non-Tg*) and Tg (*Tg*) rats on the 10th day of lactation (*L10*) and the 1st (*W1*) and 7th (*W7*) days after weaning. *Bar* 100 μm. **B** The percentages of apoptotic cells on the 15th day of pregnancy (*P15*), the 10th day of lactation (*L10*), and 1 to 7 days after weaning

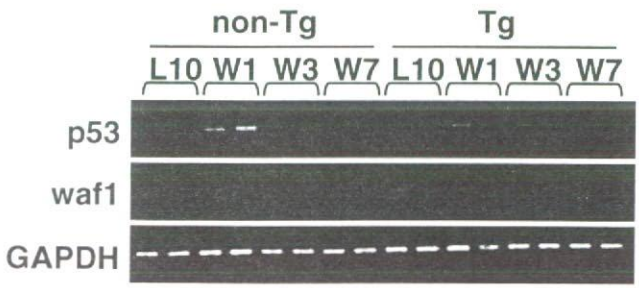


Fig. 3. Expression of p53 and $p21^{WAF1}$ genes in mammary glands during involution. The expression levels of p53 and $p21^{WAF1}$ (waf1) mRNAs in the mammary glands of non-Tg and Tg animals on the 10th day of lactation (L10) and the 1st (W1) and the 7th (W7) days after weaning were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR)

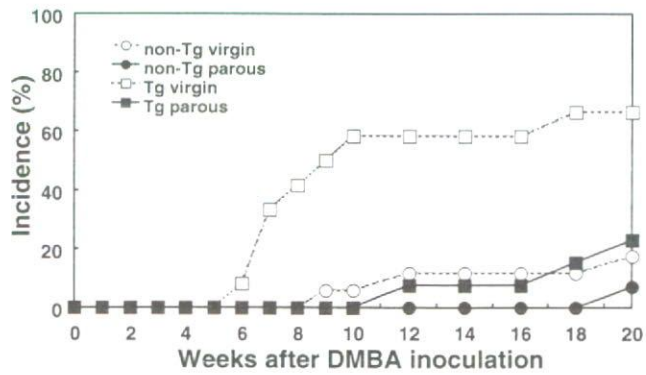


Fig. 4. Protective effect of parity against 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumorigenesis in c-Ha-*ras* Tg rats. Fifteen-week-old Tg and non-Tg parous (●, ■) or virgin (○, □) rats were given a single intragastric (i.g.) injection of DMBA. The incidence of palpable tumors was monitored for 20 weeks

previously[10–13] (Fig. 4). Twenty weeks after carcinogen treatment, parous non-Tg rats developed fewer neoplasms per animal (0.14 ± 0.53) than the age-matched virgin rats (0.59 ± 1.37). The first palpable tumor was detected at 9 weeks in the virgin rats and at 20 weeks in the parous rats (Fig. 4). Mammary tumors became palpable in 1 of 13 age-matched virgin Tg rats at 6 weeks after DMBA inoculation, and the tumor incidence gradually increased to 61.5% at 20 weeks (Fig. 4). In contrast, the first tumor became palpable in a parous Tg rat at 12 weeks, and only 28.5% (4/14) of the animals had developed lesions at 20 weeks (Fig. 4). Although there was not a significant difference in the mean weight of the mammary tumors in the two groups at the time of autopsy, significantly fewer tumors per animal were obser-

Table 2. Incidences of early lesions in the mammary glands of parous and virgin rats

	non-Tg		Tg	
	Number of rats	Incidence of early lesions[a] (%)	Number of rats	Incidence of early lesions[a] (%)
Virgin + DMBA	17	4 (23.5)	13	7 (53.8)
Parous + DMBA	14	7 (50)	14	9 (64.3)
Virgin	10	2 (20)	4	2 (50)
Parous	9	0 (0)	6	3 (50)

DMBA, 7,12-dimethylbenz[a]anthracene (DMBA)
[a] Atypical hyperplasia and small adenocarcinomas were counted as early lesions

ved in the parous Tg rats (0.50 ± 0.94) than in the age-matched virgin animals (2.00 ± 2.97). Thus, parity was protective against carcinogen-induced tumorigenesis even in this highly tumorigenic rat strain. However, microscopic surveys of mammary glands lacking gross tumors from all rats revealed that the incidence of early neoplastic lesions (atypical hyperplasia and small adenocarcinomas) did not differ between the two groups of Tg and non-Tg rats at 20 weeks (Table 2).

