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CHEMICAL CARCINOGENESIS IS ACCELARATED IN C-FOS TRANSGENIC MICE

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INDEXING WORDS

c-*fos*; transgenic mouse; chemical carcninogenesis; immunosurveillance

SYNOPSIS

Transgenic mice, having mouse proto-oncogene c-*fos* under the control of the promoter from murine major histocompatibility complex (MHC) class I gene ($H2\text{-}K^b$), have been established (c-*fos* mouse).[22] C-*fos* mouse have been shown to produce high amount of exogenous c-*fos* protein in various organs. However, they do not develop any malignant tumors. In order to examine the effect of c-*fos* protein in oncogenesis, dorsal skin or lower gingiva of the mice were scratched and painted with 9,10-dimethyl- 1,2- benzanthracene (DMBA) twice a week. C-*fos* mice showed squamous cell carcinoma in both skin and gingiva at 8 weeks after the treatment. On the contrary, control litter mate did not show any malignant change even at 11 weeks after the treatment. These results indicate that chemical carcinogenesis is accelerated in c-*fos* mice. Since the activities of immunosurveillance (e.g. natural killer (NK) cell and cytotoxic T lymphocyte (CTL)) in spleen cells from c-*fos* mice were significantly lower than those from control litter mate, effects of both the abnormal amount of c-*fos* protein and the partial defect of immunosurveillance in c-*fos* mice accelerate the induction of neoplasms in epithelial cells stimulated with DMBA.

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INTRODUCTION

The proto-oncogene c-*fos* has been isolated as the cellular homologue of the v-*fos* gene from the FBJ-murine sarcoma viruses (MSV).[9] The biological function of c-*fos* protein is still unknown. To elucidate its function, transgenic mice having mouse c-*fos* gene under the control of the promoter from murine class I major histocompatibility complex (MHC) gene (c-*fos* mice), have been established.[22] The c-*fos* mice showed hyperplasia of thymic epithelial cells and splenomegaly, indicating that disregulated expression of c-*fos* gene induces proliferation of cells. However, no malignant transformation of the cells has been generated. These results strongly suggest that c-*fos* protein itself cannot change normal cells to malignant state, and that the cells require a second signal for carcinogenesis.

In order to examine the efeect of c-*fos* protein in oncogenesis, chemical carcinogenesis was applied to c-*fos* mice. the first step of the treatment is to paint 9,10-dimethyl-1,2-benzanthracene (DMBA) on the skin (initiation). The second step is the promotion of the skin by 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Since c-*fos* mice may have one of those steps, dorsal skin or lower gingiva of the mice were painted with DMBA twice a week. C-*fos* mice showed squamous cell carcinoma in skin and gingiva as early as 8 weeks after the treatment. However malignant transformation was not generated in control litter mate even at 11 weeks after the treatment.

Immunosurveillance has been thought to regulate the carcinogenesis in animals.[2,8,24,27,30] Natural killer (NK) cells and cytotoxic T lymphocytes (CTL) play a major role in host immunosurveillance.[14,17] Since C-*fos* mice showed immune deficiencies such as decreased production of IgG class immunoglobulin[22] activities of NK cells and CTL in spleen cells from c-*fos* mice were also examined. Functional roles of proto-oncogene products and activities of immunosurveillance for carcinomagenesis in vivo will be discussed.

MATERIALS AND METHODS

Mice

C57BL/6 Cr Slc mice were purchased from the SLC (Hamamatsu,

Japan). Transgenic mice carrying mouse c-*fos* gene under the control of class I MHC gene (H-$2K^b$) promoter have already been reported.[22] This line, which had been made from (C57BL/6XSJL) F_2 mice, was backcrossed to C57BL/6 mice for 10 generations.

Pathological examination

Skins and gingivas were fixed in 10% formaldehyde for 2 days. These sections of 3 μm were stained with hematoxylin and eosin, and pathologically examined under a microscope.

