In a series of experiments we administered these chemical carcinogens to Hras128 rats and made gross pathological and histopathological assessment of lesion induction. Furthermore, transgene mutations were examined to determine whether the exogenous gene copies were targeted by the carcinogens. The results indicated that the Hras128 rat may indeed have potential for use as a medium-term assay model.

Materials and methods

Animals and chemicals

Sprague-Dawley rats (Clea Japan, Inc., Tokyo, Japan) were used for creating the human c-Ha-ras proto-oncogene transgenic rats (Hras128) (Asamoto et al., 2000), with the human c-Ha-ras proto-oncogene established by Sekiya et al. (Sekiya et al., 1985). The animals were kept under constant conditions with a 12 h light/dark cycle, a room temperature of 22 ± 2°C, and a humidity of 55 ± 10%. They were allowed access to a basal diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and tap water. All rats, transgenic and wild type littermates, were treated the same. 3-MC, B[a]P, pyrene, and AOM were purchased from Sigma Chemical Co., St Louis, USA; IQ and MeIQx from Nard Institute, Osaka, Japan; NNK from Toronto Research Chemicals Inc., Ontario, Canada; DEN from Tokyo Kasei, Co., Tokyo, Japan; and DMA and olive oil from Wako Pure Chemical Industries, Osaka, Japan. Anthracene (purity >99.9%) was provided by Dr. Matsushima of Japan Bioassay Research Center, Hadano, Japan.

The experiments were conducted according to the "Guidelines for Animal Experiments in the National Cancer Center Japan" promulgated by the Committee for Ethics of Animal Experimentation.

Experimental protocol (Figure 1)

3-MC, B[a]P, anthracene, pyrene, IQ, MeIQx, and NNK were dissolved in olive oil, and DEN, DMA, and AOM in deionized distilled water (DDW). Two hundred mg/kg of 3-MC, B[a]P, anthracene, and pyrene, 80 mg/kg of IQ and MeIQx, and 100 mg/kg of NNK and DMA were administered by gastric intubation to 7-week-old Hras128 rats and their littermates (wild type) once a week for 3 weeks. One hundred mg/kg of DEN was given once a week for 2 weeks, and 50 mg/kg of AOM once. The control group received 5 ml/kg of olive oil or DDW. Females were killed at week 12, except for the 3-MC treated group (week 10 due to a moribund condition caused by multiple mammary carcinomas), and males at week 20. Numbers, weights and sizes of all mammary tumors were then recorded.

Histological study and DNA isolation

All mammary tumors were removed and, after measurements, were immediately fixed in ice-cold acetone. Tissues were embedded in paraffin and stained with hematoxylin-eosin, followed by histopathological examination. DNA was extracted using DEXPAT (Takara, Otsu, Japan) from paraffin sections 10 m in thickness.

Mutation analysis

Mutation analysis of transgene codons 12 and 61 was performed using the PCR-restriction

fragment length polymorphism (RFLP) approach (Asamoto et al., 2002). The primers for codon 12 were hHras1F (5'-GCAGGCCCCTGAGGAGCGAT-3'), and hHras1RN (5'-AGCAGCTGCTGGCACCTGGA-3'), and for codon 61 were hHras2F (5'-AGCCCTGTCCTCCTGCAGGAT-3'), hHras2R (5'-GGCCAGCCTCACGGGGTTCA-3'), and H61/2A2 (5'-CGCATGGCGCTGTACAGCTC-3'). After 5 min at 95°C, thermocycling conditions were: 1 min at 95°C, 1 min at 60°C, 3 min at 72°C for 35 cycles, with a final extension of 10 min at 72°C. The thermal cycler was a Gene Amp PCR System 9600 (Perkin-Elmer Corp. Norwalk, USA), with MSP I (Takara, Otsu, Japan) for codon 12 and AlwN I (New England BioLabs, MA, USA) for codon 61 as restriction enzymes. After confirming mutations in codons 12 and 61 with PCR-RFLP, DNA lengths of 167 bp for codon 12 and 93 bp for codon 61 were extracted from 4% agarose gels (NuSieve GTG agarose, BMA, USA) using a Min Elute Gel Extraction Kit (QIAGEN, USA) and sequenced using Big Dye Terminator v3.1 (Applied Biosystems, Japan) and an ABI PRIZM3100-Avant Genetic Analyzer (Applied Biosystems, Japan).

