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Letter

Study on penetration of titanium dioxide (TiO_2) nanoparticles into intact and damaged skin *in vitro*

Mika Senzui[1], Toshiaki Tamura[1], Keiko Miura[1], Yoshiaki Ikarashi[2], Yoshiteru Watanabe[1] and Makiko Fujii[1]

[1]Showa Pharmaceutical University, 3-3165 Higashi-Tamagawagakuen, Machida, Tokyo 194-8543, Japan
[2]National Institute of Health Science, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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ABSTRACT — It is important for toxicological assessment of nanoparticles to determine the penetration of nanoparticle in skin qualitatively and quantitatively. Skin penetration of four different types of rutile titanium dioxide (TiO_2) (T-35, 35 nm, non-coating; TC-35, 35 nm, with almina/silica/silicon coating; T-disp, 10 x 100 nm, mixture of almina coated and silicon coated particles, dispersed in cyclopentasiloxan; T-250, 250 nm, non-coating) was determined with *in vitro* intact, stripped, and hair-removed skin of Yucatan micropigs to study the effect of dispersion and skin conditions. The TiO_2 was suspended in a volatile silicone fluid used for cosmetics, cyclopentasiloxane, at a concentration of 10%. The suspension was applied at a dose 2 μl/cm^2 for 24 hr, followed by cyanoacrylate stripping. The Ti concentration in skin was determined by ICP-MS. T-35 and T-250 easily aggregated in suspension with a mean diameter greater than 1 μm. TC-35 and T-disp showed good dispersion properties with a mean diameter in suspension of approximately 100 nm. No penetration was observed regardless of TiO_2 type in intact and stripped skin. The concentration of Ti in skin was significantly higher when TC-35 was applied on hair-removed skin. SEM-EDS observation showed that Ti penetrated into vacant hair follicles (greater than 1 mm below the skin surface), however, it did not penetrate into dermis or viable epidermis.

Key words: Nanoparticle, Skin penetration, Hair-removed skin, Stripped skin, Titanium oxide

INTRODUCTION

Titanium dioxide (TiO_2) has been used for cosmetics and is considered to be safe for topical use. Recently, TiO_2 nanoparticles (particle size < 100 nm) are used commonly in cosmetics because of their high transparency in visible wavelengths but high attenuation for UV wavelengths (Popov *et al.*, 2005). However, the safety of different conventional size particles is a concern. Safety concerns are based on not only its toxicity characteristics, but also on the potential increase in amount of absorption. In theoretical, materials with an appropriate octanol/water partition coefficient and low molecular weight (< *ca.* 500) penetrate skin through the stratum corneum (SC); therefore, penetration of inorganic particles into intact skin is not possible (Roberts *et al.*, 2002). Some studies indicate that TiO_2 and other inorganic particles, even on a nano-grade scale, do not penetrate skin (Schulz *et al.*, 2002; Pinheiro *et al.*, 2007; Nohynek *et al.*, 2008). However, some nanoparticles can penetrate viable skin (Ryman-Rasmussen *et al.*, 2006; Menzel *et al.*, 2004). Inorganic particles are often lyophobic in both water and oil, dispersed particles easily aggregate to form large (micro-grade) particles. A few studies investigating both dispersibility and skin penetration have been reported (Bennat and Müller-Goymann, 2000). The present study focused on skin penetration of TiO_2 *in vitro* with different dispersibility of TiO_2 and skin condition.

MATERIALS AND METHODS

Materials

All types of TiO_2 used in this study were rutile-type (Table 1). Cyclopentasiloxane (silicone, KF-995, Shin-Etsu Chemical, Co., Tokyo, Japan) was used as the dispersing medium. Purified water and nitric acid used in TiO_2 quantitative analysis were ultra-microanalysis grade from Wako Pure Chemicals Industries, Ltd. (Osaka,

Correspondence: Makiko Fujii (E-mail: fujii@ac.shoyaku.ac.jp)

Table 1. Titanium dioxide used in this study

Abbreviation	Primary particle size*	Coating
T-35	35 nm	uncoated
TC-35	35 nm	alumina · silica · silicone
T-disp	10 nm x 100 nm	mixture of alumina coated and silicone coated
T-250	250 nm	uncoated

All TiO_2 are rutil-type.
* from catalogue of source company.

Japan). A stock solution of titanium containing 1,000 mg/l (Kanto Chemical Co., Inc., Tokyo, Japan) was used to produce standards for calibration curves for Ti analysis. All other chemicals were of reagent grade.

Preparation of suspensions

Drops of silicone were added to a weighed amount of TiO_2 powder in a tube, followed by kneading. Additional silicone was added to bring the concentration of TiO_2 samples to 10%, followed by sonication in a bath-type sonicator (US-3, Iuchi, Tokyo, Japan) for 30 min. The T-disp was diluted with silicone for a final TiO_2 concentration of 10%, followed by sonication.

Evaluation of TiO_2 suspensions

The particle size of TiO_2 in suspension was measured using a dynamic laser scattering apparatus (DLS-8000HL, Otsuka Electrons Co., Osaka, Japan) after a thousand-fold dilution with silicone.

Skin conditions after application of TiO_2 was observed using two methods. Two µl of suspension were applied to an area of skin of approximately 1 cm^2. After drying, the skin surface was observed by digital fine scope microscopy (VC-3000, Omron, Tokyo, Japan) with a magnification of 80. The epidermis of YMP skin prepared by a heat separating method (Kligman and Christophers, 1963) was mounted on a scanning electron microscopy (SEM) stage with adhesive tape. One µl of the suspension were spread over approximately 0.5 cm^2 and dried *in vacuo*. Then, the skin sample was coated with Pt/Pd and examined using SEM (JSM-5200LV, JEOL, 20 kV).

Skin penetration

Yucatan micropig (YMP) skin was used as the model because of its similarity with human skin (Lavker *et al.*, 1991; Fujii *et al.*, 1997). YMP skin (YMP skin set, Charles River Japan, Kanagawa, Japan) removed the subdermal tissue and fat was used as full-thickness skin (intact skin). The SC was removed from intact skin with adhesive tape (Scotch 313, 3M) (stripped skin). Hair was removed from intact skin using tweezers (hair-removed skin).

Two µl of suspension were applied to an area of skin of approximately 1 cm^2. Then the skin was placed on a modified Franz-type diffusion cell. After 24 hr, the receptor phase (pH 7.1 isotonic phosphate buffer solution) was collected, the skin was removed from the diffusion cell and cut off the rim for mounting the cell. Residues on the skin surface were removed by two cyanoacrylate (Aronalfa, Toagosei, Tokyo, Japan) stripping and Ti in the skin was determined. Application amount and period were in accordance with Standard SPF Test Method of Japan Cosmetic Industry Association (1999) and OECD (2004): Test Guideline 428 (skin absorption: *in vitro* method), respectively. For some samples, the epidermis and dermis were separated by heating after cyanoacrylate stripping. A similar procedure was used for obtaining SEM pictures with energy dispersed X-ray spectrometry (SEM-EDS).

Determination of Ti

Approximately 0.1 g of skin or 1 ml of receptor phase was transferred to a Teflon digestion vessel and 5 ml of nitric acid plus 1 ml of purified water was added to each vessel. The vessels were placed in a microwave oven (MARS5, CEM Co., Matthews, NC, USA). The microwave-assisted digestion consisted of increasing the pressure to 80 psi over 20 min and then maintaining that pressure for 20 min by applying 100% power at 1,600 W. For separated epidermis and dermis, approximately 1 cm^2 of skin (*ca.* 0.01 g of epidermis, 0.3 g of dermis) was used. After digestion, the resulting solution was fixed with 20 ml of purified water. The Ti concentration in the samples was measured by ICP-MS (7500, Agilent Technologies, Santa Clara, CA, USA). The amount of Ti was calculated using a standard curve of Ti created with the peak area at mass number 47.

Ti distribution in skin

The skin sample was fixed with Karnovsky solution, dehydrated with ethanol, and replaced with resin. Horizontal cuts were made in the skin from the surface to the dermis and observations were obtained every 50 μm using SEM-EDS (JSM-6700/JED2300, JEOL, Tokyo, Japan).

Statistical analysis

The amount of Ti in skin was determined using at least 3 samples and the data subjected to analysis of variance (ANOVA) followed by Dunnett's test. A value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Particle size of TiO_2 in suspension and on the skin surface

Both oil in water (O/W) and water in oil (W/O) creams are used as sunscreen formulations with TiO_2. TiO_2 is usually formulated in the oil phase; the oil suspension using silicone, which is often used for the base of sunscreens because of volatile and repels water characteristics, was used for this study. Many types of TiO_2 particles exist, with differences in crystalline type, size, shape, and surface coating characteristics. Four types of rutile TiO_2 shown in Table 1 was used for this study. T-35 was used to represent typical nanoparticles, with a round shape and no surface coating. TC-35, which has lipophilic coating features, was chosen to produce a good dispersion in silicone. T-disp is a pre-formulated product consisting of TiO_2 dispersed in silicone. The T-250 was used for comparison because it does not form nanoparticles.

Even if the primary particle size is less than 100 nm, lyophobic colloidal dispersions are unstable and aggregation occurs easily. Particle size distributions of TiO_2 in silicone are shown in Fig. 1. Mean particle size of T-35 was 1,700 nm, which was larger than that of T-250, 1,200 nm. Although the possibility exists that large particles shielded small particles, few nanoparticles were found. In contrast, suspensions of TC-35 and T-disp contained nanoparticles with mean diameters of 80 and 130 nm, respectively. The TC-35 suspension contained large particles that were easy to precipitate.

