T Cell Deficiency Leads to Liver Carcinogenesis in Azoxymethane-Treated Rats

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There is an increasing amount of evidence suggesting that T cell deficiency contributes to tumor development. However, it is unclear whether T cell deficiency leads to liver and colon carcinogenesis. The aim of this study was to investigate the role of T cells on liver and colon carcinogenesis. Athymic F344/N Jcl-rnu/– (*nu/nu*) rats and euthymic F344/N Jcl-rnu/+ (*nu/+*) rats were administered the carcinogen azoxymethane (AOM) at a dose of 15 mg/kg body wt once a week for 2 weeks. At 48 weeks after the second carcinogen treatment, the rats were sacrificed, and livers and colons were examined. Apoptosis and cell proliferation were evaluated by DNA fragmentation and proliferating cell nuclear antigen assays, respectively. Wild-type p53 and members of the Jun and Fos oncogene families were detected by Western blotting. AOM treatment induced 100% liver tumor and 63.6% colon tumor incidence in T cell–deficient *nu/nu* rats, compared with 0% and 38.5% incidence in *nu/+* rats. T cell deficiency promoted the inhibitory action of AOM on apoptosis in both liver and colon at 48 weeks. In contrast, T cell deficiency increased cell proliferation after AOM treatment in both tissues. Wild-type p53 was reduced in both tissues of T cell–deficient rats. AOM treatment induced c-Jun and c-Fos expressions in the liver but increased only Fos B in the colon, whereas T cell deficiency enhanced c-Jun overexpression in the liver. These results suggest that T cell deficiency leads to liver carcinogenesis partly by a reduction in wild-type p53 and increasing c-Jun expression in AOM-treated rats. Exp Biol Med 231:91–98, 2006

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Introduction

Recent advances in immunology and increasing knowledge of tumor immunity have encouraged the development of various immunotherapies to eradicate tumors or to prevent their development. The expressions of major histocompatibility class (MHC) I and II, and costimulatory molecules essential for priming $CD4^+$ and $CD8^+$ T cells, are important. Despite T cell recognition, most antigenic tumors are not efficiently rejected (1, 2). The mechanisms that inhibit tumor rejection involve those mediated by the tumor itself, such as tumor-derived transforming growth factor-β, soluble MHC I chain-related molecules, and the immunoregulatory mechanisms of the host, including cytotoxic T lymphocyte (CTL) antigen-4, activation-induced cell death, $CD4^+CD25^+$ regulatory T cells, and $CD4^+$ natural killer (NK) T cells that produce interleukin-13 (3–7). In addition to these active or negative regulatory mechanisms (or both), a basic lack of interaction between the tumor and the host may lead to poor T cell priming, improper localization of antitumor T cells, and, ultimately, abrogation of antitumor immunity. Experiments using murine models have shown that abundant, activated, and tumor antigen–specific T cells fail to reject tumors, even when they can reject skin grafts or less-established tumors bearing the same antigen (8–10). Recent reports have underscored the importance of $CD4^+$ cells as regulators of the antitumor immune response (11–13). In addition, it is widely accepted that $CD8^+$ cytotoxic T lymphocytes, originally isolated from tumor-infiltrating lymphocytes *in vitro*, play a major role in tumor rejection *in vivo* (14–16).

Wild-type (wt) p53 is believed to function as a transcription factor, and activated wt p53 can induce the expression of a large number of genes, many of which evoke either cell cycle arrest or apoptosis (17–19). Wild-type p53 induces cell death through a multitude of

molecular pathways involving transcription-dependent or transcription-independent functions (or both) (20, 21). Wild-type p53 is a potent inhibitor of cell and tumor growth, and its inactivation or mutation (or both) is considered to be a prerequisite for tumor formation (22). Wild-type p53 is therefore an attractive candidate for broadly applicable cancer vaccines, which could combine the multiple tumor epitopes defined by $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ T-helper cells (23, 24).

Genes belonging to the Jun and Fos oncogene families encode nuclear proteins that are associated with a number of transcriptional complexes (25, 26). Members of the Jun gene family, including c-Jun, Jun B, and Jun D, are major components of the transcription factor AP-1, which was originally shown to mediate cell carcinogenesis. Members of the c-Fos gene family, including c-Fos, Fos B, Fra-1, and Fra-2, encode nuclear phosphoproteins that are rapidly and transiently induced by a variety of agents and that function as transcriptional regulators of several genes. Most of the Jun and Fos family members control cell life and death through their ability to regulate the expression and function of cell cycle regulators such as p53, $p21^{Cip1}$ p19, p16, and cyclin D1.