Statistics

Analysis of the incidences of mammary tumors and their sizes and multiplicities was conducted using the JMP software package (version 3.1)(SAS Institute, Cary, NC). Chi squared tests were conducted for tumor incidence data and the Dunnett's *t*-test with ANOVA for tumor size and multiplicity.

Results

Incidences and multiplicity of mammary tumors

Female rats (Table 1)

All the tumors taken (larger than 3mm in longer diameter) were adenocarcinomas with obvious invasion of surrouding mammary and stromal tissue. In female Hras128 rats, mammary tumors developed in 7 of 7 rats (100%) given 3-MC, 8/8 (100%) with B[a]P, 4/7 (57.1%) with anthracene, 3/7 (42.9%) with pyrene, 2/10 (20%) with NNK, 7/10 (70%) with IQ, 6/10 (60%) with MeIQx, 3/9 (33.3%) with DEN, 6/9 (66.7%) with AOM, and 1/9 (11.1%) with DMA. There was a significant increase in the tumor incidence in female Hras128 rats in the 3-MC and B[a]P groups at $p<0.001$, the IQ group at $p<0.01$, and the anthracene, MeIQx, and AOM groups at $p<0.05$. The pyrene group also exhibited a significantly increased number of tumors in comparison with the olive oil group ($p<0.05$). Among the littermate rats (wild type), single tumors were found in 2 rats of the 3-MC group and 1 rat of the IQ group, but there were no significant differences from the control (olive oil) group. No tumors other than mammary gland were found in Hras 128 rats. No tumors were detected in any other groups of wild type.

Male rats (Table 2)

The percentages of male rats with mammary tumors for each carcinogen were as follows: 3-MC, 87.5%; B[a]P, 62.5%; anthracene, 42.9%; pyrene, 10%; NNK, 25%; IQ, 16.7%; MeIQx, 8.3%; AOM, 25%; DEN and DMA, 0%. The incidences were significantly increased in the 3-MC, B[a]P ($p<0.001$), and anthracene ($p<0.05$) groups. The multiplicity was significantly increased in the NNK ($p<0.05$) group. Mammary tumor size (7.8±15mm) was significantly greater in the IQ group than in the olive oil group ($p<0.05$). No significant difference from controls was seen in tumor development in the littermate wild rats. In Hras128 rats, zymbal tumors occurred in 3 rats, colonic polyps in 3 rats and scrotal squamous cell papillomas in 2 rats with AOM (Figure 3A), a scrotal squamous cell papillomas in 1 rat and a malignant lymphoma in 1 rat with DMA, zymbal tumors in 2 rats with NNK, scrotal squamous cell papillomas in 2 rats with DEN, a scrotal squamous cell papilloma in 1 rat with IQ and a back skin squamous cell papilloma in 1 rat with pyrene (Table 3). Sarcomas, composed of spindle shaped tumor cells, were found only in male Hras128 rats at lower incidences. These cells were negative for antibodies for pankeratin, S-100 protein and alfa-smooth muscle antigens (Figure 3B). Sarcomas occurred in 2 rats with 3-MC, 1 rat with B[a]p, and 1 rat with MeIQx.

Mutation analysis of the transgenes

The tumors mutation results of PCR-RFLP for codons 12 and/or 61 in the Hras128 rats are shown in Table 3 and 4. Codons 12 and/or 61 in female rats were as follows: 3-MC, 84.8%; B[a]P, 75%; anthracene, 66.7%; NNK, 100%; IQ, 83.3%; and AOM, 100%. Mutations in both codons 12 and 61 were present in 18.2% of the 3-MC group and 28.6% of the B[a]P group (Table 3). Codons12 and/or 61 in male rats were 3-MC, 66.7%; B[a]P, 100%; anthracene, 66.7%; and AOM, 100%. Mutations on both codons 12 and 61 were present in 5.6% of the 3-MC group and 50% of the B[a]P group (Table 4).

Direct sequencing of mutated bands

The results of direct sequencing of DNA are summarized in Table 5. Figures in Table 5 show the numbers of mutation type in mammary tumors in Hras128 rats combined for female with male. In codon 12 there were transversion mutations of GGC to GTC and GGC to TGC (mutation underlined) at rates of 95.3% (61/64) and 4.7% (3/61), respectively. In codon 61 there were transition mutations of CAG to CGG and transversion mutation of CAG to CAT, CAG to AAG, CAG to CTG and CAG to CGT (mutation underlined) at rates of 58.3% (21/36), 33.3% (12/36), 2.8% (1/36), 2.8% (1/36) and 2.8% (1/36), respectively.