After skin application of suspensions, silicone was spread and vaporized so that only TiO_2 particles remained on the skin. Fig. 2(a) shows microscopic pictures of the surface after application of each suspension to skin followed by drying. T-250 and T-35 suspension showed aggregated white powder. After application of the TC-35 suspension, the skin was covered with white film that was thicker in furrows. Silicone spread easily on the skin and tended to collect in furrows because of low viscosity and interface tension. No particles were observed but the skin was slightly white after application of T-disp suspension. The SEM pictures showed large agglomerated TiO_2 (about 5 μm) for T-35 and T-250, although their primary particle sizes were different. The TC-35 also aggregated into particles of approximately 1 μm, although many small particles stuck to the skin. The T-disp formed uniformly agglomerated particles that differed from other preparations. TiO_2 particles appeared to be covered with dispersing agent (Figs. 2(b), (c)).

Skin penetration of TiO_2

Cosmetics and sunscreens are usually used only on intact skin. However, skin can be injured slightly by objects or through physical force. Thus, skin penetration of TiO_2 was investigated *in vitro* with intact skin and with stripped skin as a model of injured skin. Previous reports have indicated that hair follicles are important in the skin penetration of nanoparticles (Lekki *et al.*, 2007; Zvyagin *et al.*, 2008). Therefore, hair-removed skin was used to represent skin damaged by hair-removal treatments often done for the pursuit of beauty.

After 24-hr application, the skin surface was stripped with cyanoacrylate to remove surface TiO_2. A tape stripping technique is often used to remove residual materials on skin; however, materials in furrows or on hair follicles cannot be removed by this technique (Pflücker *et al.*, 1999). In this study, the amount of Ti varied greatly when

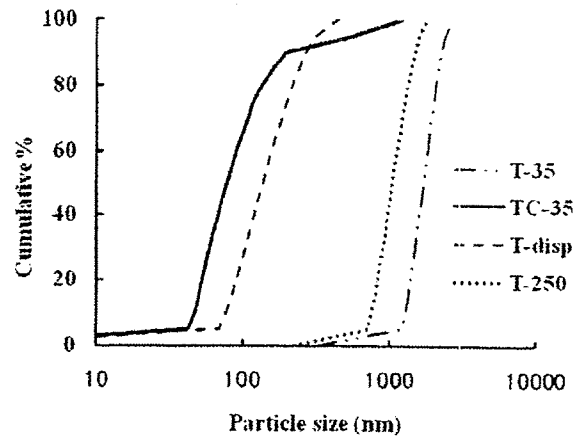

Fig. 1. Particle size of TiO_2 dispersed in silicone. Particle size was measured by dynamic laser scattering using a thousand-fold dilution of 10% TiO_2 silicone suspension.

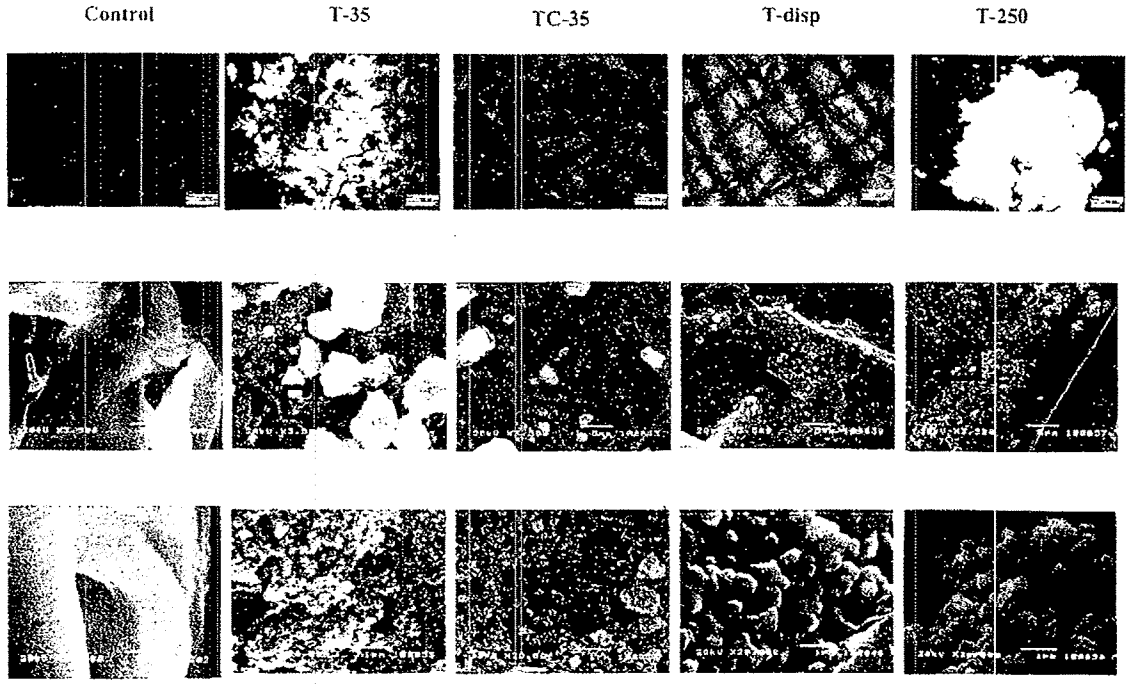

Fig. 2. The conditions of TiO_2 after skin application of a 10% silicon suspension of TiO_2 at a dose of 2 μl/cm². The skin surface was observed using a digital fine scope at a magnification of (a) 80. The surface of the epidermis was observed by SEM at a magnification of (b) 3,500 and (c) 20,000.

Ti remained in furrows (data not shown), so cyanoacrylate stripping was used. In this procedure, the surface SC layer of intact skin and hair-removed skin, and some hairs of intact skin and stripped skin were also removed.

Silicone is used to control formulation properties. Ti concentration in the receptor phase was similar in all skin conditions and formulations applied (Fig. 3(a)). Fig. 3(b) shows Ti concentration values in skin. For intact and stripped skin, no significant difference in Ti concentration was found between the control and suspension applications, which indicates TiO_2 did not penetrated into the skin regardless of particle size and even when the SC, which is the skin's primary barrier, was removed. For hair-removed skin, Ti concentration in skin after application of TC-35 suspension was significantly higher than that of the control, and after application of T-disp suspension, tended to be high. The Ti concentration in the dermis was no different than that of the control. Ti concentration in the epidermis after application of TiO_2 nanoparticles tended to be greater than that of the control, but the difference was not significant (Fig. 4). The epidermis consists of SC, viable epidermis and hair follicles. The horizontal sections from hair-removed skin after application of TC-35 suspension were observed using SEM-EDS. One of two SEM-EDS images showed the presence of Ti in the empty hair follicle after removal of the hair shaft 1 mm from the surface (Fig. 5(a)). Ti was detected in the hair follicle pocket, but not in the surrounding viable skin (Fig. 5(b)). We also found the similar distribution of 20 nm FITC-polystyrene (data not shown). The radius of a hair follicle is 0.05-0.2 mm (Otberg *et al.*, 2004), which would allow solvent to enter if the hair shaft and sebum did not fill the follicle space. When fluid enters a small space by capillary action, small particles of Ti in fluid may be able to enter the follicle. Large particles cannot be moved by such a small force, but TC-35 well dispersed in solvent might enter a follicle more easily than other types of TiO_2. For T-disp, the dispersing agent had some effect, resulting in particles left on the skin after drying of the suspension.

In conclusion, TiO_2 does not penetrate into viable skin, even if the particle size is less than 100 nm and the SC is damaged. However, immediately after hair removal, some TiO_2 particles penetrated relatively deeply into the

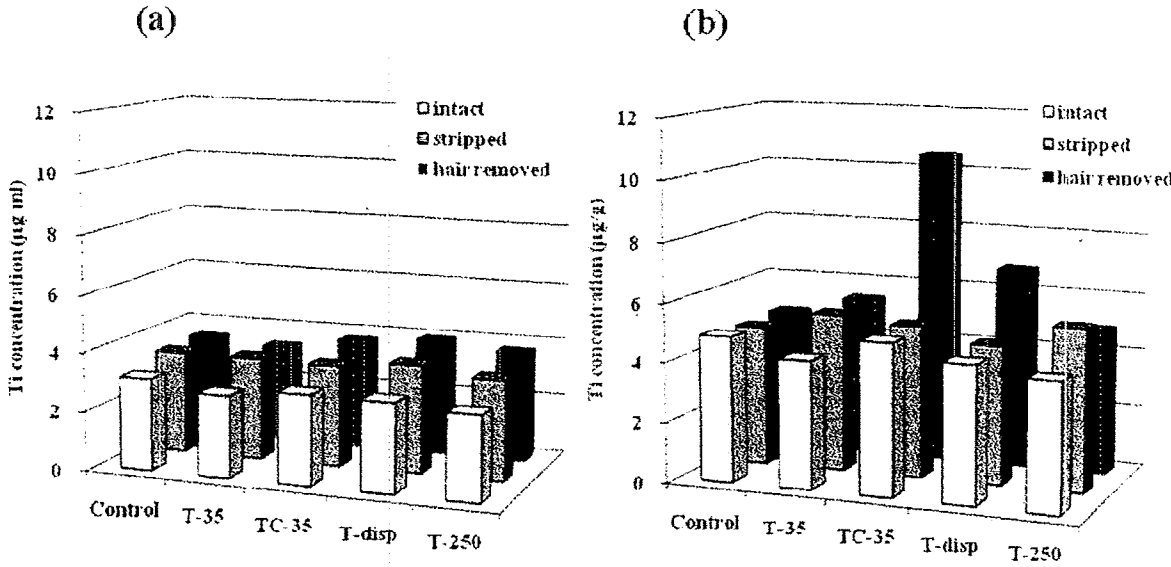

Fig. 3. Ti concentration in receptor phase (a) and in skin (b) after 24 hr application of a 10% silicone suspension of TiO_2 at a dose of 2 μl/cm² on intact skin, stripped skin, and hair-removed skin.
Silicon applied as a control. TiO_2 on the skin surface was removed by cyanoacrylate stripping.