Although large numbers of studies have suggested that T cell deficiency contributes to cancer development, it is unclear how T cell deficiency is involved in carcinogenesis and cancer development in some cell types. Azoxymethane (AOM), a potent colon carcinogen, was used in this study. AOM is metabolized by the liver to form the genotoxic carcinogen methylazoxymethanol, and then travels into the intestinal tract via bile fluid. AOM induces high levels of DNA damage in both the colon and the liver, yet tumors are formed almost exclusively in the colon (27). However, in the present study, AOM induced a 100% incidence of liver carcinogenesis in T cell–deficient rats, but only a 63.6% incidence of colon carcinogenesis. The aim of the study was to investigate why T cell deficiency leads to liver carcinogenesis after AOM treatment, and how p53 and the Jun oncogene family play roles in this carcinogenesis.

Materials and Methods

Animals and Experimental Procedures. Athymic F344/N Jcl-rnu/– (*nu/nu*) rats and euthymic F344/N Jcl-rnu/+ (*nu/+*) rats were purchased from CLEA Japan (Tokyo, Japan). The rats were housed in plastic cages in a room illuminated from 0800 to 2000 hrs (12:12-hr light:dark cycle), and allowed *ad libitum* access to water and a CE-2 basal diet (CLEA Japan). The CE-2 basal diet included 8.9% moisture, 25.4% crude protein, 4.4% crude fat, 4.1% crude fiber, and 6.9% crude ash. At 5 weeks of age, the rats received ip injections of either AOM (15 mg/kg body wt; Sigma, St. Louis, MO) dissolved in 1 ml of physiological saline once a week for 2 weeks, or 1 ml of physiological saline once a week for 2 weeks.

To examine the liver and colon for carcinogenesis, at 48 weeks after the second injection of AOM, animals were anesthetized and then sacrificed. Livers and colons were removed, cut into several sections, fixed in 10% buffed formalin, and embedded in paraffin. Four-micrometer sections were cut and stained with hematoxylin-eosin and silver impregnation. In the liver, neoplastic lesions >2 mm were counted as tumors. In the colon, neoplastic lesions >5 mm were counted as tumors.

DNA Fragmentation Assay. The amount of fragmented DNA was determined as previously described (28–31). The liver tissues were homogenized in 10 volumes of a lysis buffer (pH 8.0) consisting of 5 m*M* Tris-HCl, 20 m*M* EDTA (Sigma) and 0.5% (w/v) t-octylphenoxypolyethoxyethanol (Triton X-100; Sigma). One-milliliter aliquots of each sample were centrifuged at 27,000 *g* for 20 mins to separate the intact chromatin (pellet) from the fragmented DNA (supernatant). The supernatant was decanted and saved, and the pellet was resuspended in 1 ml of Tris buffer (pH 8.0) consisting of 10 m*M* Tris-HCl and 1 m*M* EDTA. The pellet and supernatant fractions were assayed for DNA content using a diphenylamine reaction. The results were expressed as a percentage of fragmented DNA divided by total DNA. Six animals were studied in each group.

DNA Ladders Assay. The total DNAs from liver and colon were extracted sequentially using a phenol-chloroform–isoamyl alcohol mixture (25:24:1, v/v/v) to remove proteins and then purified as previously described (28). Resolving agarose gel electrophoresis was preformed using a 1.5% gel containing 1.0 μg/ml ethidium bromide. Depending on the experiment, 20 μg of total DNA per well was loaded. DNA standards were included to identify the size of DNA fragments. The DNA ladders were observed under ultraviolet fluorescent light.

Western Blotting Analysis. Total protein and cytosolic fractions of liver and colon tissues were purified as previously described (29–32). Equal quantities of protein were electrophoresed in a sodium dodecyl sulfate–polyacrylamide gel, then electroblotted onto a nitrocellulose membrane (Trans-Blot; Bio-Rad, Hercules, CA). After blocking with phosphate-buffered saline containing 0.1% polyoxyethylene sorbitan monolaurate (Tween-20; Sigma) and 5% skim milk at 4°C overnight, the membranes were incubated with a primary antibody for 1 hr. The primary antibodies used were rabbit polyclonal antiproliferating cell nuclear antigen (PCNA) antibody (1:1000; Santa Cruz Biotech, Santa Cruz, CA), rabbit polyclonal anti-c-Jun antibody (1:1000; Santa Cruz Biotech), rabbit polyclonal anti-Jun B antibody (1:1000; Santa Cruz Biotech), rabbit polyclonal anti-Jun D antibody (1:500; Santa Cruz Biotech), rabbit polyclonal anti-c-Fos antibody (1:200; Santa Cruz Biotech), rabbit polyclonal anti-Fos B antibody (1:500; Santa Cruz Biotech), rabbit polyclonal anti-Fra-1 antibody (1:200; Santa Cruz Biotech), rabbit polyclonal anti-Fra-2 antibody (1:200; Santa Cruz Biotech), and mouse monoclonal anti-β-actin antibody (1:5000; Sigma). The membranes were then incubated with a secondary antibody; namely, horseradish peroxidase-conjugated anti-mouse or