Discussion

The present study demonstrated that the mammary tissue of our transgenic rats is sensitive to the carcinogenic actions of chemicals such as IQ, MeIQx, NNK and AOM, the last two not normally inducing breast tumors (Masumura et al., 2003; Reddy et al., 1975; Thorup et al., 1995). Furthermore, positive results were also obtained with 3-MC and B[a]P, along with their parent compounds, pyrene, rated as Group 3 in the IARC Monograph series (1983), and anthracene. It should be noted that anthracene, which has been generally considered as a non-carcinogen, also gave positive results in a 2-year chronic feeding test (personal communication from Dr. Matsushima of the Japan Bioassay Research Center).

Although the mouse model harboring the same human c-Ha-ras proto-oncogene as in our Hras128 rats has been extensively examined for susceptibility to various carcinogens and has found application as a middle-term assay system with lung tumors as the end-point lesions, the experimental protocol required at 26 weeks (Mitsumori et al., 1998; Yamamoto et al., 1998). The duration with the current model, 12 weeks for females and 20 weeks for males has clear advantages in terms of practical application. Indeed, based on our recent observation of development of mammary cancers 15 and 20 days after the administration of MNU (Matsuoka et al., 2003), it may be possible to shorten the experimental period by histopathological detection of early carcinomas in abdominal mammary glands.

Tumors observed in Hras128 were mammary and squamous cell papilloma in the back and the scrotum skin. Histological types of mammary carcinomas were tubular with a cribriform arrangement, solid tubular or papillary tubular (Figure 2), all of which are similarly found after treatment with N-methyl-N-nitrosourea and, importantly, resemble those found in humans (Asamoto et al, 2000). Acinar cell type tumors were not observed. Areas of differing morphology were often found mixed within the same mammary tumors. Furthermore, there was no tendency for specific types to be localized in different mammary glands. No treatment related incidence of any specific histological type or localization was observed. Fibrosarcomas, composed of spindle-shaped irregular shaped tumor cells, were found only in male Hras128 rats at lower incidence. Metastasis from adenocarcinomas was not found. Although we have conducted histological examinations of all major organs, including the esophagus, forestomach, tongue and urinary bladder, which were also found to be highly susceptible to chemical carcinogens in Hras128 rats, no tumors were found, possibly due to the relatively shorter duration of the observation period and low doses of carcinogens. It appears that carcinomas induced in Hras128 rats are not as variable as those observed in transgenic mice (Cardiff et al., 2000).

Since high incidences of the transgene mutation are observed in the mammary tumors in this transgenic rat induced by typical mammary carcinogens (Asamoto et al., 2000), it is clearly of

interest whether the same situation might exist with regard to various other carcinogens. Our present studies clearly indicated that the transgenes, but not the endogenous rat c-Ha-ras gene, demonstrate mutations at relatively high incidence, suggesting an important role in carcinogenesis. Although the number of tumors used for mutation analysis was low except for the B[a]P and 3-MC cases, the results are highly suggestive that the compounds commonly cause mutation of the transgenes. The c-Ha-ras gene was also observed in mice with the same transgene (Ando et al., 1992).

In our recent studies, such mutations were already evident in endbuds (Hamaguchi et al., 2004), postulated tissue targets of carcinogens (Russo et al., 1983; Russo et al., 1979), before obvious proliferative change occurred. Thus it is possible that test compounds including non-mammary carcinogens might also cause mutation of the transgenes, a possibility which we are presently exploring. In the present study, most mutations were of transversion type in codon 12, GGC to GTC predominating, irrespective of the chemical carcinogen. Clearly, it is necessary to analyze whether transversion clustering is dependent on the carcinogen administered or the organ in which the tumor appears.

Establishment of short-term assay models is essential in order to reduce the cost and increase the number of compounds which can be tested (Tennant et al., 1995; Tsuda et al., 1999). From our present review, the human c-Ha-ras proto-oncogene transgenic rat is a good candidate for this purpose. The assay model is advantageous because the end-point is frank mammary carcinomas which can be grossly observed. Furthermore, this model can be used for the assay of modifying agents including chemopreventive compounds (Matsuoka et al., 2003) and also non-genotoxic promoting agents (Fukamachi et al., 2004; Tsuda et al., 2005). Given the number of compounds released in our environment, further validation studies using Hras128 rats will be necessary.