Isolation of total RNA from various organs

Total RNA was isolated from various organs of mice by a guanidin method reported elsewhere.[6] Briefly, various organs were taken from mice and frozen by liquid nitrogen immediately. They were homogenized with 4M guanidine thiocynate solution by a Polytron (Switzerland). The total RNA was pelleted through a 5.7M CsCl cusion by ultra-centrifugation at 36, 000 rpm used SW60 rotor for 16 hours at 15C. The RNA was disolved in RNase free water and quantified by a spectro-photometer.

RNase protection assay

RNase protection assay to detect the exogenous c-*fos* RNA was performed as described by Ruther et al.[21] Briefly, 10 μg of total RNA was hybridized overnight at 65C with ^{32}P labeled RNA probe in 80% formamide hybridization buffer. Unprotected RNA was digested by RNase A and RNase T_1 at 37C for 1 hour. The protected RNA fragments were separated by electrophoresis on a 6% plyacrylamide denaturing gel and exposed to X-ray film for 48 hours at -70C with intensifier screen.

Assay for natural killer (NK) cell activity

A 4-hour ^{51}Cr release assay was performed using YAC-1 cells for target cells, as described in detail elsewhere.[25,29] Briefly, single mononuclear cells from spleen were used for effector cells. 5×10^4 radio-labeled target cells were co-cultured with 2.5×10^5 (effector/target ratio = 50), or 1.25×10^5 (effector/target ratio = 25) effector cells in 96 wells rounds bottomed micro titer plate (Corning #25850) with 200 μl RPMI1640 medium (GIBCO) containing

10% fetal calf serum (FCS) (FILTRON) in 5% CO_2 at 37C for 4 hours. The gamma ray ratio-activity in 150 µl of a cultured supernatant was counted by an auto-gamma scintillation counter (Aloka, ARC-300). Maximum release of ^{51}Cr from the target cells was examined by treating the cells with 10% Triton X-100. The percent specific lysis for individual assay was calculated from the following formula for triplicate samples.

%specific lysis = (tested release - spontaneous release
/maximum release - spontaneous release) x 100

Assay for allo-specific cytotoxic T lymphocyte (CTL) activity.

All-specific CTL assay was performed as described by Ishikawa et al.[7.15.31] Briefly, 4 x 10^7 of spleen cells from c-*fos* mouse (H-2^b) were co-cultured with 8 x 10^5 of M12.4 stimulator cells (H-2^d),[11] had been treated with 40 µg/ml of Mitomycin C (Kyowa Hakko Kogyo Co., Ltd.) for 2 hours at 37C, in 5 ml RPMI 1640 enriched with 10% FCS in macroculture plate (36 mm) (Corning #25810) in 5% CO_2 at 37C for 5 days. ^{51}Cr labeled M 12.4 cells were used for CTL targets. 2 x 10^4 of ^{51}Cr labeled M12.4 cells were mixed with graded numbers of precultured spleen cells in 96 wells round-bottomed micro titer plate in 200 µl of RPMI1640 medium containing 10% FCS, and incubated for 10 hours. The released isotope in 150 µl of a culture supernatant was measured by the gamma-counter.

RESULTS

Exogenous c-fos RNA was detected in total RNA isolated from skin and gingiva

The expression of exogenous c-*fos* RNA was examined in 10 µg of total RNA from various organs by a RNase protection assay. As shown in Fig. 1, high expression of the c-*fos* RNA was detected in total RNA from skin, thymus and spleen. The gingiva expressed about a quarter of the RNA from spleen. The skin expressed more than double amounts of the RNA from the gingiva.

Squamous cell carcinoma was induced in c-fos mice painted with DMBA

In order to examine the effect of c-*fos* protein on carcino-

genesis, dorsal skins or lower gingivas of c-*fos* mice were scratched and painted with 1% 9,10-dimethyl-1,2-benzanthracene (DMBA) acetone solution twice a week. These skins and gingivas were isolated and fixed with 10% formaldehyde for pathological studies. All of the skins from the c-*fos* mice (n=4) showed marked down growth in the epithelium and atypical epithelium replacing hair follicle and horney cyst with cellular atypism in the treated area at 5 weeks after the treatment (Fig. 2A), compared with normal skins from control litter mate (n=3) (Fig. 2B). 4 out of 4 c-*fos* mice showed squamous cell carcinoma in the treated region of the skins at 8 weeks after the treatment (Fig. 3A). Whereas, none of the control litter mate (n=4) showed malignant changes. They expressed only reactive changes (Fig. 3B).