Because the mutation rate of the human c-Ha-*ras* transgene is markedly higher than that of the endogenous rat gene, the transgene was utilized as a probe to monitor *ras* mutations, which are thought to occur during the initiation step of carcinogenesis. Restriction fragment length polymorphism analysis detected mutations in codon 12 of the human Ha-*ras* transgene in most of the normal epithelial samples from the rats, regardless of their parity status (data not shown). Therefore, the initiation steps occurred with equal frequency in the virgin and parous mammary glands of Tg rats.

Conclusion

Several hypotheses have been proposed to account for parity-induced protective effects against mammary carcinomas. One of the best characterized mechanisms involves the p53 tumor suppressor. Kuperwasser et al. discovered that the nuclear localization and functional activation of normal p53 arises from pregnancy and hormone stimulation of the mammary gland.[32] Subsequently, p53 was shown to be a mediator of pregnancy-induced and hormone-induced resistance to mammary carcinogenesis in mice.[17,18] Pregnancy does not protect p53-null mammary epithelium against DMBA-induced tumorigenesis.[18] Nuclear p53 is sustained at a high level until 3–6 days after carcinogen exposure in mammary glands pretreated with E_2 and progesterone, thereby inducing a protective effect.[17,33] Nuclear p53 decreases 10 days after carcinogen treatment,[17,34] and the levels in virgin and parous animals are the same 20 weeks after DMBA inoculation (data not shown). Therefore, p53 appears to transiently contribute to the protection of mammary glands against chemical carcinogen-induced tumorigenesis through the elimination of DNA-damaged cells.

Several laboratories have reported that the short-term administration of ovarian steroids (estrogens and progesterone) or human chorionic gonadotropins not only before but also after carcinogen treatment decreases the incidence of mammary carcinomas in rodents.[17,35,36] Moreover, we have demonstrated that pregnancy following the carcinogen exposure also reduces mammary carcinogenesis in rats.[37] In the present study as well as previous studies, the same total number and incidence of microscopic lesions were observed at autopsy in virgin and parous rats, although pregnancy drastically reduced mammary cancer incidence and multiplicity[37] (also, Matsuoka et al., unpublished data). Therefore, both pregnancy and the hormones may have the potential to protect mammary glands from carcinogenesis at the promotion phase as well as at the initiation phase.

Pregnancy protects Tg rats from the development of mammary carcinomas by influencing carcinogenesis during the promotion phase. As mammary tumorigenesis in the Tg strain is also suppressed by soy isoflavones,[3] the Tg mammary gland may serve as a model system for elucidating the mechanisms underlying these preventive effects. We hope that the interventional studies introduced in the review series of this issue will provide clues to conquer breast cancer.

Acknowledgments We are grateful to Dr. G. D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO) for providing the antisera to 17-β-estradiol (GDN 244) and progesterone (GDN 337). This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (KAKENHI) from the Ministry of Education, Science, Sports, and Culture of Japan, a Grant-in-aid for the Second-term Comprehensive 10-Year Strategy for Cancer Control, a Grant-in-aid for Cancer Research, a Health and Labor Science Research Grant for Research on Food and Chemical Safety from the Ministry of Health, Labor and Welfare of Japan, and a Grant-in Aid for The Long-range Research Initiative from the Japan Chemical Industry Association (CC05-01).