Acknowledgements

This work was supported in part by a Grant-in-Aid for the Second-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare, Japan, by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid for Scientific Research (KAKENHI) on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan, by a Grant-in Aid for The Long-range Research Initiative from Japan Chemical Industry Association (CC05-01), and by Surveillance Study for Appropriate Assessment of Refined Petroleum Products by the Ministry of Economy, Trade and Industry. The authors would like to thank Dr. T. Matsushima of the Japan Bioassay Center, Hadano, for generous provision of purified anthracene and Dr. Samuel M. Cohen of University of Nebraska Medical Center for his kind advice in preparation of the manuscript. T. Ohnishi was the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research under the Second Term Comprehensive 10-Year Strategy for Cancer Control, when he was at Experimental and Chemotherapy Division of National Cancer Center Research Institute.

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Figure legends

Figure 1. Experimental protocol for the assay of test compounds using Hras128 and littermate wild type rats.
0 is the start of administration by i.g. and rats are killed at 12 or 20 weeks of this study.

Figure 2.
Macroscopic and histological appearance of tumors found in Hras 128 rats. A, From left, B[a]P-, IQ-, and olive oil-treated female rats. B, Fibroadenoma in a DEN treated female rat. C, Papillary tubular carcinoma in a B[a]P treated female rat. D, Solid tubular (left half) and tubular (right half) carcinoma in an IQ treated female rat. E, Solid tubular carcinoma with a cribriform pattern in an IQ treated female rat. F, Tubular carcinoma with loose fibrosis in a MeIQx treated female rat.
Figure 3
Histological appearance of non-adenocarcinoma tumors. A, Squamous cell papilloma of the scrotum in a Hras128 rat treated with AOM showing papillary formation and keratinization. B, Sarcoma of a mammary gland in a male Hras128 rat treated with B[a]P. Note the spindle-shaped tumor cells showing a storiform arrangement.

Table 1. Incidence and multiplicity of mammary tumors in Hras128 and non-transgenic female rats

	Treatment	No. of rat	Incidence (%)[a]	Diameter(mm)[b]	Microscopic Data		
					Adenoma[b]	Adenocarcinoma[b]	Total[b]
Hras128	3-MC	7	7(100)***	28±8.5**	0.3±0.5	5.3±3.9**	5.6±3.8**
	B[a]P	8	8(100)***	20±8.4**	0.1±0.4	6.8±3.5***	6.9±3.6***
	Anthracene	7	4(57.1)*	9.8±14	0.4±0.5	0.1±0.4	0.6±0.5*
	Pyrene	7	3(42.9)	8.0±8.3	0.1±0.4	0.4±0.8*	0.6±0.8
	NNK	10	2(20)	1.3±9.3	0	0.2±0.6	0.6±0.6
	IQ	10	7(70)**	14±10*	0	2.0±1.9**	2.0±1.9**
	MeIQx	10	6(60)*	7.6±7.1	0	0.8±0.9*	0.8±0.9*
	DEN	9	3(33.3)	4.8±8.3	0.1±0.3	0.2±0.4	0.3±0.5
	DMA	9	1(11.1)	4.5±8.6	0	0.3±0.7	0.2±0.7
	AOM	9	6(66.7)#	13±12#	0	0.6±0.5#	0.6±0.5#
	Olive Oil	11	2(18.2)	6.5±14	0.2±0.4	0	0.2±0.4
	DDW	8	1(12.5)	4.3±8.8	0	0.3±0.7	0.3±0.7
Non-Tg	3-MC	7	2(28.6)	7.2±13	0	0.3±0.5	0.3±0.5
	B[a]P	7	0	0	0	0	0
	Anthracene	8	0	0	0	0	0
	Pyrene	7	0	0	0	0	0
	NNK	12	0	0	0	0	0
	IQ	14	1(7.1)	0.7±2.7	0.1±0.3	0	0.1±0.3
	MeIQx	12	0	0	0	0	0
	DEN	9	0	0	0	0	0
	DMA	9	0	0	0	0	0
	AOM	9	0	0	0	0	0
	Olive Oil	12	0	0	0	0	0
	DDW	8	0	0	0	0	0

a, Adenoma and carcinoma combined; b, Number count / rat, Mean ± SD, *P<0.05, **P<0.01, ***P<0.001 as compared to olive oil of Hras128 rat, #P<0.05 as compared to DDW of Hras128