Table 1. T Cell Deficiency Responses to Liver and Colon Tumor Incidences in AOM-Treated Rats at 48 Weeks

	Tumor incidence[a]	
	F344/N Jcl-rnu/+	F344/N Jcl-rnu/−
Vehicle		
Liver	0% (0/6)	0% (0/6)
Colon	0% (0/6)	0% (0/6)
AOM		
Liver	0% (0/13)*	100% (11/11)*,#
Colon	38.5% (5/13)*	63.6% (7/11)*,#

[a] Tumor incidence is expressed as a ratio of rats with tumor/total rats. Results were analyzed statistically using the chi-square test.
*$P < 0.01$ compared to the vehicle-treated rats, #$P < 0.05$ compared to the F344/N Jcl-rnu/+ rats.

anti-rabbit IgG (1:2000; Santa Cruz Biotech), and developed by chemiluminescence using enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK).

For wt p53 assessment, equal quantities of protein were electrophoresed in a nondenatured polyacrylamide gel at 10%. To avoid protein denaturation, the immunoassay was conducted without boiling and the inclusion of SDS in the samples (33, 34). The immunoblotting was assayed using a primary mouse monoclonal anti-wt p53 antibody (1:1000; Santa Cruz Biotech). This antibody recognizes only wt p53, but not mutant p53, under nondenaturing conditions.

Densitometric assessment of the autoradiogram bands was conducted using Image Gauge VDS (Fujifilm, Tokyo, Japan). The intensity of each band was quantified by measuring the absolute integrated optical intensity, which estimates the volume of the band in the lane profile, and expressed as the ratio to the β-actin band intensity. Three animals were evaluated in three separate experiments.

Statistical Analysis. The results are expressed as the mean ± SD. Data were evaluated by ANOVA in which multiple comparisons were performed by the method of least significant difference. Tumor incidence was expressed as the percentage of animals with liver or colon tumors, and the results were analyzed statistically using the chi-square test. Differences were considered significant if the probability of the difference occurring by chance was less than 5 in 100 ($P < 0.05$).

Results

T Cell Deficiency Leads to Liver and Colon Carcinogenesis in AOM-Treated Rats. At 48 weeks after the second injection of AOM, tumorigenesis in the colon and liver was evaluated (Table 1). No colon or liver tumors were observed in either *nu/+* or *nu/nu* rats after vehicle treatment. After AOM treatment, *nu/nu* rats showed a higher incidence of colon adenocarcinomas than *nu/+* rats (Table 1). The number of colon tumors in *nu/nu* rats was about 2.1-fold higher than in *nu/+* rats at 48 weeks after AOM treatment (data not shown). All these colonic tumors were well to moderately differentiated adenocarcinomas without metastasis or vascular/lymphatic invasion (Fig. 1).