The gingiva from a c-*fos* mouse treated with DMBA for 5 weeks showed slight acanthosis and elongation of rate ridge in the epithelium (Fig. 4A), compared with the normal gingiva from a control litter mate (Fig. 4B). The gingiva from a c-*fos* mouse at 8 weeks after the treatment demonstrated well differentiated squamous cell carcinoma (Fig. 5A), while a control litter mate showed slight acanthosis and focal subcutaneous inflammation in epithelium (Fig. 5B). Invasive well differentiated squamous cell carcinoma was induced in the gingiva from a c-*fos* mouse at 11 weeks after the treatment (Fig. 6A). On the contrary, a control mouse had only slight hyperkeratosis and focal inflammatory change in the subcutaneous tissue. Obvious malignant changes in the legion were not observed (Fig. 6B). These results indicate that squamous cell carcinoma is induced in c-*fos* mice earlier than in normal litter mate by the DMBA treatment.

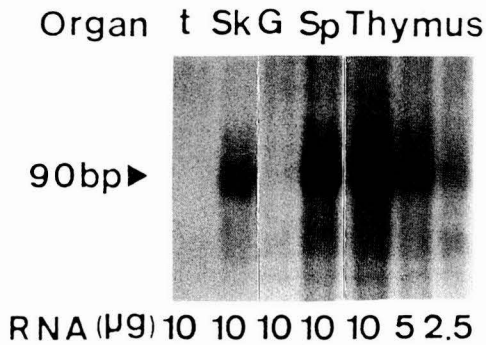
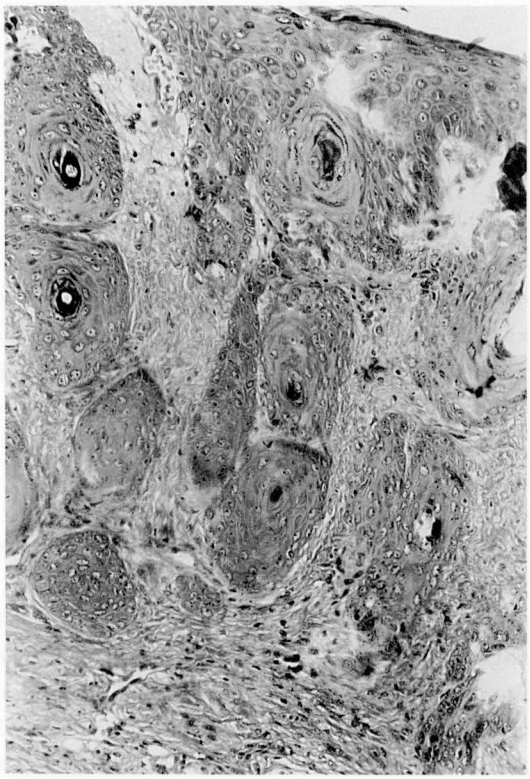
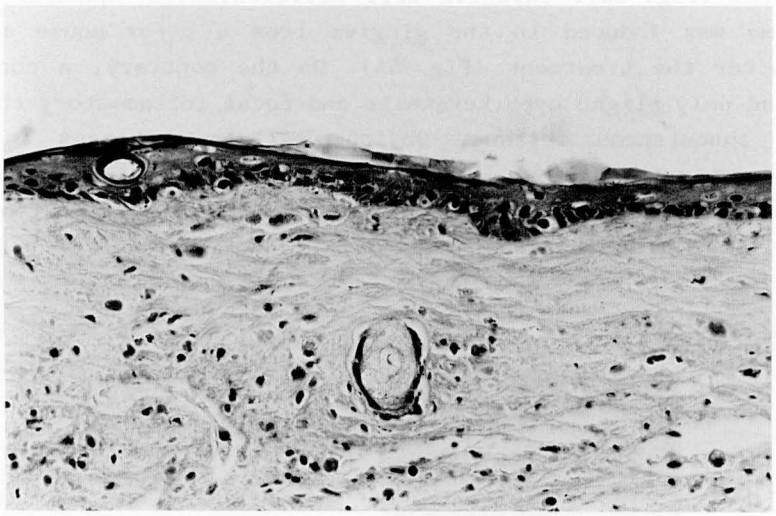


Figure 1
Exogenous c-*fos* RNA was detected in total RNA from the skin and gingiva of c-*fos* mice.
The amount of exogenous c-*fos* RNA were examined in 10 μg of tRNA (t), spleen (Sp), skin (Sk), thymus and gingiva (G) from c-*fos* mouse by a RNase protection assay using a probe which detects the exogenous c-*fos* transcripts in the length of 90 bases.