Table 2. Incidence and multiplicity of mammary tumors in Hras128 and non-transgenic male rats

	Treatment	No. of rat	Incidence (%)[a]	Diameter(mm)[b]	Microscopic Data		
					Adenoma[b]	Adenocarcinoma[b]	Total[b]
Hras128	3-MC	8	7(87.5)***	38±18***	0	4.1±2.9***	4.1±2.9***
	B[a]P	8	5(62.5)***	28±9.8***	0.1±0.4	1.0±0.9**	1.1±1.1**
	Anthracene	7	3(42.9)*	5.5±7.5*	0.3±0.5*	0.1±0.4	0.4±0.5*
	Pyrene	10	1(10.0)	3.0±9.7	0	0.1±0.3	0.1±0.3
	NNK	12	3(25)	3.1±8.1	0.3±0.5*	0	0.3±0.5*
	IQ	12	7(70)**	7.8±15*	0	0.3±0.6	0.3±0.6
	MeIQx	12	6(60)*	4.0±14	0	0.1±0.3	0.1±0.3
	DEN	10	3(33.3)	0	0	0	0
	DMA	10	1(11.1)	0	0	0	0
	AOM	8	6(66.7)#	13±8.5	0	0.3±0.7	0.3±0.7
	Olive Oil	12	2(18.2)	0	0	0	0
	DDW	8	1(12.5)	14.5	0	0.13	0.13
Non-Tg	3-MC	7	1(14.3)	2.7±7.2	0	0.1±0.4	0.1±0.4
	B[a]P	8	0	0	0	0	0
	Anthracene	6	0	0	0	0	0
	Pyrene	10	0	0	0	0	0
	NNK	10	0	0	0	0	0
	IQ	10	0	0	0	0	0
	MeIQx	10	0	0	0	0	0
	DEN	10	0	0	0	0	0
	DMA	10	0	0	0	0	0
	AOM	9	0	0	0	0	0
	Olive Oil	10	0	0	0	0	0
	DDW	8	0	0	0	0	0

a, Adenoma and carcinoma combined; b, Number count / rat, Mean ± SD, *P<0.05, **P<0.01, ***P<0.001 as compared to olive oil of Hras128 rat.

Table 3. Tumors other than mammary glands in Hras128 and non-transgenic rats

	Animals	No. of rat	Number of rats with tumor: Zymbal gland tumor	Colonic adenoma	Scrotum and back skin papilloma	Malignant Lymphoma
Hras128	Female	105	0	0	0	0
	Male	114	5(4.4%)	3(2.6%)	7(6.1%)	1(0.9%)
Non-Tg	Female	117	0	0	0	0
	Male	108	0	0	0	0

Table 4. Transgene mutation rate of mammary tumors in Hras128 female rats

Test compound	Codon12 (%)	Codon 61 (%)	Codon 12 and/or 61 (%)
3-MC	20/33 (60.6)	14/33 (42.4)	28/33 (84.8)
B[a]P	17/28 (60.7)	12/28 (42.9)	21/28 (75.0)
Anthracene	1/3 (33.3)	1/3 (33.3)	2/3 (66.7)
Pyrene	1/2 (50.0)	0/2 (0)	1/2 (50.0)
NNK	1/2 (50.0)	1/2 (50.0)	2/2 (100)
IQ	8/12 (66.7)	2/12 (16.7)	10/12 (83.3)
MeIQx	1/3 (33.3)	0/3 (0)	1/3 (33.3)
DEN	0/3 (0)	0/3 (0)	0/3 (0)
DMA	0/2 (0)	1/2 (50.0)	1/2 (50.0)
AOM	3/5 (60.0)	2/5 (40.0)	5/5 (100)