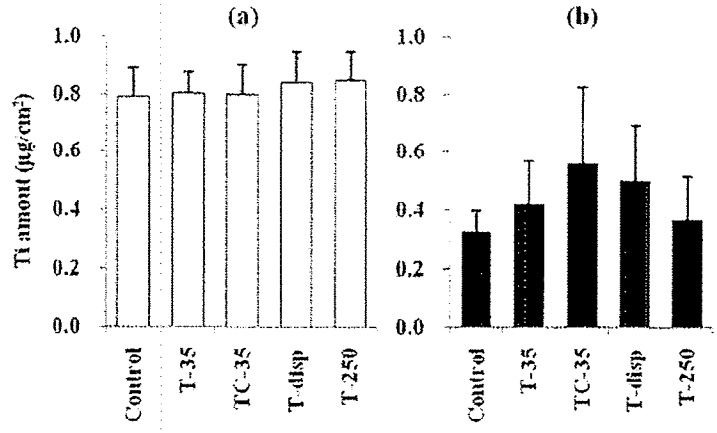

Fig. 4. Amount of Ti in (a) dermis and (b) epidermis after 24 hr application of a 10% silicone suspension TiO_2 at a dose of 2 μl/cm² to hair-removed skin.
Silicone was applied as a control. TiO_2 on the skin surface was removed by cyanoacrylate stripping, followed by separation of the dermis and epidermis by heat.

skin, possibly by entering the empty hair follicle. This was an *in vitro* study, so no inflammation was induced by the hair removal procedure. Inflammation could affect these results, therefore, further *in vivo* studies on viable skin with the hair removed are necessary.

There might be various nanoparticls to determine skin penetration, thus *in vitro* method is important for screening. If the materials have possibility to use on hair

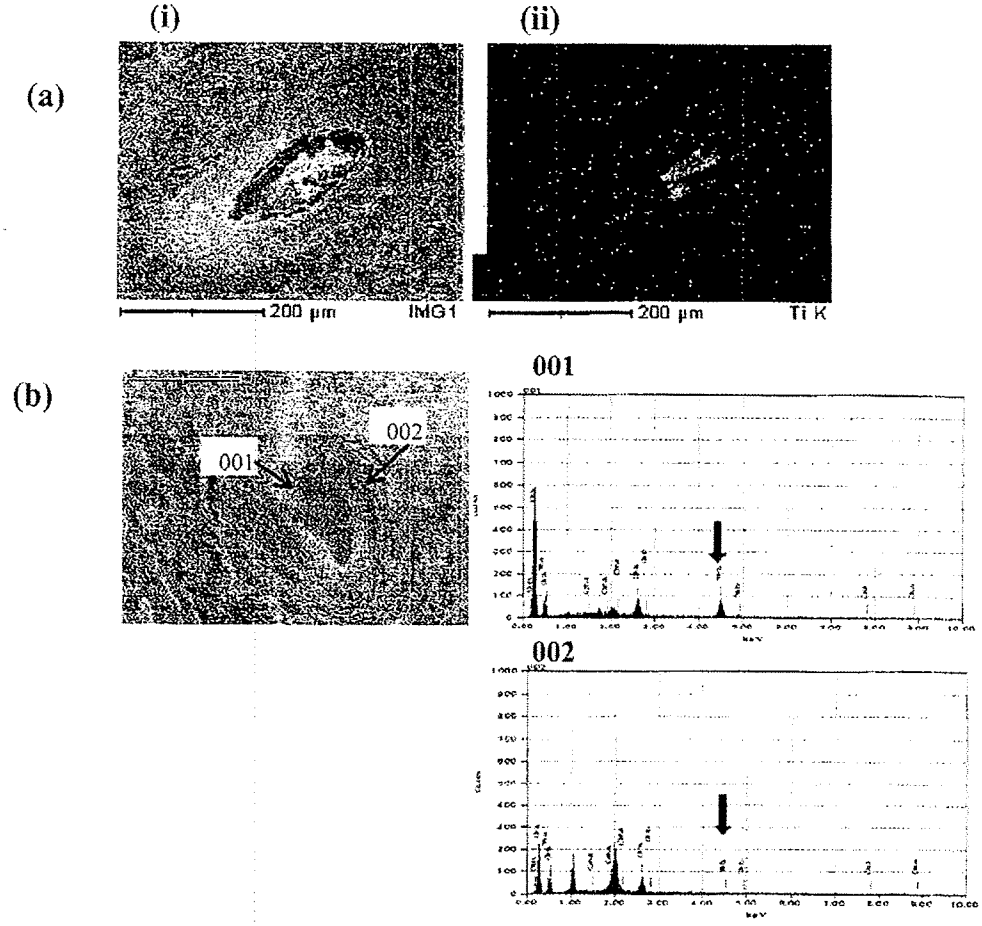

Fig. 5. SEM-EDS images (a) and elemental analysis (b) of a horizontal section of skin after 24 hr application of a 10% silicone suspension of TC-35 at a dose of 2 μl/cm² to hair-removed skin.
(a) at a depth of 1,050 μm from the skin surface, (i) SEM images, (ii) Ti distribution; (b) at a depth of 1,250 μm from the skin surface, (001) the hair follicle, (002) the dermis in contact with the hair follicle.

removed skin, hair removed skin should be taken into safety assessment of nanoparticles as well as stripped skin as a model of damaged skin. Also, this results indicate that the split skin (thickness 200~400 μm) which OECD Test guideline 428 recommended for skin absorption study *in vitro* have possibility to overestimate the skin permeation of nanoparticles because hair follicle is cut and nanoparticle in hair follicle is into receptor phase.

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Possible enhancing activity of diacylglycerol on 4-nitroquinoline 1-oxide induced carcinogenesis of the tongue in human c-Ha-*ras* proto-oncogene transgenic rats

Hiroyuki Tsuda [a,c,*], Masaaki Iigo [c], Nobuo Takasuka [c], Shinobu Ueda [d], Yutaka Ohshima [a], Katsumi Fukamachi [a], Tomoyuki Shirai [b], Sachiko Hirano [c], Eiji Matsuda [c], Keiji Wakabayashi [c]

[a] *Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan*
[b] *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*
[c] *Cancer Prevention Basic Research Project, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan*
[d] *Section for Studies on Metastasis, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan*

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Abstract

1,2-Diacylglycerol (1,2-DAG) is involved in cell proliferation as an activator of protein kinase C (PKC) and has been shown to stimulate growth of cancer cells, raising the possibility of a role in tumor promotion. Ingested DAG oil, containing 70% 1,3-DAG and 30% 1,2-DAG, is digested and considered to be safe as edible oil. However, DAG may directly contact with oral cavity mucosa in undigested form. The present study was conducted to examine the effects of DAG oil on carcinogenesis in c-Ha-*ras* proto-oncogene transgenic (Tg) rats administered 4-nitroquinoline 1-oxide (4NQO, 10 ppm) in their drinking water for 10 weeks for initiation of mainly upper digestive organs. DAG oil added in basal diet at 5.5%, 2.75%, 1.38% and 0% with total fat made up to 5.5% with triacylglycerol (TAG) was administered during the initiation and post-initiation period. The study was terminated at week 12 (Tg females) and 20 (Tg males, wild females and males). The fatty acid composition of DAG oil was similar to TAG (linoleic acid 46.6% and oleic acid 38.9%). In Tg male rats, DAG oil administration was associated with significant increase ($P < 0.05$) in the incidence of squamous cell carcinomas (SCC) of the tongue (5.5% DAG, 43.8%; 2.75% DAG, 20%; 1.38% DAG, 14.3%; 0%, 12.3%) with the Cochran–Armitage trend test and also number of tumors in coefficients for linear contrast trend tests. Tongue SCC induction of wild males and all females was not significant. The present results suggest that DAG oil may have enhancing and/or promotion potential for tongue carcinogenesis in male Tg featuring elevated *ras* expression.
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Keywords: Diacylglycerol; Tongue; Tumor; Rat; *ras*

1. Introduction

Diacylglycerol (DAG) oil composed of 1,3-DAG (70%) and 1,2-DAG (30%) has been used for cooking oil in Japan and the USA. A small amount of DAG exists in various vegetable oils. Orally administered DAG oil is not carcinogenic to rats in a chronic feeding study (Soni et al., 2001) possibly because it undergoes hydrolysis by lipase to yield free fatty acids and monoacylglycerols in the small intestine

Abbreviations: DAG, diacylglycerol; 4NQO, 4-nitroquinoline 1-oxide; Tg, transgenic; TAG, triacylglycerol; SCC, squamous cell carcinomas; PKC, protein kinase C; TG, triglycerides; FFA, free fatty acids; T-Cho, total cholesterol; LP, lipoprotein; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase.