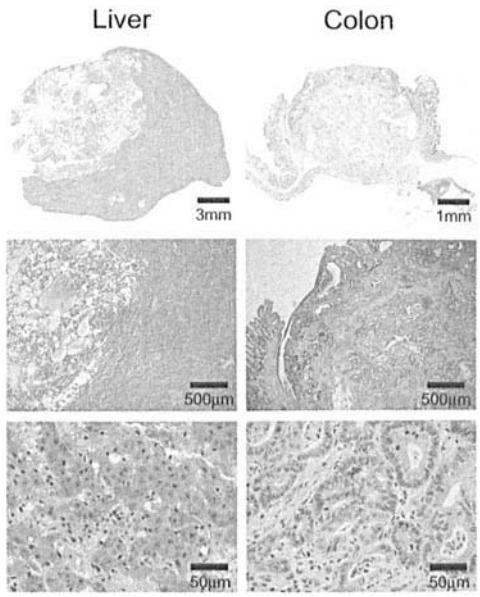
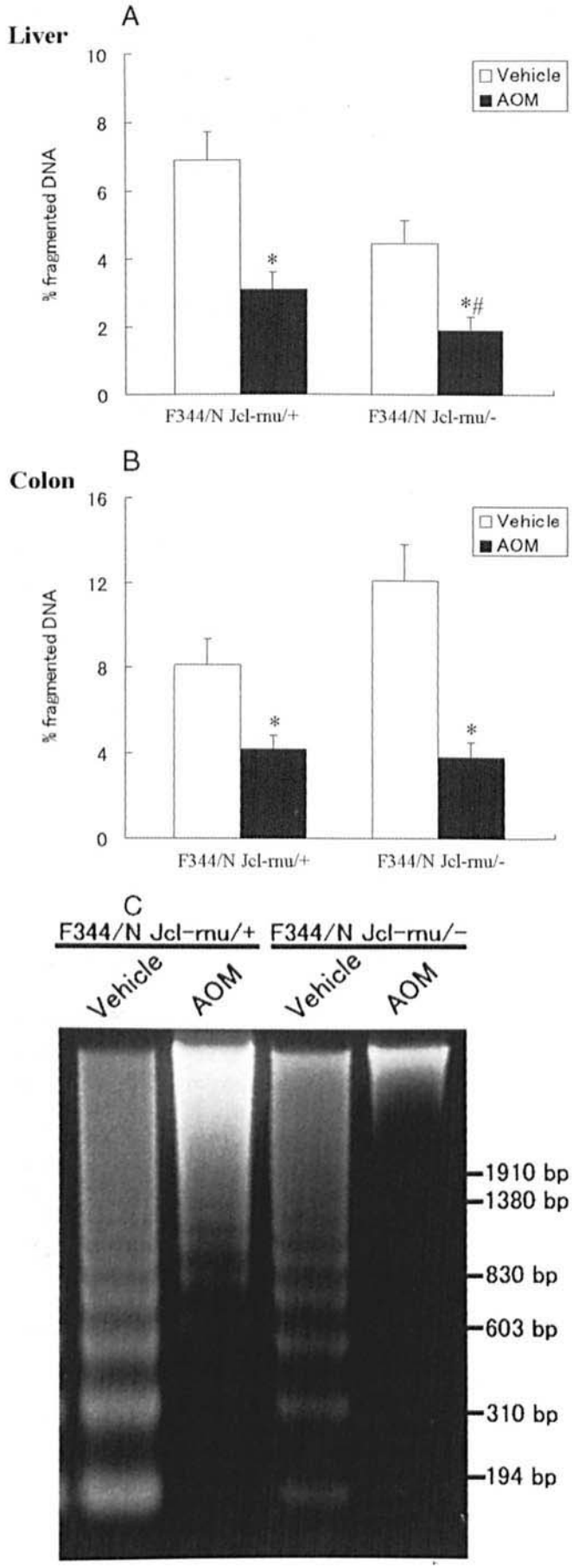


Figure 1. Histological findings of liver and colon tumors in AOM-treated T cell–deficient rats at 48 weeks. The liver tumor is composed of cells with large, irregular nuclei, high nuclear/cytoplasmic ratios, and prominent nucleoli. The liver tumor cells are growing in a trabecular pattern, attempting to mimic the cell plates of the normal liver. The colon tumor is a moderately differentiated adenocarcinoma with an invasive growth pattern.

Surprisingly, T cell deficiency in *nu/nu* rats showed 100% incidence of liver neoplastic tumor formation compared with a 0% incidence in *nu/+* rats with AOM treatment. The tumor cells grew in a trabecular pattern, attempting to mimic the cell plates of the normal liver (Fig. 1). The tumor cells contained large, irregular nuclei, high nucleus/cytoplasm ratios, and prominent nucleoli. Although multifocal preneoplastic foci of the liver were observed in AOM-treated *nu/+* rats, these lesions were smaller than 0.3 mm in diameter. The results suggest that T cells may have an inhibitory action for hepatocarcinogenesis in AOM-treated rats.