References

1. Asamoto M, Ochiya T, Toriyama-Baba H, Ota T, Sekiya T, Terada M, Tsuda H (2000) Transgenic rats carrying human c-Ha-*ras* proto-oncogenes are highly susceptible to *N*-methyl-*N*-nitrosourea mammary carcinogenesis. Carcinogenesis (Oxf) 21: 243–249
2. Tsuda H, Fukamachi K, Ohshima Y, Ueda S, Matsuoka Y, Hamaguchi T, Ohnishi T, Takasuka N, Naito A (2005) High susceptibility of human c-Ha-*ras* proto-oncogene transgenic rats to carcinogenesis: a cancer-prone animal model. Cancer Sci 96:309–316
3. Matsuoka Y, Fukamachi K, Hamaguchi T, Toriyama-Baba H, Kawaguchi H, Kusunoki M, Yoshida H, Tsuda H (2003) Rapid emergence of mammary preneoplastic and malignant lesions in human c-Ha-ras proto-oncogene transgenic rats: possible

application for screening of chemopreventive agents. Toxicol Pathol 31:632–637
4. Tsuda H, Asamoto M, Ochiya T, Toriyama-Baba H, Naito A, Ota T, Sekiya T, Terada M (2001) High susceptibility of transgenic rats carrying the human c-Ha-*ras* proto-oncogene to chemically-induced mammary carcinogenesis. Mutat Res 477:173–182
5. Han BS, Fukamachi K, Takasuka N, Ohnishi T, Maeda M, Yamasaki T, Tsuda H (2002) Inhibitory effects of 17-beta-estradiol and 4-*n*-octylphenol on 7,12-dimethylbenz[a]anthracene-induced mammary tumor development in human c-Ha-*ras* proto-oncogene transgenic rats. Carcinogenesis (Oxf) 23:1209–1215
6. Hamaguchi T, Matsuoka Y, Bechberger J, Ohnishi T, Fujita K, Naus CC, Kusunoki M, Tsubura A, Tsuda H (2006) Establishment of an apoptosis-sensitive rat mammary carcinoma cell line with a mutation in the DNA-binding region of p53. Cancer Lett 232:279–288
7. Hamaguchi H, Matsuoka Y, Kawaguchi H, Fukamachi K, Takasuka N, Ueda S, Shimizu K, Ohki M, Kusunoki M, Sakakura T, Yoshida H, Tsuda H (2004) Terminal endbuds and acini as the respective major targets for chemical and sporadic carcinogenesis in the mammary glands of human c-Ha-*ras* protooncogene transgenic rats. Breast Cancer Res Treat 83:43–56
8. Kelsey JL, Gammon MD (1991) The epidemiology of breast cancer. CA Cancer J Clin 41:146–165
9. Rosner B, Colditz GA, Willett WC (1994) Reproductive risk factors in a prospective study of breast cancer: the Nurses' Health Study. Am J Epidemiol 139:819–835
10. Russo J, Russo IH (1980) Susceptibility of the mammary gland to carcinogenesis. II. Pregnancy interruption as a risk factor in tumor incidence. Am J Pathol 100:497–512
11. Thordarson, G, Jin E, Guzman RC, Swanson SM, Nandi S, Talamantes F (1995) Refractoriness to mammary tumorigenesis in parous rats: is it caused by persistent changes in the hormonal environment or permanent biochemical alterations in the mammary epithelia? Carcinogenesis (Oxf) 16:2847–2853
12. Sivaraman L, Stephens LC, Markaverich BM, Clark JA, Krnacik S, Conneely OM, O'Malley BW, Medina D (1998) Hormone-induced refractoriness to mammary carcinogenesis in Wistar-Furth rats. Carcinogenesis (Oxf) 19:1573–1581
13. Guzman RC, Yang J, Rajkumar L, Thordarson G, Chen X, Nandi S (1999) Hormonal prevention of breast cancer: mimicking the protective effect of pregnancy. Proc Natl Acad Sci U S A 96: 2520–2525
14. Medina D, Smith GH (1999) Chemical carcinogen-induced tumorigenesis in parous, involuted mouse mammary glands. J Natl Cancer Inst 91:967–969
15. Grubbs CJ, Farnell DR, Hill DL, McDonough KC (1985) Chemoprevention of *N*-nitroso-*N*-methylurea-induced mammary cancers by pretreatment with 17-beta-estradiol and progesterone. J Natl Cancer Inst 74:927–931
16. Grubbs CJ, Juliana MM, Whitaker LM (1988) Short-term hormone treatment as a chemopreventive method against mammary cancer initiation in rats. Anticancer Res 8:113–117
17. Sivaraman L, Conneely OM, Medina D, O'Malley BW (2001) p53 is a potential mediator of pregnancy and hormone-induced resistance to mammary carcinogenesis. Proc Natl Acad Sci U S A 98:12379–12384