* Corresponding author. Address: Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. Tel.: +81 52 853 8991; fax: +81 52 853 8996.
E-mail address: htsuda@med.nagoya-cu.ac.jp (H. Tsuda).

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in rats so that DAG itself is not absorbed in the lower gastro-intestinal tract (Osaki et al., 2005). DAG oil is also reported not mutagenic (Kasamatsu et al., 2005). Furthermore, in DMBA-induced mammary carcinogenesis in rats, DAG oil did not enhance mammary tumor development more than edible TAG (Sugano et al., 2002). Accordingly, safety of the DAG oil has been generally recognized.

1,2-DAG has been shown to stimulate protein kinase C (PKC) an enzyme that plays a key role in growth control and signal transduction (Reddy et al., 1996; Sozeri et al., 1992). These functions are similar to a potent tumor promoting agent, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which contains a 1,2-DAG-related structure in its molecule and is reported to directly activate PKC (Castagna et al., 1982). PKC is normally activated by endogenous 1,2-DAG released from membrane phospholipids (Hurley et al., 1997; Nishizuka, 1984) and exogenous 1,2-DAG can also stimulate intracellular signaling pathways (Friedman et al., 1989), resulting in mitosis in a certain carcinoma cells *in vitro* (Liscovitch and Cantley, 1994). 1,2-DAG can also mediate cellular responses through a binding site unrelated to PKC that possesses a C1 domain like Ras guanyl nucleotide-releasing protein (RasGRP) (Kazanietz, 2002) and activated RasGRP has been implicated in activation of the Ras effector Raf-1 and thereby activation of the MAP kinase cascade (Lorenzo et al., 2000). Thus, increased levels of exogenous 1,2-DAG may be considered a tumor-promoting risk factor in many organs.

Several studies have shown that a high-fat diet results in increased fecal concentrations of 1,2-DAG (Choe et al., 1992; Pickering et al., 1995). Moreover, fecal bacteria in the colon can produce 1,2-DAG (Vulevic et al., 2004) which could impact directly on colon carcinogenesis (Friedman et al., 1989). Furthermore, direct absorbance by the tongue and other tissues in the oral cavity is conceivable so that carcinogenesis in these sites could be affected. To determine whether DAG oil enhances tongue carcinogenesis induced by 4-nitroquinoline 1-oxide (4NQO) (Suzui et al., 1995; Tang et al., 2004), the present study was therefore performed with Tg rats which over-express *ras* and are highly susceptible to induction of mammary, urinary bladder, esophagus, skin and tongue (Suzuki et al., 2006) tumors by chemical carcinogens (Asamoto et al., 2000; Park et al., 2004; Tsuda et al., 2005).

2. Materials and methods

2.1. Animals and experimental protocol

A total of 372 Tg and wild type rats of both sexes derived from Sprague–Dawley strain bred by CLEA (CLEA Japan Inc., Tokyo, Japan) at 7 weeks of age were maintained in plastic cages in an air conditioned room at 22 ± 2 °C and 55 ± 10% humidity with free access to standard AIN-93G basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water. The experimental protocol is shown in Fig. 1. After 1 week of acclimatization, they were randomly allocated to six groups (each with male and female Tg and wild-type sub-groups). The rats in Groups 1–4 received deionized water containing 10 ppm 4NQO (Wako Pure Chemical

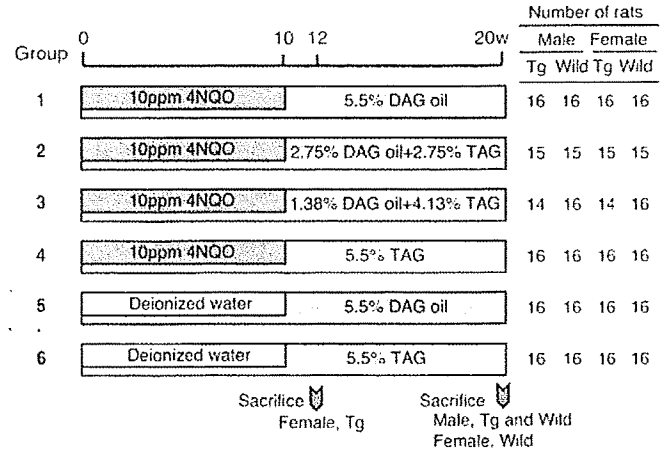

Fig. 1. Experimental protocol. Group 1–4 rats were treated with 4NQO (10 ppm) and Groups 5 and 6 were given the vehicle for the initial 10 weeks. Group 1–3 rats were fed DAG oil added to the AIG-93G basal diet at 5.5%, 2.75% and 1.38% (1:2 serial dilution), respectively. Fat content was adjusted to 5.5% in all groups by adding TAG as appropriate. Group 4 was treated with TAG alone for comparison of modification effects on tumor induction (control group). The experiment was terminated at week 12 for female Tg rats and at week 20 for female wild type and all the male rats. The effective number of animals in each group is shown on the left. M, male; F, female.

Ind., Osaka, Japan) for 10 weeks (see legend for Fig. 1), as well as basal diets containing DAG and/or TAG (total fat, 5.5%) (Kao Corporation, Tokyo, Japan) throughout the initiation and post-initiation periods. Groups 5 and 6 were fed DAG oil and TAG without 4NQO treatment. Body weights were monitored every other week throughout the experiment, and food and water intakes were measured every other week for the initial 10 weeks then once a month. Gross observation and palpation were regularly performed to monitor the development of tongue and mammary tumors throughout the experimental period. The surviving animals were sacrificed by exsanguination under deep anesthesia with diethyl ether, 12 weeks (Tg females) and 20 weeks (Tg males, wild males and females) after the start of the experiment. The experiments were conducted according to the "Guidelines for Animal Experiments in National Cancer Center" of the Committee for Ethics of Animal Experimentation of the National Cancer Center, Japan. At necropsy, all major organs except the brain were removed and assessed for any macroscopic lesion development. The oral cavity and tongue were carefully inspected and the lumen of the esophagus, stomach and intestines were inflamed with in 10% buffered formalin for fixation. The tongue, lung, liver, and kidney issues were also fixed in 10% buffered formalin, embedded in paraffin blocks, and sectioned (2.5 μm) for hematoxylin and eosin staining.

2.2. Diets

DAG oil and TAG were supplied and added into powdered diet by Kao Corp., (Tokyo, Japan). The rats were fed dry powdered AIN93-based purified diets containing 5.5% fat (Groups 1 and 5, 5.5% DAG oil; Group 2, 2.75% DAG oil + 2.75% TAG; Group 3, 1.38% DAG oil + 4.18% TAG; Groups 4 and 6, 5.5% TAG) as shown in Fig. 1. Fatty acid compositions in DAG oil and TAG were almost the same (linoleic acid 46.6% and oleic acid 38.9% of total fatty acids). The diet was prepared 2 weeks before use and stored in the cold room at 4 °C. Chemical structures of 1,3-DAG, 1,2-DAG and TAG, and fatty acid composition are shown in Fig. 2.

2.3. Analysis of biochemical parameters of the blood

For biochemical analysis, coagulated blood was centrifuged and serum was collected and stored in a deep freezer at −80 °C until measurement of the following parameters: triglycerides (TG), free fatty acids (FFA), total

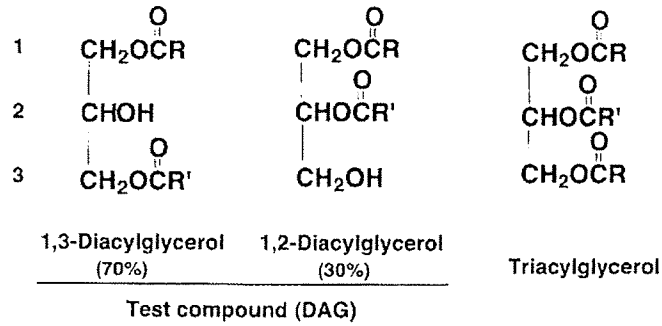

Fig. 2. Structure and composition of the test compound DAG oil and control oil TAG. Both have oleic acid (R) at 38.9% and linoleic acid (R′) at 46.6%. DAG oil is a mixture of 1,3-DAG and 1,2-DAG at 70:30 ratio with R and R′ at random position and TAG has R′ in mostly "2" position.

cholesterol (T-Cho), lipoprotein (LP), glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT).

2.4. Statistical analysis

Statistical analyses of the incidences of lesions were performed using the Fisher's exact-test and the Cochran–Armitage trend test. Multiplicity, number of tumor per rat, of the lesion was analyzed by coefficients for linear contrast trend test. The data for body and liver weights, and biochemical parameters were analyzed by the Dunnet's test. Statistical significance was concluded with P-values less than 0.05.

3. Results

There were no significant compound-related effects noted on food consumption, body and organ weights. Data for tongue tumor incidences and multiplicities in the different groups for Tg and wild males are summarized in Tables 1–4. The macroscopic appearances of tongue tumors in Groups 1 and 4 are illustrated in Fig. 3. Representative histological features of SCC are shown in Fig. 4. In male Tg rats, incidences of tongue SCC were 43.8, 20, 14.3 and 12.3% in groups treated with 5.5, 2.75, 1.38 and 0% DAG oil, respectively (Table 1). Similarly, quantitative values (number/rat) of tongue SCC were 0.44, 0.20, 0.14 and 0.13, and combined values of squamous cell papilloma (papilloma) with SCC were 0.69, 0.73, 0.21 and 0.31 (Table 2). The values were significantly increased with linear trend test by coefficient for linear contrast ($P < 0.05$). In female Tg and wild type rats (not shown), incidences of tongue papilloma and SCC (Tables 3 and 4) were not correlated with the treatment.