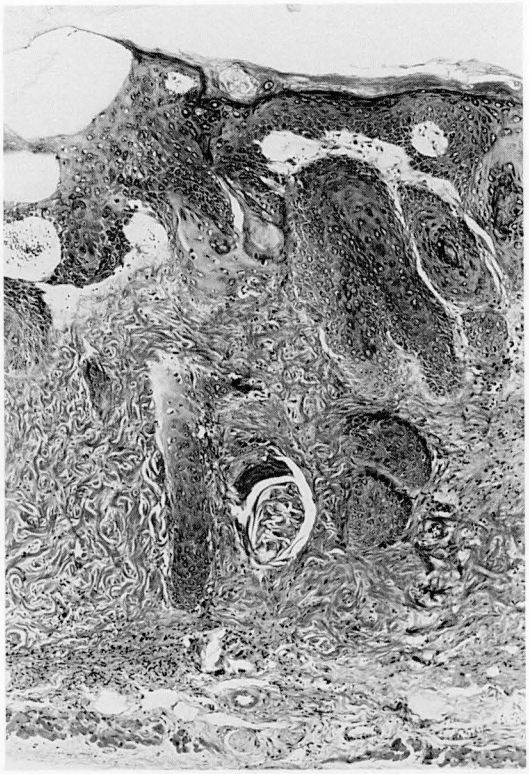


A



B

Figure 2 The skin lesion at 5 weeks after the treatment of DMBA.
A: c-*fos* mouse. The epithelium showed marked down growth, atypical epithelium replacing hair follicle and horney cyst with cellular atypism, but no evidence of invasive growth.
B: litter mate. The epithelium showed slight acanthosis and parakeratosis. Inflammatory cells infiltrated in subcutaneous tissue, an no malignant cell was observed.



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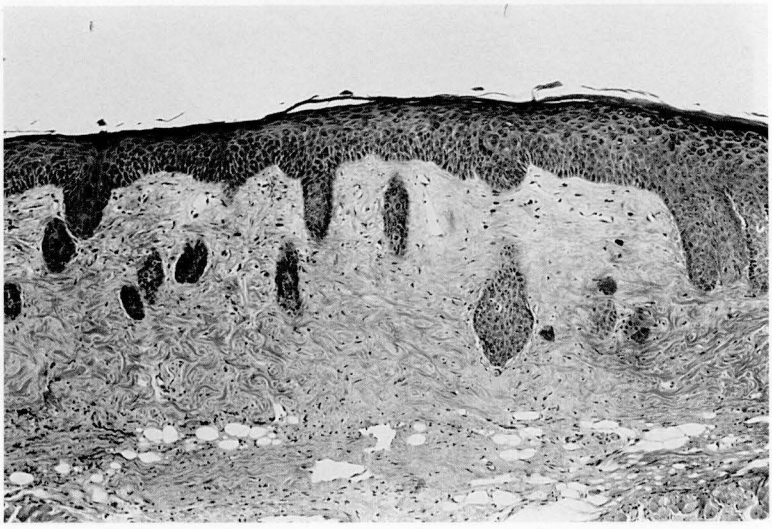
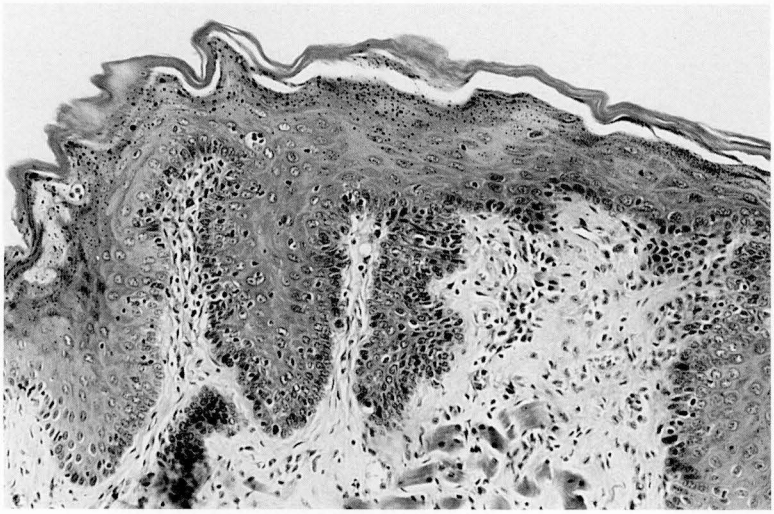