18. Medina D, Kittrell FS (2003) p53 function is required for hormone-mediated protection of mouse mammary tumorigenesis. Cancer Res 63:6140–6143
19. Ries S, Biederer C, Woods D, Shifman O, Shirasawa S, Sasazuki T, McMahon M, Oren M, McCormick F (2000) Opposing effects of Ras on p53: transcriptional activation of mdm2 and induction of p19ARF. Cell 103:321–330
20. Ryan KM, Phillips AC, Vousden KH (2001) Regulation and function of the p53 tumor suppressor protein. Curr Opin Cell Biol 13:332–337
21. Russo J, Russo IH (1987) Biological and molecular bases of mammary carcinogenesis. Lab Invest 57:112–137
22. Thompson HJ, McGinley JN, Wolfe P, Singh M, Steele VE, Kelloff GJ (1998) Temporal sequence of mammary intraductal proliferations, ductal carcinomas in situ and adenocarcinomas induced by 1-methyl-1-nitrosourea in rats. Carcinogenesis (Oxf) 19:2181–2185
23. Russo J, Russo IH (1996) Experimentally induced mammary tumors in rats. Breast Cancer Res Treat 39:7–20
24. Singh M, McGinley JN, Thompson HJ (2000) A comparison of the histopathology of premalignant and malignant mammary gland lesions induced in sexually immature rats with those occurring in the human. Lab Invest 80:221–231
25. Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR, van Zwieten MJ (1990) Comparative study of human and rat mammary tumorigenesis. Lab Invest 62:244–278
26. Russo J, Russo IH (2000) Atlas and histologic classification of tumors of the rat mammary gland. J Mamm Gland Biol Neoplasia 5:187–200
27. Taya K, Greenwald GS (1981) In vivo and in vitro ovarian steroidogenesis in the pregnant rat. Biol Reprod 25:683–691
28. Li M, Liu X, Robinson G, Bar-Peled U, Wagner KU, Young WS, Hennighausen L, Furth PA (1997) Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. Proc Natl Acad Sci U S A 94:3425–3430
29. Jerry DJ, Pinkas J, Kuperwasser C, Dickinson ES, Naber SP (1999) Regulation of p53 and its targets during involution of the mammary gland. J Mamm Gland Biol Neoplasia 4:177–181
30. Peli J, Schroter M, Rudaz C, Hahne M, Meyer C, Reichmann E, Tschopp J (1999) Oncogenic Ras inhibits Fas ligand-mediated apoptosis by downregulating the expression of Fas. EMBO J 18:1824–1831
31. Kazama H, Yonehara S (2000) Oncogenic K-Ras and basic fibroblast growth factor prevent Fas-mediated apoptosis in fibroblasts through activation of mitogen-activated protein kinase. J Cell Biol 148:557–566
32. Kuperwasser C, Pinkas J, Hurlbut GD, Naber SP, Jerry DJ (2000) Cytoplasmic sequestration and functional repression of p53 in the mammary epithelium is reversed by hormonal treatment. Cancer Res 60:2723–2729
33. Rajkumar L, Guzman RC, Yang J, Thordarson G, Talamantes F, Nandi S (2001) Short-term exposure to pregnancy levels of estrogen prevents mammary carcinogenesis. Proc Natl Acad Sci U S A 98:11755–11759
34. Sivaraman L, Medina D (2002) Hormone-induced protection against breast cancer. J Mamm Gland Biol Neoplasia 7:77–92
35. Medina D, Peterson LE, Moraes R, Gay J (2001) Short-term exposure to estrogen and progesterone induces partial protection against *N*-nitroso-*N*-methylurea-induced mammary tumorigenesis in Wistar–Furth rats. Cancer Lett 169:1–6
36. Swanson SM, Whitaker LM, Stockard CR, Myers RB, Oelschlager D, Grizzle WE, Juliana MM, Grubbs CJ (1997) Hormone levels and mammary epithelial cell proliferation in rats treated with a regimen of estradiol and progesterone that mimics the preventive effect of pregnancy against mammary cancer. Anticancer Res 17: 4639–4645
37. Yang J, Yoshizawa K, Nandi S, Tsubura A (1999) Protective effects of pregnancy and lactation against *N*-methyl-*N*-nitrosourea-induced mammary carcinomas in female Lewis rats. Carcinogenesis (Oxf) 20:623–628