Table 1
Incidence of tongue tumors (Tg, male)

Group	Treatment	No. of rats	Incidence (%)		
			Papilloma	SCC	Papilloma + SCC
1	4NQO + 5.5%DAG	16	4 (25.0)	7 (43.8)*	9 (56.3)
2	4NQO + 2.75%DAG + 2.75%TAG	15	6 (40.0)	3 (20.0)*	8 (53.3)
3	4NQO + 1.38%DAG + 4.13%TAG	14	1 (7.1)	2 (14.3)*	3 (21.4)
4	4NQO + 5.5%TAG	16	3 (18.8)	2 (12.3)*	5 (31.3)
5	5.5%DAG	16	0	0	0
6	5.5%TAG	16	0	0	0

Papilloma, squamous cell papilloma; SCC, squamous cell carcinoma.
* $P < 0.05$, linear trend by Cochran-Armitage trend-test.

Table 2
Multiplicity of tongue tumors (Tg, male)

Group	Treatment	No. of rats	No./rat[a]		
			Papilloma	SCC	Papilloma + SCC
1	4NQO + 5.5%DAG	16	0.25 ± 0.45	0.44 ± 0.51*	0.69 ± 0.70*
2	4NQO + 2.75%DAG + 2.75%TAG	15	0.53 ± 0.74	0.20 ± 0.41*	0.73 ± 0.80*
3	4NQO + 1.38%DAG + 4.13%TAG	14	0.07 ± 0.27	0.14 ± 0.36*	0.21 ± 0.43*
4	4NQO + 5.5%TAG	16	0.19 ± 0.40	0.13 ± 0.34*	0.31 ± 0.48*
5	5.5%DAG	16	0	0	0
6	5.5%TAG	16	0	0	0

Papilloma, squamous cell papilloma; SCC, squamous cell carcinoma.
* $P < 0.05$, linear trend by a test using coefficients for linear contrast.
[a] Mean ± SD.

Table 3
Incidence of tongue tumors (wild, male)

Group	Treatment	No. of rats	Incidence (%)		
			Papilloma	SCC	Papilloma + SCC
1	4NQO + 5.5%DAG	16	1 (6.3)	1 (6.3)	2 (12.5)
2	4NQO + 2.75%DAG + 2.75%TAG	15	0	2 (13.3)	2 (13.3)
3	4NQO + 1.38%DAG + 4.13%TAG	16	0	0	0
4	4NQO + 5.5%TAG	16	0	0	0
5	5.5%DAG	16	0	0	0
6	5.5%TAG	16	0	0	0

Papilloma, squamous cell papilloma; SCC, squamous cell carcinoma.

Table 4
Multiplicity of tongue tumors (wild, male)

Group	Treatment	No. of rats	No./rat[a]		
			Papilloma	SCC	Papilloma + SCC
1	4NQO + 5.5%DAG	16	0.06 ± 0.25	0.06 ± 0.25	0.13 ± 0.34
2	4NQO + 2.75%DAG + 2.75%TAG	15	0	0.13 ± 0.35	0.13 ± 0.35
3	4NQO + 1.38%DAG + 4.13%TAG	16	0	0	0
4	4NQO + 5.5%TAG	16	0	0	0
5	5.5%DAG	16	0	0	0
6	5.5%TAG	16	0	0	0

Papilloma, squamous cell papilloma; SCC, squamous cell carcinoma.
[a] Mean ± SD.

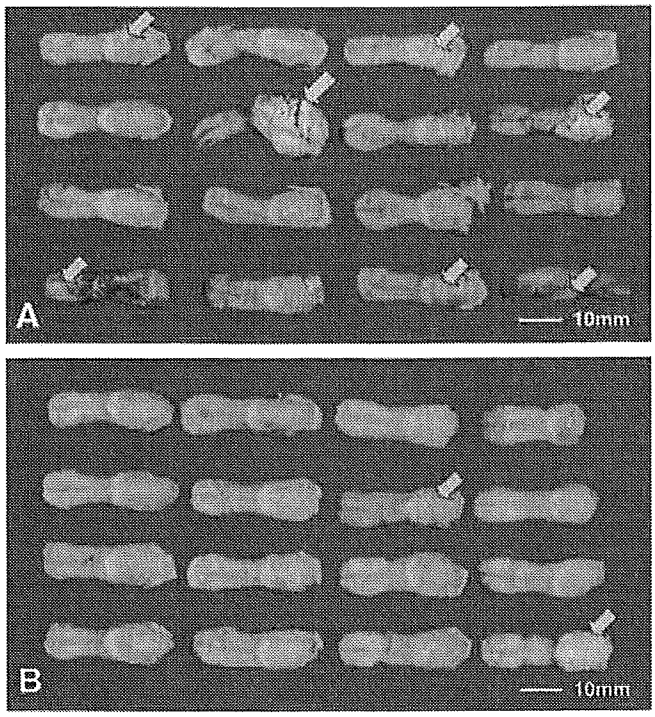

Fig. 3. Macroscopic appearance of tongue carcinomas; (A) 4NQO and 5.5%DAG (Group 1) and (B) 4NQO alone (Group 4). Arrows indicate carcinoma lesions.

In the forestomach of male Tg rats, incidences of the papilloma were 12.5%, 26.7%, 21.4%, and 6.3% in the groups receiving diets containing 5.5%, 2.75%, 1.38% and 0% DAG oil, respectively. These differences were not significant. Incidence and multiplicity of papilloma in male wild type rats and female Tg and wild type rats did not vary with the treatment.

In the mammary glands of male Tg rats, incidences of adenocarcinoma were 37.5%, 26.7%, 28.6% and 31.3% in the groups treated with 5.5%, 2.75%, 1.38% and 0% DAG oil, respectively. The female Tg rat group was sacrificed at week 12 because of early palpable tumor development, the incidence in the all group reaching almost 70%. These differences were not significant. Also in other organs, there was no significant variation in quantitative data for neoplastic lesion development.

Data for serum parameters are summarized in Tables 5 and 6. The levels of TG and GOT were significantly lowered with 5.5% DAG oil in male Tg and wild rats ($P < 0.05$). However, differences were not consistent across the carcinogen initiated and non-initiated groups and there was no clear dose dependence.

4. Discussion

Tg rats carrying three copies of the human c-Ha-ras proto-oncogene are highly susceptible to various carcino-

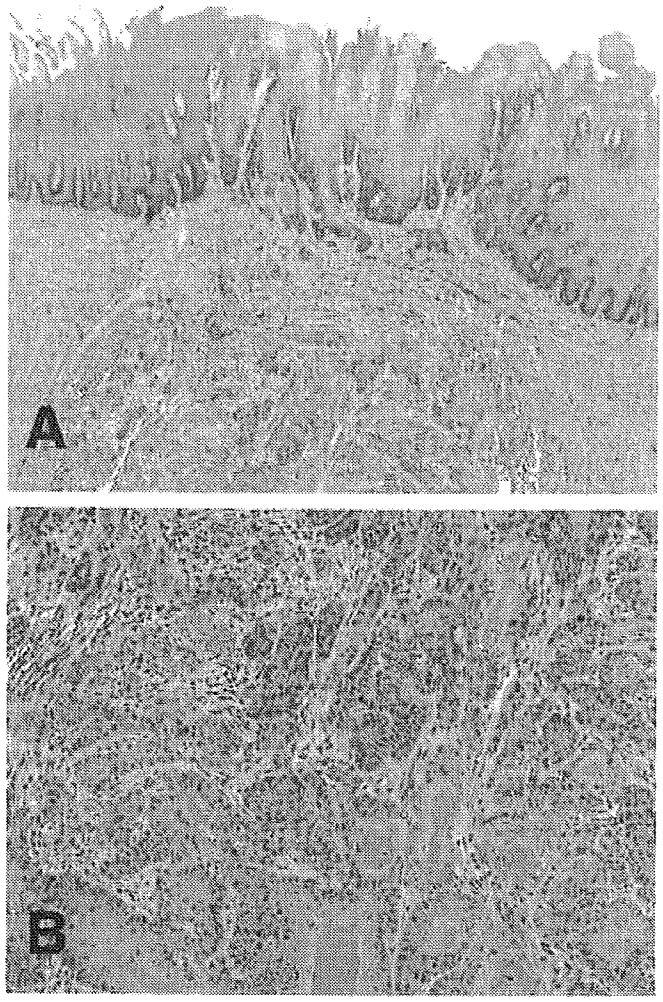

Fig. 4. Squamous cell carcinoma induced in a rat in Group 1; (A) lower magnification and (B) a higher magnification showing obvious invasion of submucosal and muscular tissue.

gens, developing mammary, tongue, skin and esophagus tumors (Tsuda et al., 2005). The present study provided evidence that DAG oil may indeed exert enhancing potential on SCC development in the tongue of male Tg rats after treatment with 4NQO. Both tumor incidence and multiplicity were significantly increased in trend analysis in the treatment groups, without obvious effects on food consumption or organ or body weights, pointing to a direct effect on the tongue epithelium.

The enhancing effect of DAG on 4-NQO-induced tongue carcinogenesis was observed only in male Tg rats. The susceptibility of the Tg rats to 4NQO-induced tongue (Suzuki et al., 2006) and DMBA-induced skin (Park et al., 2004) carcinogenesis is higher in males than in females. As estradiol inhibited development of skin tumors in male Tg rats (Han et al., 2002), the sex hormone may affect carcinogenesis of skin and maybe tongue. The mechanism remains to be clarified, but sex-dependence of tongue, mammary and skin susceptibility might be involved.