B

Figure 3 The skin lesion at 8 weeks after the treatment of DMBA.
A: c-*fos* mouse. Well differentiated carcinoma was observed invaded to basal cell membrane with cellular atypism.
B: litter mate. The epithelium showed parakeratosis, and inflammatory cells were observed in subcutaneous tissue, but epithelial cell had no cellular atypism.



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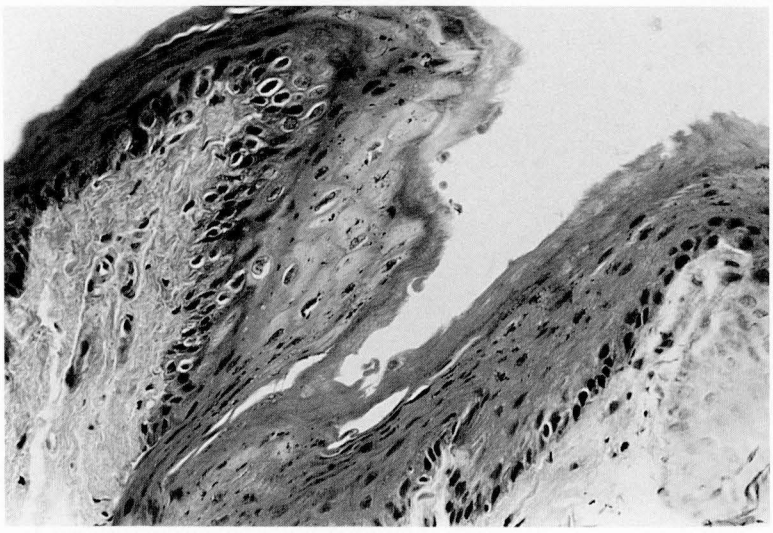
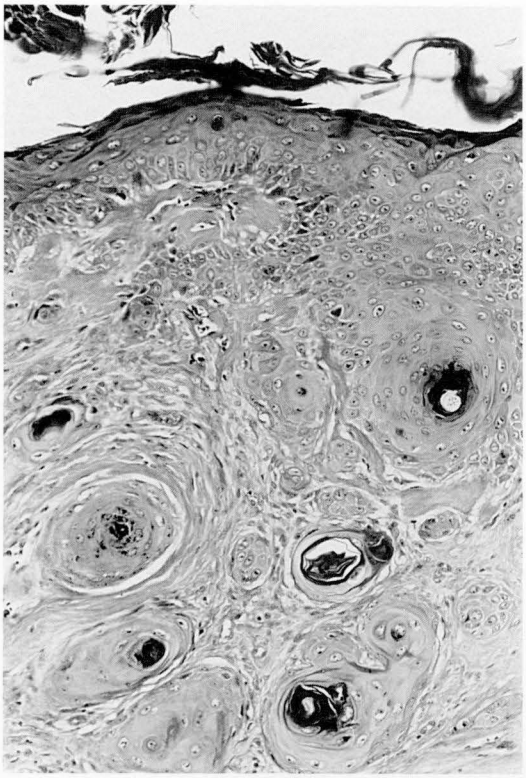