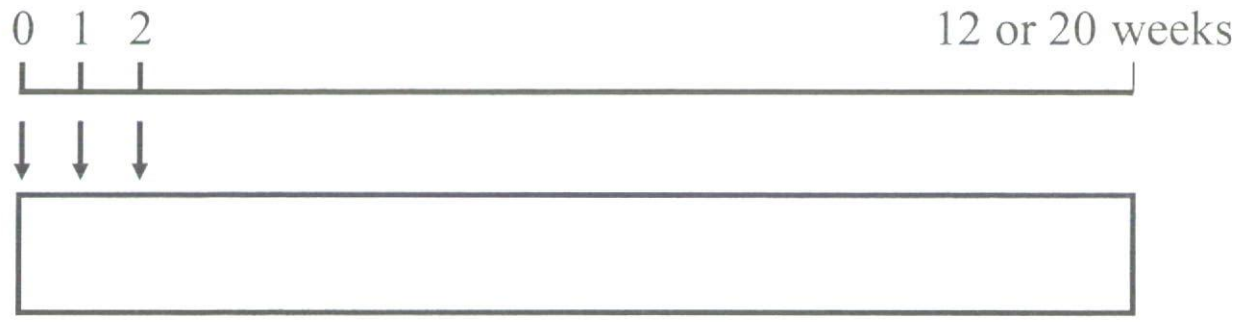
Table 5. Transgene mutation rate of mammary tumors in Hras128 male rats

Test compound	Codon12 (%)	Codon 61 (%)	Codon 12 and/or 61 (%)
3-MC	2/18 (11.1)	11/18 (61.1)	12/18 (66.7)
B[a]P	4/4 (100)	2/4 (50.0)	4/4 (100)
Anthracene	0/3 (0)	2/3 (66.7)	2/3 (66.7)
Pyrene	1/1 (100)	0/1 (0)	1/1 (100)
NNK	-	-	-
IQ	1/2 (50.0)	0/2 (0)	1/2 (50.0)
MeIQx	0/2 (0)	0/2 (0)	0/2 (0)
DEN	-	-	-
DMA	-	-	-
AOM	4/7 (57.1)	3/7 (42.9)	7/7 (100)

Table 6. Transgene mutation type of codon 12 and 61 in Hras128 rats

	Codon12 (GGC)		Codon 61 (CAG)				
Test compound	GTC	TGC	CGG	CAT	AAG	CTG	CGT
3-MC	20	2	9	4	1	0	0
B[a]P	20	1	6	4	0	0	0
Anthracene	1	0	3	0	0	0	0
Pyrene	2	0	0	0	0	0	0
NNK	1	0	0	1	0	0	0
IQ	9	0	1	1	0	0	0
MeIQx	1	0	0	0	0	0	0
DEN	0	0	0	0	0	0	0
DMA	0	0	0	0	0	1	0
AOM	7	0	2	2	0	0	1

Figure 1



Animal: 7-week-old male and female Hras128 rats

↓ Test compound administration with gastric intubation (i.g.)
Administration by i.g. start at 0 and rats are killed
at 12 or 20 weeks

200 kg/kg x 3 of 3-MC, B[a]P, Anthracene and pyrene
80 mg/kg x 3 of IQ and MeIQx
100 mg/kg x 3 of NNK and DMA
100 mg/kg x 2 of DEN
50 mg/kg x 1 of AOM
5 ml/kg x 3 of olive oil or deionized distilled water (DDW)