［2］食物摂取，水の飲用

はじめに

最近では，ナノの大きさを持つ物質の特性を利用して，意図して摂取することを目的として製造された製品が出回り始めてきている。一方，摂取を積極的な目的としないまでも，食品などの口から体内に入るものの原材料の一部や包装材またはその原料にナノマテリアルが使用されている。これらの状況を考えると，今後，われわれの生活環境からナノ物質を口から摂取する機会が増加する可能性が高くなっていくと推測される。吸入や皮膚からの曝露に比べ，経口摂取の場合は，不特定多数の人たちが継続して曝露されることを想定しなければならず，配慮していかなければならない。

これらのナノマテリアルが口から入る場合には，吸入などの気道から入る場合や，皮膚の表面から取り込まれる場合と異なり，食品や水と同時に摂取され，その消化・吸収の過程と密接にかかわっており，直接表層の細胞と接する密度が少ないことが特徴の一つと考えられる。しかし，実際のところ，ナノ粒子が口から体内に入った場合の吸収や排泄等の挙動については，ほとんど情報がないのが現状である。これは，これまでは化学物質としての挙動の解析を中心として研究されてきた経緯が背景としてある。ナノ粒子は，粒子の大きさが極端に小さい物質であること，固有の化学的な性質に加え，単位重量あたりの表面積の大きさが異なること，表面電荷の異なることがあるなど，これまでの検討課題として考慮されてこなかった観点を考慮しなければならないであろう。すなわち，ナノマテリアルに関する課題が明らかとなってきた現時点で取り組みが始まったといってもよいであろう。このため，本稿では，現在得られている知見を概観した上で，課題を明らかとし，課題解明への取り組みをどのように考えていくべきかを整理したい。

1．吸収機構の解明の課題

まず，ナノ粒子が超微粒子の形態で摂取されても，食品が消化される過程でどのような形態変化をとるか明らかとしなければならないであろう。ナノ粒子の表面状態や水への分散性を反映して，口腔，食道，胃，十二指腸，小腸，大腸と消化管を通過する過程で，共存する食品などの変化，水分含量，消化管部位の相違などの環境の変化にしたがい，ナノ粒子のままで存在するのか，凝集体を形成するのか，凝集体としてどの部位で変化するのか，それぞれ状態が異なってくる。それにしたがって吸収のされ方も異なってくるであろう。例えば，超微粒子の形態であれば細胞間を経て体内に入る可能性もある。凝集体の大きさによっては，細胞と接触して細胞内に入る可能