T Cell Deficiency Enhances Suppression of Apoptosis and Promotion of Cell Proliferation in the Liver and Colon After AOM Treatment. Percentages of fragmented DNA in total DNA in livers and colons are shown in Figure 2A and B. Azoxymethane treatment significantly inhibited cell DNA fragmentation in the liver and colon. After AOM treatment, the percentage of fragmented DNA in the liver was significantly reduced in both *nu/nu* rats (61.1%) and *nu/+* rats (45.8%) compared with their respective vehicle treatments ($P < 0.01$ for each). The DNA fragmentation in the liver was significantly suppressed in *nu/nu* rats (2.1% ± 0.3%) compared with *nu/+* rats (3.8% ± 0.4%) after AOM treatment ($P < 0.01$). In the colon, the percentage of fragmented DNA was reduced 48.1% (in *nu/+* rats) and 68.6% (in *nu/nu* rats) after AOM



treatment, compared with rats treated with vehicle ($P < 0.01$). The DNA ladders assay also showed that liver apoptosis was significantly inhibited in AOM-treated rats at 48 weeks, and the inhibitory effect was significantly enhanced in *nu/nu* rats compared with *nu/+* rats (Fig. 2C). In the colon, results of DNA ladders were similar to those observed in the liver (data not shown).

Cell proliferation was evaluated by assessing PCNA (Fig. 3A, B). After AOM treatment, the PCNA of liver tissue was increased about 1.8-fold in F344/N Jcl-rnu/+ rats and 4.2-fold in *nu/nu* rats compared with their respective vehicle treatments ($P < 0.01$ for each). In colon, the PCNA was about 2.9-fold in *nu/+* rats and 3.4-fold in *nu/nu* rats after AOM treatment, compared with rats treated with vehicle treatment ($P < 0.01$ for each).

T Cell Deficiency Reduces wt p53 Protein Expression in Liver and Colon. The expression of wt p53 protein was determined by Western blotting analysis in total proteins and cytosolic fractions (Fig. 4A, B). In both liver and colon, total wt p53 was significantly reduced in *nu/nu* rats compared with *nu/+* rats. AOM treatment did not inhibit total wt p53 in *nu/+* rats, but cytosolic wt p53 was significantly increased in both liver and colon compared with vehicle-treated rats. The data show that wt p53 generation partly depends on T cells in these rats. The results suggest that T cell deficiency responds to liver and colon carcinogenesis via reducing wt p53. Although AOM treatment did not inhibit total wt p53 in *nu/+* rats, significantly increased cytosolic wt p53 resulted in inactivation of wt p53 in liver and colon.

T Cell Deficiency Enhances c-Jun Overexpression in the Liver After AOM Treatment. The transcription factor Jun and Fos oncogene families have been implicated in several cellular processes, including proliferation, cell survival, and cell transformation. We investigated the expressions of Jun and Fos oncogene family members in liver and colon (Fig. 5A, B). Azoxymethane treatment significantly increased c-Jun and c-Fos expressions in liver tissue in both *nu/+* and *nu/nu* rats, but did not induce Jun B, Jun D, Fos B, Fra-1, or Fra-2 expression. In the colon, AOM treatment significantly increased the amount of Fos B, but it did not increase the Jun family members, c-Fos, Fra-1, and Fra-2 in either *nu/+* or *nu/nu* rats. In T cell–deficient *nu/nu* rats, c-Jun expression was significantly increased in liver, but not in colon, compared with that of *nu/+* rats after AOM treatment. The results indicate that T cell deficiency enhanced c-Jun overexpression in the liver, but it did not

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Figure 2. T cell deficiency suppresses liver and colon apoptosis in AOM-treated rats at 48 weeks. (A and B) The percentage of fragmented DNA in the liver and colon. The values are the means ± SD of six rats for each group. *$P < 0.01$ vs. vehicle-treated rats; #$P < 0.01$ vs. F344/N Jcl-rnu/+ rats. (C) DNA ladders in the liver. Twenty micrograms of total DNA per well was loaded. The ladder, which is characteristic of apoptosis, is clearly shown.