Figure 2

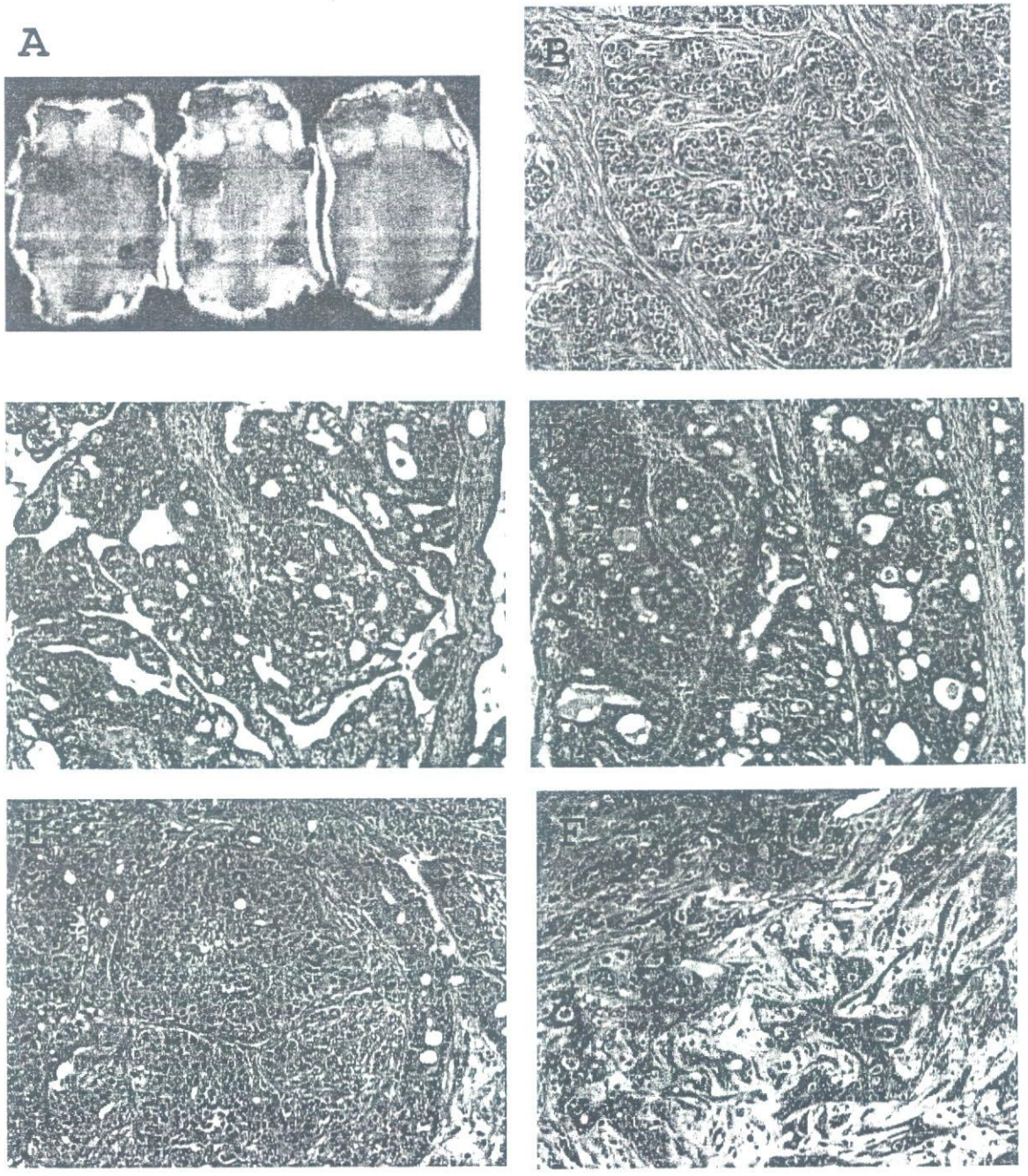
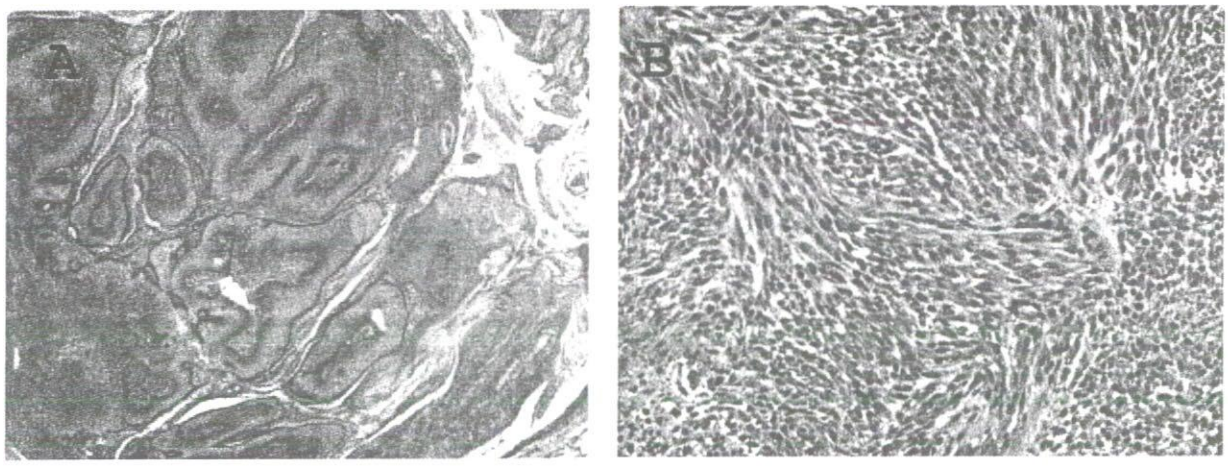


Figure 3



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Food and Chemical Toxicology 45 (2007) 1013–1019
www.elsevier.com/locate/foodchemtox

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

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Received 17 December 2005; accepted 11 December 2006

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.

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.

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doi:10.1016/j.fct.2006.12.008