B

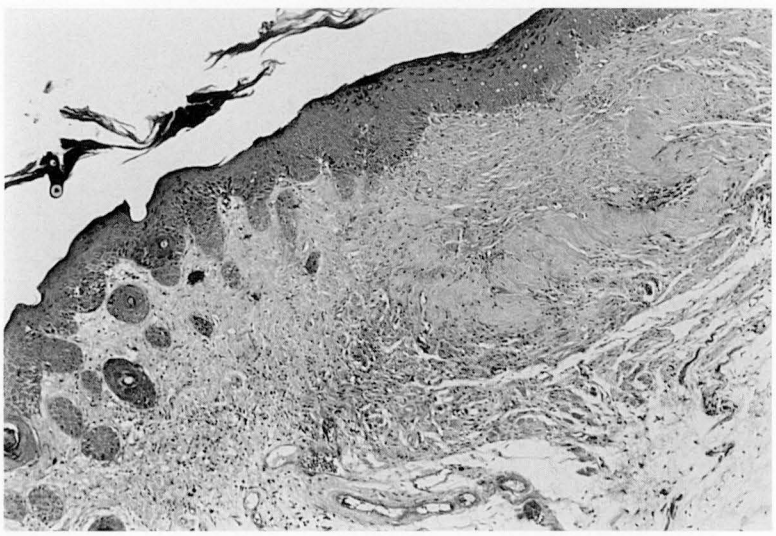
Figure 4 The gingiva lesion at 5 weeks after the treatment of DMBA.

A: c-*fos* mouse. The tissue showed slight acanthosis and elongation of rate ridge in the epithelium. No malignant change was observed.

B: litter mate. Only slight reactive changes were observed.



A

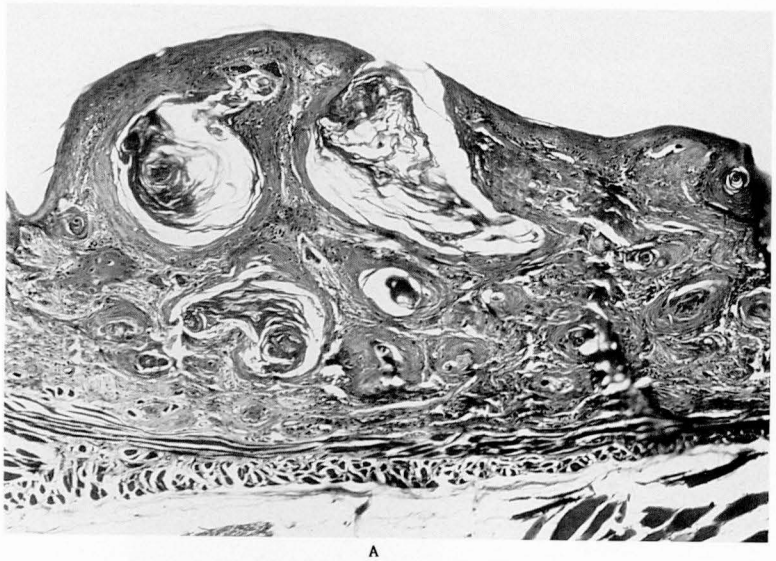


B

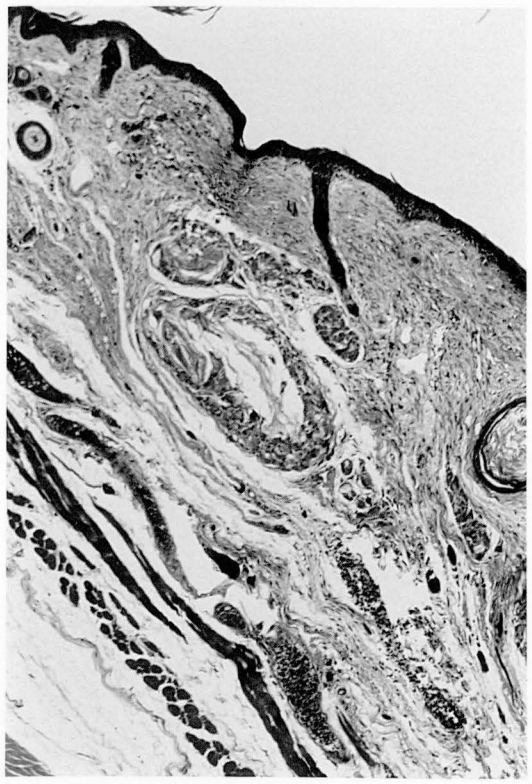
Figure 5 The gingiva lesion at 8 weeks after the treatment of DMBA.