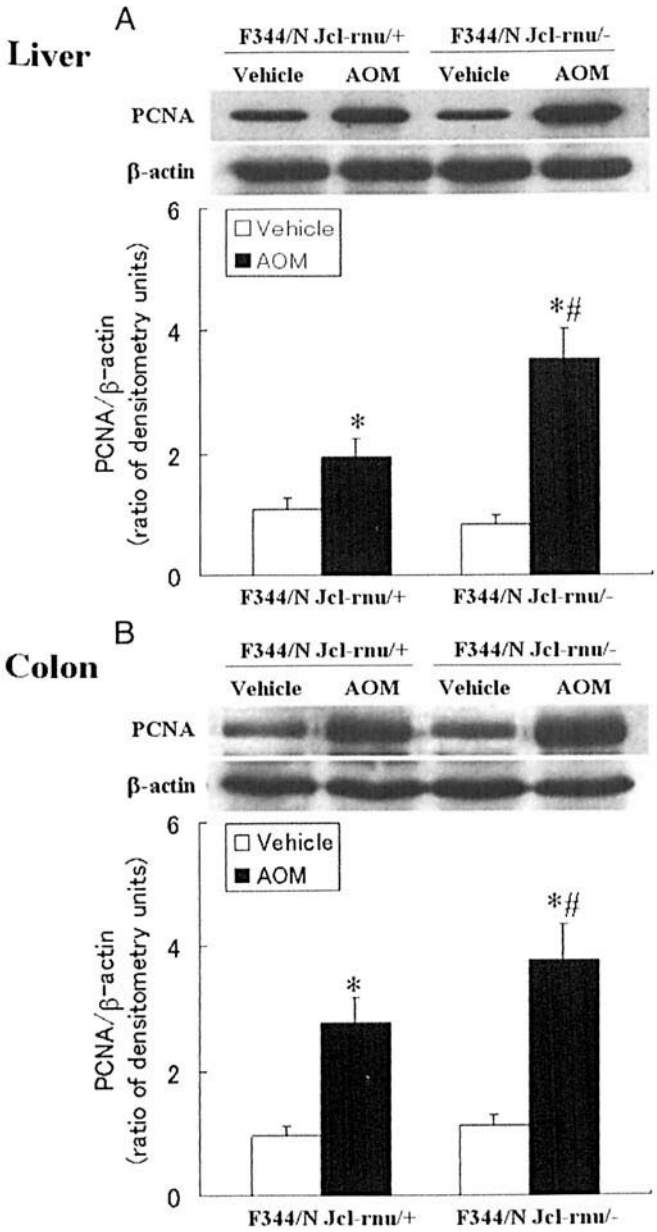

Figure 3. T cell deficiency enhances AOM-induced cell proliferation in rat liver and colon at 48 weeks. PCNA was evaluated in the total protein using Western blotting analysis. The values are the means ± SD of three rats in three separate experiments for each group. $*P < 0.01$ versus vehicle-treated rats; $\#P < 0.01$ versus F344/N Jcl-rnu/+ rats.

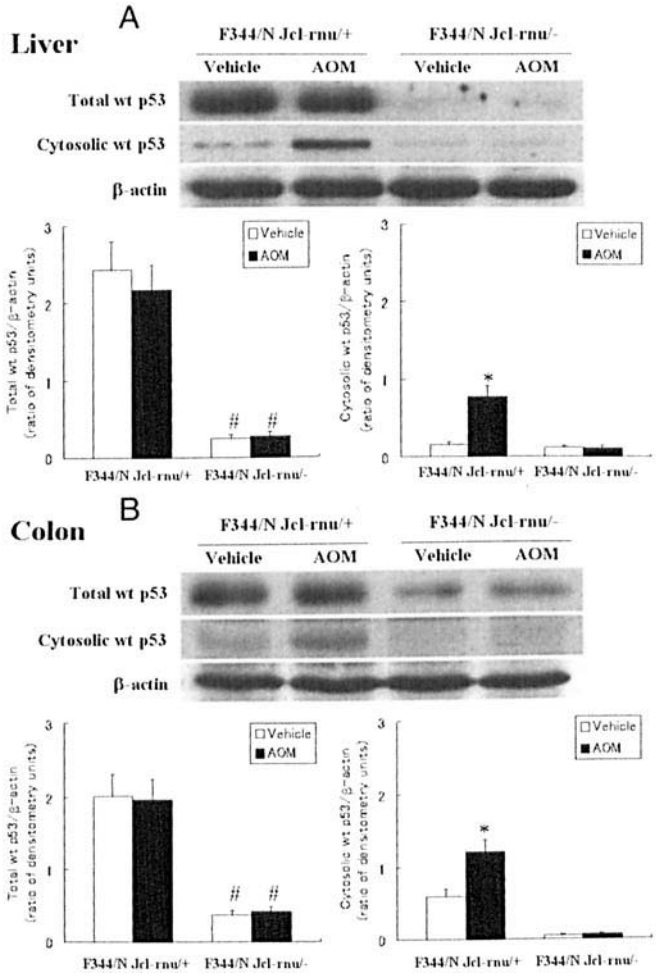

Figure 4. T cell deficiency leads to a reduction in wt p53 in rat liver and colon. Equal quantities of protein were electrophoresed in a nondenatured sodium dodecyl sulfate–polyacrylamide gel, and then immunoblotting was assayed using a primary mouse monoclonal anti-wt p53 antibody (1:1000; Santa Cruz Biotech). This antibody recognizes only wt p53, but not mutant p53, under nondenaturing conditions. The values are the means ± SD of three rats in three separate experiments for each group. $*P < 0.01$ versus vehicle-treated rats; $\#P < 0.01$ versus F344/N Jcl-rnu/+ rats.

enhance Fos B overexpression in the colon after AOM treatment.