As shown in Tables 1 and 2, the formation of papillomas was not significantly affected by low doses of DAG, however, higher doses of DAG promoted progression from papilloma to SCC. The incidence of SCC and the multiplicity of SCC and papilloma + SCC were significantly increased (linear trend test, $P < 0.05$). These statistical analysis show that DAG oil administration enhances dose dependent 4NQO-induced tongue carcinogenesis in male Tg rats.

Earlier studies of 2-year feeding of 2.65% DAG oil plus 2.65% edible oil or 5.3% DAG oil to Sprague–Dawley rats demonstrated no effects, with reference to clinical signs, body and organ weights, food consumption, hematology, blood chemistry, or microscopic non-neoplastic changes as compared to controls. In our study, these physical findings and biochemical parameters in the blood also did not show a clear difference between DAG and TAG oil feeding groups.

No significant increase in mammary carcinoma development was observed between DAG and high edible oil, linoleic and oleic safflower oil, fed groups, although a significant increase was noted when the DAG group was compared to a rapeseed and soybean oil fed group (Soni et al., 2001). Recently, negative results for carcinogenic potential were reported with chronic feeding tests of DAG oil in rats, mice and dogs (Chengelis et al., 2006a,b; Chengelis et al., 2006c). At the dietary level of 7% DAG oil, irrespective of the source of DAG (composition of 1,2-DAG and 1,3-DAG not given), effects were

Table 5
Blood chemical analysis of male Tg rats

Group	Treatment	TG	FFA	T-Cho	LP	GOT	GPT	BUN	CR
1	4NQO + 5.5%DAG	130 ± 80*	1084 ± 461	105 ± 17	6.0 ± 0.7	126 ± 22*	25 ± 18	52 ± 77	0.48 ± 0.27
2	4NQO + 2.75%DAG + 2.75%TAG	157 ± 48	570 ± 170	105 ± 10	5.8 ± 0.4	77 ± 32	18 ± 6	14 ± 2	0.34 ± 0.03
3	4NQO + 1.38%DAG + 4.13%TAG	239 ± 100	1119 ± 470	110 ± 9	7.0 ± 0.8	134 ± 31	35 ± 25	20 ± 4	0.35 ± 0.07
4	4NQO + 5.5%TAG	302 ± 143	666 ± 68	108 ± 17	6.6 ± 1.1	75 ± 14	18 ± 4	16 ± 1	0.31 ± 0.04
5	5.5%DAG	160 ± 66#	691 ± 154	97 ± 3	6.8 ± 0.8	104 ± 27#	22 ± 8	18 ± 3	0.35 ± 0.04
6	5.5%TAG	139 ± 35	836 ± 177	121 ± 17	6.8 ± 0.4	126 ± 43	28 ± 11	17 ± 3	0.31 ± 0.02

* $P < 0.05$ as compared to 4NQO + 5.5%TAG group (Group 4).
\# $P < 0.05$ as compared to 5.5%TAG group (Group 6).

Table 6
Blood chemical analysis of male Wild-type rats

Group	Treatment	TG	FFA	T-Cho	LP	GOT	GPT	BUN	CR
1	4NQO + 5.5%DAG	147 ± 55	571 ± 78	83 ± 19	6.6 ± 0.9	81 ± 29	16 ± 8	17 ± 3	0.36 ± 0.05
2	4NQO + 2.75%DAG + 2.75%TAG	191 ± 57	624 ± 151	102 ± 15	6.4 ± 0.5	94 ± 45	42 ± 71	16 ± 1	0.32 ± 0.02
3	4NQO + 1.38%DAG + 4.13%TAG	207 ± 61	758 ± 112	112 ± 20	6.8 ± 0.8	105 ± 40	20 ± 8	17 ± 3	0.34 ± 0.06
4	4NQO + 5.5%TAG	136 ± 52	573 ± 117	88 ± 6	6.4 ± 0.5	97 ± 33	21 ± 17	19 ± 2	0.38 ± 0.04
5	5.5%DAG	165 ± 58[#]	742 ± 155	109 ± 8	7.0 ± 1.2	122 ± 43	28 ± 13	23 ± 2	0.33 ± 0.05
6	5.5%TAG	234 ± 104	1266 ± 508	109 ± 24	8.8 ± 5.7	133 ± 63	25 ± 12	20 ± 2	0.40 ± 0.12

[#] $P < 0.05$ as compared to 5.5%TAG group (Group 6).

similar to those of TAG on mammary carcinogenesis. Thus the tumor incidence and multiplicity did not demonstrate inter-group variation during a 90-day feeding period (Sugano et al., 2002).

With our wild-type rats, no consistent trend was noted across the sexes but yields of tumors were relatively low. Thus, the over expression of *ras* appeared to be of importance. DAG has been reported to act as an effector of the clonal expansion of mutated *ras* containing cells and a mechanism whereby an increase in endogenous DAG could contribute to clonal expansion of cells containing *ras* has been proposed (Mills et al., 1993). In another study, a TPA-independent irreversible decrease and alteration of the subcellular distribution of PKC-alpha and beta 2 in DMBA-initiated/TPA-promoted skin papillomas was been found (Mills et al., 1992). This effect occurred in the absence of any elevation in the total pool of endogenous DAG. Thus, alterations of PKC isozymes could also be important early events in multistage carcinogenesis.

DAG can cause conversion of GDP to GTP by two Ras exchange factors, PKC and RasGRP1, leading to mitogen-activated protein (MAP) kinase phosphorylation and cellular proliferation (Marais et al., 1998; Roose et al., 2005). Moreover, over expression of PKC species is known to be important for induction of squamous cell tumors in mice (Reddig et al., 2000). It is possible that the administered DAG was directly absorbed and activated pathways involved in carcinogenesis. Further studies to examine this possibility are obviously required. Regarding the possible role of *ras* mutations, it is of interest to note that exposure to DAG in populations carrying genetic alterations, such as smokers, may enhance the risk of oral cancer development (Das et al., 2000; Mishima et al., 1998; Scully et al., 2000; Xu et al., 1998).

While dietary 1,3-DAG has been found to reduce fat deposition in the viscera and body of rats (Meng et al., 2004), such alteration was not evident in the present study. Furthermore, no anti-obesity or lipid-lowering effects of DAG oil were reported (Sugimoto et al., 2003a,b). Thus, it appears that the metabolic effects of DAG oil were not essential for tumor development in the present study, in line with the lack of consistent influence on serum parameters.

In conclusion, the present study indicates that DAG oil, a mixture of 1,3-DAG and 1,2-DAG, is capable of enhancing tongue carcinogenesis by 4NQO in male Tg rats over expressing *ras*. Although DAG oil alone has not been demonstrated to be carcinogenic to the rat, the risk that DAG oil in combination with carcinogens in foodstuffs may act on the tongue and possibly pharyngeus, and esophagus in man indicates that further studies with an increased number of animals, higher dose and longer duration are warranted.

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Review

Rat mammary preneoplasia and neoplasia: a model for human breast cancer research

Yoichiro Matsuoka[1,*], Hiroaki Kawaguchi[2], Hiroki Yoshida[2], Hiroyuki Tsuda[3] and Airo Tsubura[1]

[1]Second Department of Pathology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Japan. [2]Department of Tumor Pathology, Field of Oncology, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan. [3]Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

ABSTRACT

An experimental system that biologically and histologically mimics human breast cancer is needed to understand the pathogenesis of the disease and to build strategies for its prevention and cure. The experimental system should be a tool to assess the influence of host factors, such as age, reproductive history, and genetic background, as well as environmental factors. Rat mammary gland carcinogenesis is the model that most closely fulfills these conditions.

KEYWORDS: carcinogenesis, experimental model, mammary carcinoma, preneoplasia, rat

INTRODUCTION

Breast cancer remains the most frequent type of cancer in women worldwide. The incidence of breast cancer in Western countries is leveling off and may have recently started to decline, especially in women younger than 40 years [1]. However, the incidence of this disease is definitely increasing throughout Asia.

A possible genetic contribution to breast cancer risk is indicated by the increased incidence of breast cancer among women with a family history of breast cancer and by the observation of families in which multiple family members develop breast cancer. Other risk factors for breast cancer include: age, previous breast disease, reproductive and menstrual history, estrogen therapy, radiation exposure, diet, and alcohol intake. While many epidemiological studies have addressed the effects of these risk factors in women, an experimental system that closely mimics human breast cancer is needed to directly investigate the factors that contribute to breast cancer risk. The rat mammary gland treated with carcinogens is one of the most widely studied and useful models of mammary carcinogenesis. In this review, we describe the development of rat mammary glands, induction and pathogenesis of the mammary tumors, and utility of the induced tumors for human breast cancer research.

*Corresponding author
matsuoyo@takii.kmu.ac.jp

Development of rat mammary gland

The proliferation and differentiation of the mammary gland involves a variety of hormones and growth factors, such as estrogen, progesterone, prolactin, hepatocyte growth factor, growth hormone, and IGF-1 [2-4]. The functional and structural development of the gland itself can be divided into seven stages: embryonic, postnatal, juvenile, puberty, pregnancy, lactation, and involution.