A: c-*fos* mouse. Invasive well differentiated squamous cell carcinoma containing cancer pearls was found. This carcinoma invaded under the epithelium.

B: litter mate. No malignant changes were occurred, although slight hyperkeratosis and focal inflammatory change was ovserved in subcutaneous tissue.



A



B

Figure 6 The gingiva lesion at 11 weeks after the treatment of DMBA.

A: c-*fos* mouse. Well differentiated squamous carcinoma broadly invaded under the epithelium.

B: litter mate. Only slight hyperkeratosis and focal inflammatory change were shown in the subcutaneous tissue. No obvious malignant changes were observed.

Immunosurveillance was deteriorated in c-fos mice

Tumor cells have been thought to be recognized by immune compitente cells which are potentially active in limiting or eliminating those tumor cells. This mechanism is designated as immunosurveillance. Natural killer (NK) cells and cytotoxic T lymphocytes (CTL) are the effector cells for the immunosurveillance. Since c-*fos* mice demonstrated immune abnormalities, NK cell activity in c-*fos* mice was examined against YAC-1 cells by using the 4 hr ^{51}Cr release assay described in method section. Table 1 showed that the NK cell activity of spleen cells from c-*fos* mice was significantly lower (less than 50%) than that from control litter mates.

Cytotoxic T lymphocytes (CTL) activity of c-*fos* mice was also examined. Allo-specific CTL was induced by cultivating spleen cells from c-*fos* mice ($H\text{-}2^b$) with mitomycin C (MMC) treated M12.4 B lymphoma cells ($H\text{-}2^d$) for 5 days. The in vitro induced allo-CTL activity was measured against M12.4 cells as target cells by 10-hr ^{51}Cr release assay described in method section. As shown in Table 2, the allo-specific CTL activity in spleen cells from c-*fos* mice was distinctly lower (less than 50%) than that from control litter mate. These results support the evidence of quick carcinogenesis in c-*fos* mice by the DMBA treatment.

Table 1 Natural killer (NK) cell activity.

Effector cells	% specific lysis [1]	
	E/T ratio [2]	
	50	25
c-*fos* [3]	8.73±1.17 [4]	6.83±0.62
control [5]	13.90±0.95	12.65±2.50

1) calculated by the formula described in method

2) E/T ratio=Effector cell number/Target cell number

3) spleen cells from c-*fos* mouse

4) mean±SD were calculated for three samples

5) spleen cells from control litter mate

DISCUSSION

The mechanism of carcinogenesis is still unknown. Recent observations of proto-oncogene studies indicate that oncogene product plays a key role in oncogenesis.[26] That is, high expression of proto-oncogene in transgenic mice induces malignant tumors.[1,12,23,28] We have also studied the functional role of c-*fos* proto-oncogene in carcinogenesis by making transgenic mice. C-*fos* gene is the cellular homologue of v-*fos* gene in FBJ-murine sarcoma virus that generate osterosarcoma. Transgenic mice with exogenous c-*fos* gene under the control of human metallo-thionein (MT_{IIa}) gene promoter expressed high amounts of c-*fos* protein in various organs and showed osteohyperplasia without any malignant changes.[21] Since 3' nontranlated region of c-*fos* RNA was important for the stability of the RNA,[16] the MT_{IIa} /c-*fos* gene in which the 3' nontranslated region was replaced by a long terminal repeat (LTR) of the FBJ-sarcoma virus was used for making different transgenic mice.[23] These mice generated chondrosarcoma, indicating that high expression of the stable c-*fos* mRNA induced bone tumor. However, no other kind of tumor was observed in the mice in spite of high expression of the stable c-*fos* mRNA in many other organs. Therefore, c-*fos* gene product can immortalize only bone cells. Other organs may require another stimulation besides c-*fos* protein for carcinogenesis.