Discussion

Recent reports have underscored the importance of $CD4^+$ cells as regulators of the antitumor immune response (11–13). It is widely accepted that $CD8^+$ cytotoxic T lymphocytes, originally isolated from tumor-infiltrating lymphocytes *in vitro,* play a major role in tumor rejection *in vivo* (14–16). It is common sense among tumor immunologists that NK, CTL, and Th1 cells play a crucial role in the powerful elimination of tumor cells. NK cells function not only as surveillants in the early stages of tumor development, but they are also helpers in the priming process role played by CTLs and Th1 cells by producing interferon (IFN)-γ (35, 36). Therefore, the effectual interaction of innate NK cells with acquired Th1 cells and CTLs may result in the complete regression of tumors in a number of experimental systems. In contrast, a decline in NK activities, including cytotoxicity and IFN-γ production at an early stage of tumor development leads to ineffective generation of antitumor immunity and allows tumors to grow well (37, 38). Therefore, T cells play an important role in carcinogenesis inhibition.

Liver tumor development in rat models with T cell deficiency have been established to study the molecular mechanisms of T cell function in hepatocarcinogenesis.

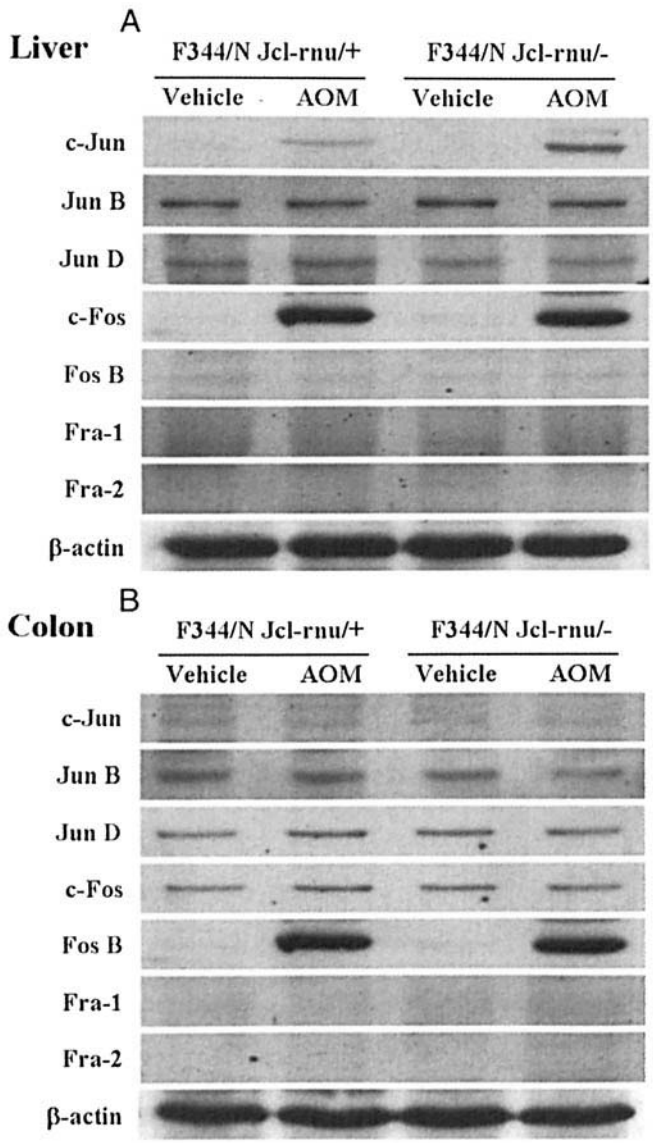

Figure 5. T cell deficiency enhances c-Jun overexpression in the liver after rats were treated with AOM. AOM treatment significantly increases the c-Jun and c-Fos levels in the liver tissue, but not in the colon. T cell deficiency enhances c-Jun overexpression in the liver after AOM treatment. Three rats were studied in three separate experiments for each group.