Embryonic stage

By day 11 of gestation, two parallel ridges of ectoderm lateral to the midline extend from the

shoulder to the inguinal region, forming the mammary streaks in the fetal rat. Downgrowths of ectodermal epithelium from the mammary streaks form the 12 primary buds from which the 6 pairs of mammary glands develop. There is one pair located in the cervical region, two pairs in the thoracic region, one pair in the abdominal region, and two pairs in the inguinal region [5].

Postnatal stage

The major development of the mammary gland in the rat occurs between birth and puberty. By the end of the first week, each mammary gland consists of a single primary or main lactiferous duct with three to five secondary ducts. During the second week, the secondary ducts branch dichotomously into third, fourth, and fifth generations of ducts.

Juvenile stage

Starting at about the fourth week, growth of the mammary ducts increases significantly. The growth rate of the gland now exceeds the previous isometric rate. Ductal arborization is initiated from the highly proliferative terminal end buds (TEBs) found at the tips of the ductal branches [6] (Figure 1), and ductal morphogenesis and lumen formation is accomplished by a highly regulated process of cell proliferation and cell death [7]. The number of TEBs reaches a maximum at 20 days of age and then decreases as the TEBs differentiate into terminal ductules and alveolar buds (ABs) [5]. TEBs are influenced by systemic steroid hormones and aid the ducts in linear growth as well as the regulation of branching patterns. The ductal pattern is created by the penetration of TEBs through the stromal fat pad. The TEB consists of two histologically distinct cell types. The body cells give rise to mammary epithelial cells while the cap cells are the precursors of the myoepithelial cells [7].

Puberty

Puberty, defined as the onset of estrous cycles, commences in the rat between 35 and 42 days of age. At the onset of puberty, each AB develops into 10 to 12 alveoli to form a lobule, which accumulates progressively over multiple estrous cycles [5, 8]. By 85 days of age, virgin female rats have a relatively constant number and proportion of TEBs, ABs, and lobules, although histological changes in size and secretory development occur within the lobules during the estrous cycle [9-11].

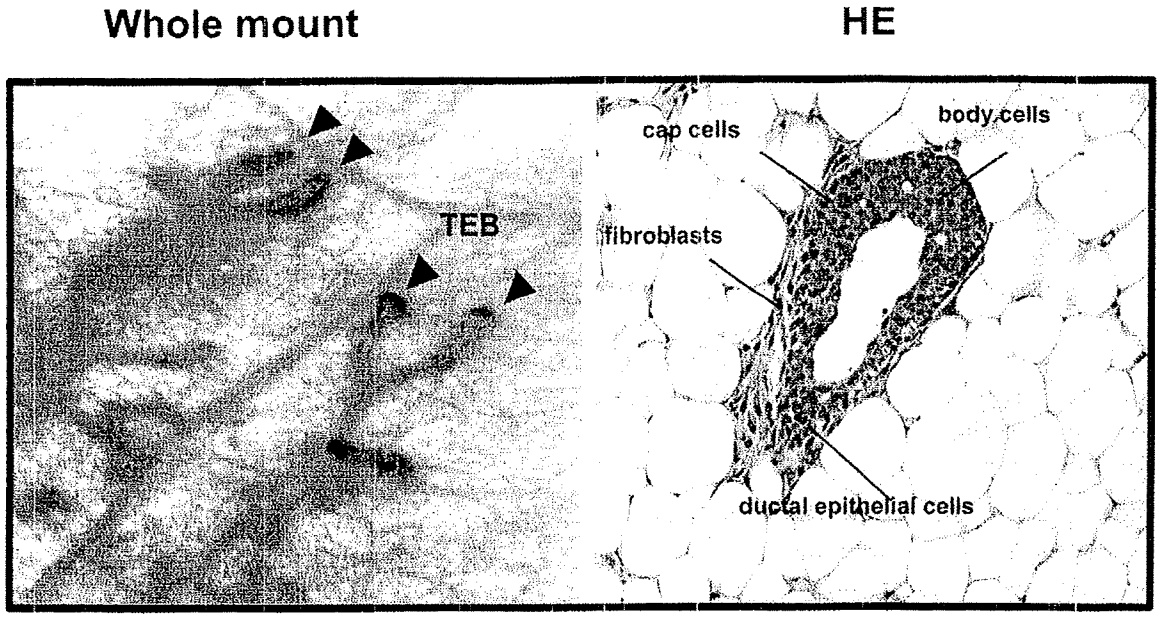

Figure 1. Terminal end buds
Whole mount preparation (left) and hematoxylin-eosin (HE) staining (right) of 49-day-old virgin rat mammary gland showing terminal end buds (TEBs).

Cellular structures of mature mammary gland

The mammary gland can be divided into two compartments: the epithelial (or parenchymal) compartment and the stromal (or mesenchymal) compartment. At the cellular level, the parenchymal compartment of the mammary gland is composed of two different types of epithelial cells with distinct morphologies, functions, and proliferative activities. These cell types are the luminal epithelial cells, which are located along ducts, ductules, terminal ducts, and alveoli, and the myoepithelial cells.

The luminal epithelial cells are cuboidal or columnar in shape [12], and can be defined immunohistochemically by the expression of keratins 8, 18, and 19 [13, 14]. These cells develop the majority of mammary carcinomas [15].

The myoepithelial cells lie between the luminal epithelial cells and the basement membrane [16-19]. The shape, thickness, and continuity of these cells vary during development and between individual epithelial structures in the mammary glands. The myoepithelial cells synthesize and secrete the continuous basement membrane that separates the epithelium from the stromal compartment [12, 20]. The myoepithelial cells express higher levels of cell adhesion receptors and adhesion-associated molecules than the luminal epithelial cells [19]. The former cells can be immunohistochemically distinguished from the latter cells by the expression of intermediate filament proteins (vimentin, keratin 5, and keratin 14) and contractile proteins (myosin and smooth muscle actin) [13, 14, 21].

Pregnant stage

During pregnancy, there is a rapid and continuous increase in the mammary gland epithelium resulting in growth of the lobules and the ducts [12, 22]. The alveoli develop and increase in size and number until the space between ducts is almost completely filled with them. The number of alveoli per unit area shows a large increase from day 5 to day 10 of pregnancy and peaks at day 20 of gestation [12]. During pregnancy, the mammary gland is influenced by estrogen, progesterone, and other placental hormones. The duration of pregnancy is usually 21 days in the rat.

Lactational stage

Lactation is the production and secretion of milk. The initiation of lactation appears to be induced by a decrease in estrogen and progesterone. About 20% of total mammary growth occurs during the first 14 days of lactation. Several hormones, such as prolactin, insulin, and glucocorticoid, are involved in the maintenance of lactation.

Involution

After weaning, there is involution of the glands with a three-fold reduction in the size of lobules; however, the number of lobules remains high, and the gland never returns to the same level of differentiation as seen in virgin female rats of the same age [5]. Involution has been attributed to several factors, including falling levels of circulating prolactin upon the cessation of suckling, mild ischemia as a result of milk engorgement and compression of the vasculature, factors in milk that promote cell death, physical distension of the luminal epithelium, and increased activity of basement membrane-degrading enzymes [12]. Mammary involution comprises two distinct phases. The first phase is apoptosis among the secretory epithelial cells. The second phase is characterized by the degradation of the alveolar structures and the mammary basement membrane and extracellular matrix [23-27]. The apoptosis-induced signals and the loss of survival factors may exert significant control over mammary gland involution.

Induction of mammary lesions in rats

While many strains of rats develop spontaneous tumors later in life, they respond to chemical carcinogens and radiation with faster development of both hormone-dependent and hormone-independent mammary tumors. For the specific induction of mammary tumors in rats via genotoxic mechanisms, the most commonly used agents are 7,12-dimethylbenz[a]anthracene (DMBA) and N-methyl-N-nitrosourea (NMU). A single dose of DMBA (2.5-20 mg) or NMU (25 or 50 mg/kg body weight) by gavage or by intravenous or subcutaneous routes, respectively, induces mammary tumors with latencies of 8 to 21 weeks [28, 29]. Other chemicals such as benzo[a]pyrene, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 1,2-dibromo-3-chloropropane can induce mammary carcinomas with lower incidences [5, 30, 31] (Table 1). Sublethal doses of different types of radiation, including x-rays and neutrons,

Table 1. Induction of mammary tumors by chemical or physical agents.

Carcinogen	Lesion Type
Genotoxic Agents	
DMBA	Ductal / Alveolar/ Mesenchymal?
NMU	Ductal / Mesenchymal?
benzo[a]pyrene[30]	Ductal
2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP)	Ductal / Mesenchymal
1,2-Dibromo-3-chloropropane[116]	Ductal
1,2-Dibromoethane[117]	Alveolar/ Mesenchymal?
Ochratoxin A[118]	Alveolar/ Mesenchymal?
Radiation (x-rays, γ-rays, neutrons)	Ductal / Mesenchymal
Non- Genotoxic Agents	
Estrogens[119, 120]	Ductal

induce mammary tumors within a year [32, 33] (Table 1). Induced tumors are histologically benign and malignant, and they generally have features in common with human tumors.

Chemically-induced mammary preneoplastic and neoplastic lesions

With chemical carcinogens, the earliest visible histological changes in the rat mammary glands are focal or multifocal hyperplasias primarily within the terminal ductule or AB or both [34-36]. The 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 ABs in the rat mammary glands [35-39]. These observations indicate that there are two different pathogenetic pathways: one pathway for malignant lesions and another pathway for benign lesions. In addition, benign lesions tend to appear later than the malignant ones, indicating that the former are not precursors of the latter [35, 40].