Table 2 Allo-specific cytotoxic T lymphocyte (CTL) activity.

Effector cells	% specific lysis[1]		
	E/T ratio[2]		
	80	40	20
c-*fos*[3]	44.0±3.1[4]	30.2±2.8	15.4±1.3
control[5]	73.0±4.8	34.5±4.4	26.1±5.0

1) calculated by the fomula described in the method
2) E/T ratio=Effector cell number/Target cell number
3) spleen cells from c-*fos* mouse
4) mean±SD were calculated for three samples
5) spleen cells from control litter mate

New transgenic mice carrying exogenous c-*fos* gene regulated by promotor of class I MHC gene (c-*fos* mouse) were established.[22] Skin and gingivaof the c-*fos* mice expressed large amount of exogenous c-*fos* RNA as shown in Fig. 1. In order to examine the effect of another stimulation on carcinogenesis in c-*fos* mice, skin or gingiva of c-*fos* mice were painted with DMBA. C-*fos* mice generated squamous cell carcinoma at 8 weeks after the treatment in both skin and gingiva. In contrast, control litter mate showed no tumor even at 11 weeks after the treatment in gingiva. Sabes et al. have also reported that the earliest carcinogenesis in buccal pouch of hamster treated with DMBA was induced at 11 weeks after the treatment.[18,24] Therefore, carcinogenesis in c-*fos* mice treated with DMBA was accelereated.

Chemical carcinogenesis in murine system usually uses two step method, initiation by DMBA and promotion by 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Hennings et al. have reported that carcinomas are normally induced at 5-6 months after the DMBA/TPA treatment.[13] Brown et al. have reported that multiple carcinomas were induced in BALB/c mice, infected with Harvey mucrine sarcoma viruses as initiation, treated with TPA as promotion for 5 months.[5] Since only DMBA treatment could generate squamous cell carcinoma in c-*fos* mice at 8 weeks after the treatment, c-*fos* protein may play a role for promotion in c-*fos* mice.

The osteosarcoma induced in newborn mice by transducing retroviruses FBJ-MSV showed elevated levels of v-*fos* expression. Chondrosarcoma generated in MT_{IIa}-c-*fos* transgenic mice also expressed high amount of exogenous c-*fos* gene, indicated that high amount of c-*fos* expression was essential for the outgrowth of the cells and tumor development. The gingiva of c-*fos* mice, however, did not show distinct amount of exogenous c-*fos* RNA compared with the skin (see Fig. 1). Although, precancerous lesions were observed in the skin (Fig. 2A) compared with only reactive changes in the gingiva (Fig. 4A) at 5 weeks after the treatment, both of the organs developed squamous cell carcinoma (Fig. 3A and 5A) at 8 weeks after the treatment. These observations suggest that c-*fos* protein in squamous cells does not play a major role for their carcinogenesis, and that other functional abnormalities in c-*fos* mice accelerate carcinogenesis in DMBA-stimulated cells.

Cellular proto oncogenes can be inactivated by point mutations or chromosomal translocations. Several investigators. have

reported tht analysis of cellular oncogenes (c-*onc*) of tumors induced by some physical or chemical carcinogens.[4,10,20,33] In those reports, skin tumors induced by DMBA have associated with a specific A-T transversion at the second nucleotide of codon 61 of the Harvey-*ras* (Ha-*ras*) gene. From these views, tumors in c-*fos* mice may have some mutations, gene amplification or activation of other oncogenes. Furthermore molecular investigation in tumor will be needed.

Immunosurveillance is thought to be a major defense mechanism for in vivo carcinogenesis. The frequency of neoplasms in the T cell deficient mice is unequivocally greater than that in normal siblings.[32] The 'beige' mouse which misses NK cells is also more susceptible to primary tumor development by chemical carcinogens.[19] C-*fos* mice also showed deteriorated activity of immunosurveillance. The activities of NK cells and allo-specific CTL in spleen cells from c-*fos* mice were less than half of those litter mate as shown in table 1 and table 2. These facts indicate that the acceleration of carcinogenesis in c-*fos* mice induced by the DMBA treatment is due to the synergistic effects of overexpression of c-*fos* gene and partial deterioration of immunosurveillance.

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