Multiple studies in experimental animals showed that cellular rather than humoral immune responses were responsible for tumor rejection (11–16, 39). The chemical, AOM, is considered a potent colon carcinogen, and induces high levels of DNA damage in both colon and liver, yet tumors are formed almost exclusively in the colon (27). However, in the present study, AOM induced a 100% incidence of liver carcinogenesis in T cell–deficient rats, but only a 63.6% incidence of colon carcinogenesis. The data demonstrate that liver tumor formation was strongly induced in T cell–deficient rats after AOM treatment. Tumor development was correlated, at least in part, with increased levels of c-Jun and decreased levels of p53, resulting in the suppression of apoptosis and the promotion of cell proliferation. These results suggest that T cell deficiency led to AOM-induced liver carcinogenesis.

Tumor growth is determined not only by increased cell proliferation, but also by decreased tumor cell apoptosis. Our results showed that AOM treatment significantly inhibited cell apoptosis and promoted cell proliferation in both the liver and the colon. After AOM treatment, liver and colon cell apoptosis was significantly suppressed in *nu/nu* rats compared with *nu/+* rats. T cell deficiency also significantly increased cell proliferation in both the liver and colon after AOM treatment. These results suggest that T cell deficiency response to AOM-induced liver and colon carcinogenesis via inhibiting cell apoptosis and promoting cell proliferation.

Wild-type p53 has multiple functions as a tumor suppressor, including cell cycle arrest in response to DNA damage, induction of apoptosis, and DNA repair (40–43). In its normal state, the tumor suppressor action of wt p53 is present in the nucleus and mediated by specific DNA binding and protein-protein interactions within the nucleus, although a secondary event sometimes occurs in which wt p53 is lost (44). Abnormal cytosolic wt p53 localization has been observed in a number of cancer cell lines, and this cytosolic wt p53 is stable and inactive (45). We previously demonstrated that AOM treatment significantly increased cytosolic wt p53 and inhibited wt p53-mediated mitochondria-dependent apoptosis in colon carcinogenesis (32). Loss of wt p53 function and p53 deficiency is associated with increased susceptibility to AOM-induced colon cancer (32, 46). p53 is frequently mutated in human cancer (47, 48); however, recent evidence shows that colon cancer induced by the model rodent carcinogen AOM does not carry a p53 mutation (49–51). The AOM-induced cancer model may be well suited for studying tumor promotion events that precede wt p53 disruption. The present data show that T cell deficiency significantly reduced wt p53 formation in the liver and colon. Although AOM treatment did not inhibit total wt p53 in F344/N Jcl-rnu/+ rats, significantly increased cytosolic wt p53 resulted in inactivation of wt p53 in the liver and colon. These results suggest that T cell deficiency responses to liver carcinogenesis via reducing wt p53 after AOM treatment. Cytotoxic T lymphocytes specific for wt p53 peptides have been shown to react against a wide range of tumors, but not normal cells, and a successful $CD8^+$ CTL-based immunotherapy for cancer is dependent on active $CD4^+$ T-helper cells (52, 53). Wild-type p53 generation may partly depend on T cells. However, it is unclear how T cells induce p53 generation.

The oncogenicity of c-Jun is probably due to its cooperation with the ras oncogene during tumor cell proliferation, and its effect on the antiproliferation activity of wt p53 by direct repression of p53 transcription (54–56). Overexpression of c-Fos also plays important roles in cell proliferation and development (57, 58). In this study, we found that c-Jun and c-Fos were significantly induced in the liver rather than in the colon after AOM treatment, and that

T cell deficiency significantly increased c-Jun expression in liver. In addition, we also observed that AOM treatment increased Fos B formation in the colon, but not in the liver. A previous study suggested that wt p53 is a negative regulator of c-Jun expression (55). Taken together, these results suggest the possibility that liver carcinogenesis due to T cell deficiency is partly related to a reduction in wt p53, and that this reduction in wt p53 results in c-Jun overexpression in the liver after AOM treatment.

In conclusion, T cell deficiency can contribute to liver carcinogenesis in AOM-treated rats. Wt p53 was reduced in both liver and colon in T cell–deficient rats, suggesting that T cell deficiency response to liver and colon carcinogenesis is related to a reduction in wt p53 formation. Azoxymethane treatment significantly increased c-Jun and c-Fos in the liver, but not in the colon, and T cell deficiency enhanced this AOM-induced c-Jun overexpression. The results suggest that reduction of wt p53 and overexpression of c-Jun may be involved in the mechanism of liver carcinogenesis.

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