Ductal hyperplasia pathway

A common type of chemically-induced preneoplastic lesion found in rats is ductal hyperplasia, which is 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. In histological sections, intraductal proliferation becomes larger (Figure 2) and leads to the formation of micro-adenocarcinomas. When young virgin rats (21 to 50 days old) are inoculated with DMBA or NMU, ductal hyperplasia is detectable within 14 days after inoculation, and intraductal carcinomas are detectable after 20 days. Locally invasive carcinomas develop from these intraductal lesions to form palpable tumors approximately 13 weeks after the injection [28, 36].

In contrast to conventional strains, a rat strain carrying the human c-Ha-ras protooncogene is highly susceptible to mammary chemical carcinogens [41, 42]. When the transgenic rats are intravenously injected with 50 mg/kg body weight of NMU at 50 days of age, atypical ductal hyperplasias develop in 44% of the animals by day 15, and small invasive carcinomas form in almost all animals by day 20. Adenocarcinomas become palpable in all animals by day 56 [43]. This rat model can be used for short-term screening of chemopreventive agents, as well as mid-term screening of promoting agents for mammary carcinogenesis.

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 ABs. Transformed TEBs and terminal ducts evolve into ductal hyperplasias, carcinomas in situ, and invasive carcinomas [35, 38, 44]. In humans, ductal hyperplasias with or without atypia and

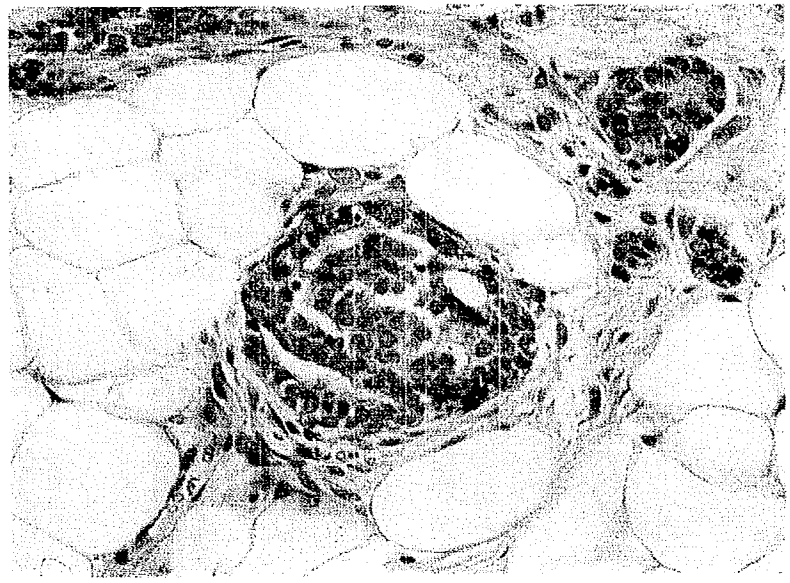

Figure 2. Intraductal epithelial hyperplasia
An enlarged terminal ductal structure exhibiting intraductal proliferations of epithelium.

atypical lobular hyperplasia are considered to be risk factors for subsequent development of invasive breast carcinomas [45-47]. However, the comparison between the pathogenetic pathways of mammary carcinogenesis in rats and humans is only tentative, because the role of the TEBs in humans is unknown. The TEB in the human female is a prepubertal structure, and the biology and differentiation of this structure from prepuberty to puberty needs to be studied. An important difference between the pathogenetic pathways in rats and humans is at the level of the terminal ductal lobular unit. The TEB in the rat would be equivalent to the intralobular terminal duct in the human, the area which is most susceptible to neoplastic growth [28]. Observations of early carcinomas in the human breast are needed to facilitate a clearer understanding of the pathogenetic scheme.

Alveolar hyperplasia pathway

Alveolar hyperplasia is a term commonly applied to enlarged lobules consisting of relatively normal alveoli that resemble the normal prelactating mammary gland [28, 37]. However, minimal or mild degrees of alveolar hyperplasia are difficult to distinguish from the normal state. In rats, the cause and biological behavior of alveolar hyperplasia are unknown, but the lesion is thought to be a precursor of adenoma and/or fibroadenoma (Figure 3). Chemically-induced alveolar hyperplasia does not appear to give rise to adenocarcinomas in rats. By clear contrast, the principal preneoplastic lesion in mouse mammary glands is alveolar hyperplasia or hyperplastic alveolar nodule [48, 49], which can be induced by mouse mammary tumor viruses [50-52], chemical carcinogens [53], X-irradiation [54], and prolonged hormone stimulation [55]. Also, in humans, atypical lobular hyperplasia is thought to progress to invasive lobular carcinoma via lobular carcinoma in situ.

Histopathology of neoplastic lesions

Rat mammary tumors have been classified by several authors [28, 38, 56, 57], and benign tumors, such as intraductal papilloma, papillary cystoadenoma, adenoma, and malignant tumors, such as papillary carcinoma, cribriform carcinoma (Figure 4), comedo carcinoma, and tubular carcinoma, have been recognized. Most of the neoplastic lesions found in the rat mammary glands have their counterpart in human pathology, with the exceptions of human-specific lesions such as lobular carcinoma and Paget's disease. Although lobular carcinoma, in situ or invasive, has not been described in the conventional strains of rats [44], the alveolar epithelia can transform to give rise to carcinomas under a certain genetic

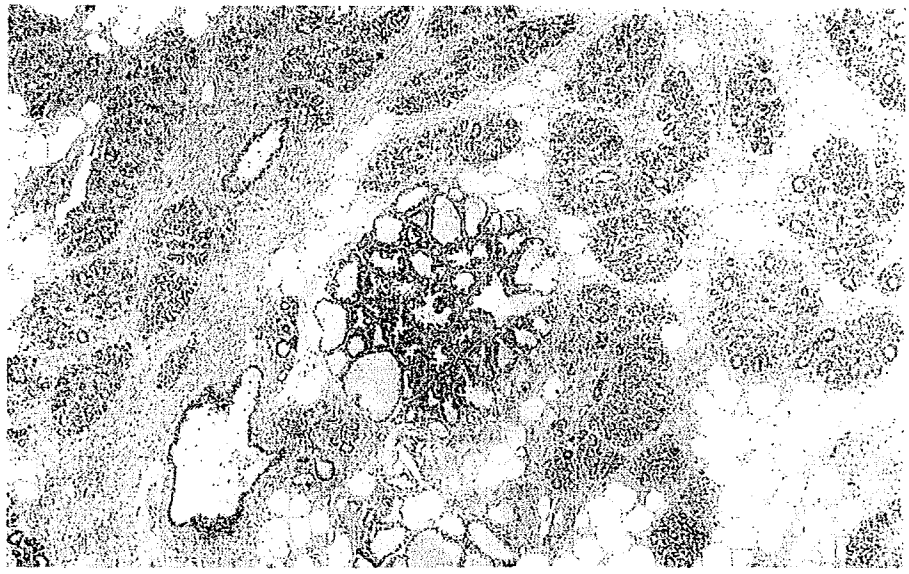

Figure 3. Fibroadenoma with alveolar hyperplasia
Note the proliferation of epithelium resembling alveolar buds and also the papillary growth pattern.

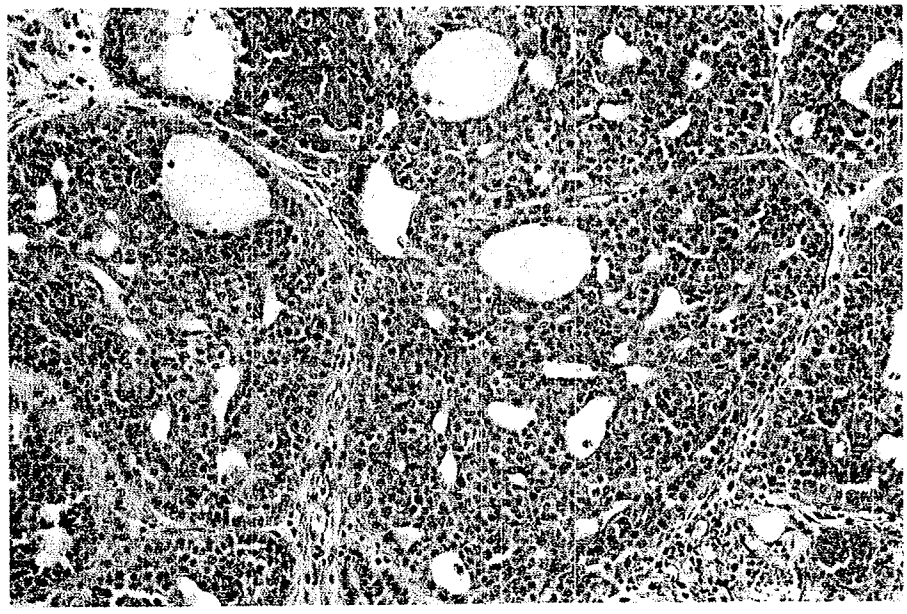

Figure 4. Cribriform carcinoma
Ductal carcinoma with areas of cribriform pattern.

background such as human c-Ha-ras transgenic rats [39] (Figure 5).

Molecular pathology of neoplastic lesions

Mutation analyses of oncogenes and tumor suppressor genes in sporadic human breast cancer and chemically-induced rat mammary carcinomas have revealed both similarities and differences in the mutation spectra of the two types of tumors. Ha-ras mutations are commonly observed with an incidence of 18% to 80% in the rat carcinomas induced by DMBA, NMU, or 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), whereas mutations in Ki-ras, p53, and brca1 are rarely