Jpn. J. Cancer Res. **92**, 880–885, August 2001

Enhanced Formation of Azoxymethane-induced Colorectal Adenocarcinoma in γδ T Lymphocyte-deficient Mice

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T cell receptor (TCR) γδ-positive T lymphocytes, which are localized mostly within the intraepithelial space of intestinal epithelium, have been suggested to play a role in maintaining the normal configuration of intestinal epithelium. However, the role of TCRγδ-positive T lymphocytes in the formation and progression of colorectal adenocarcinoma that originates from colorectal epithelial cells remains to be elucidated. In this study, TCRαβ and TCRγδ-positive T lymphocyte-deficient mice (homozygous *TCRα* and *TCRδ*-gene knockout mice) and the background wild-type mice were administered azoxymethane, and the formation of macroscopic tumors and microscopic aberrant crypt foci in colorectal mucosa were compared among the three types of mice. Well-differentiated adenocarcinoma appeared 5 months after 5 administrations of azoxymethane (10 mg/kg weight) only in a few *TCRδ*-gene knockout mice and the frequency of the carcinoma-bearing mice was increased at 7 and 9 months after the administration. Aberrant crypt foci were also detected in the colorectal mucosa of *TCRδ*-gene knockout mice to a greater extent than in colorectal mucosa of *TCRα*-gene knockout mice 1 month after the azoxymethane administration. These results suggest that TCRγδ-positive T lymphocytes, which are present mainly in the intraepithelial space, play a role in suppression of the formation and progression of colorectal adenocarcinoma in mice.

Key words: TCRγδ — Knockout mouse — Azoxymethane — Colon — Adenocarcinoma

Previous research has demonstrated the presence of lymphocytes in the space between intestinal epithelial cells (intestinal intraepithelial lymphocytes: iIEL) and most of the iIEL were shown to carry T cell receptor (TCR) γδ molecules (γδ T cells) on the surface.[1)] γδ T cells have been suggested to differentiate in the intestinal mucosa[2)] and to play a unique role in concert with surrounding cells such as helper T cells, cytotoxic T cells (CTL), macrophages, natural killer cells (NK cells) and epithelial cells.[3,4)] γδ T cells have been reported to secrete a cytokine which supports the elimination of impaired epithelial cells and to maintain the normal configuration of the intestinal epithelium.[5,6)] However, the precise functions of γδ T cells have not been elucidated.

Some recent studies have described the effect of γδ T cells on intestinal tumor cells. γδ T cells regulated the cytotoxic activities of NK cells and CTL, of which the targets were intestinal tumor cells.[7)] The frequency of tumor-infiltrated γδ T cells was lowered in human well-to-moderately differentiated colorectal adenocarcinoma,[8)] suggesting a relationship between γδ T cells and the formation and progression of colorectal adenocarcinoma. Notably, γδ T cells in which the variable region on the TCRδ chain is Vδ1 (Vδ1-γδ T cells) were reported to be predominant in iIEL, to recognize the major histocompatibility complex class I-related molecules A and B (MICA and MICB) and to have cytotoxic activity against intestinal tumor cells.[9)] The above report also suggests that γδ T cells in iIEL may affect the formation and progression of colorectal adenocarcinoma.

The present study was designed to examine the effect of γδ T cells on the formation and progression of chemically induced colorectal adenocarcinoma using γδ T cell-deficient mice. Azoxymethane (AOM) was administered to wild-type C57BL/6 mice, αβ T cell-deficient mice (*TCRα*-gene knockout mice) and γδ T cell-deficient mice (*TCRδ*-gene knockout mice) and the formation of macroscopic tumors and microscopic aberrant crypt foci in colorectal mucosa was examined. The effect of γδ T cells on the formation and progression of colorectal adenocarcinoma is discussed in the light of the results of the present study.

MATERIALS AND METHODS

Mice Homozygous *TCRα*-gene knockout mice,[10)] homozygous *TCRδ*-gene knockout mice[11)] and wild-type C57BL/6 (the background of the knockout mice) were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were bred and maintained in a clean air system (EBAC-S, Clea Japan Inc., Tokyo). Two- to 3-month-old mice (body weight: 15–20 g) were used for experiments.

Induction of colorectal tumors and aberrant crypt foci For the induction of colorectal tumors, mice were administered AOM (10 mg/kg weight) intraperitoneally (i.p.) once

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a week for 1–5 weeks. In the preliminary experiments, 4–5 administrations of AOM were required for the formation of colorectal tumors and 5 administrations of AOM were used in further experiments. The colorectal part was resected at 3.5, 5, 7 and 9 months after the last administration and the formation of macroscopic tumors in mucosa was examined. The macroscopic tumor and a small piece of colorectal tissue were cut and fixed in 10% formalin. Thin sections of paraffin-embedded tissues were analyzed under a microscope after staining. For the induction of aberrant crypt foci (ACF), mice were administered AOM (10 mg/kg weight) once a week for 1–4 weeks. The formation of ACF was examined 1–1.5 months after the last administration by the method previously described. Briefly, the oral terminus of the colon and anal terminus of the rectum were knotted with strings and the whole colorectal part was resected. Then, 10% formalin was infused into the intestinal cavity with a syringe and left for 5 min. After the fixation, the mucosal side of the colorectal tissue was exposed by cutting longitudinally one side of the tissue. The tissue was spread on filter paper and fixed again with 10% formalin overnight. The surface of the fixed colorectal mucosa was stained with 0.2% methylene blue in 0.1 *M* phosphate buffer (pH 7.4) and the number of ACF was counted under a microscope at a low magnification.

Morphological analysis of colorectal tissue Whole colorectal tissue was obtained from each mouse and small pieces were resected from normal and tumor parts of the tissue. The pieces were fixed with 10% formalin or periodate-lysine-2% paraformaldehyde (PLP) fixatives.[12] Thin sections prepared from formalin-fixed tissue were stained with hematoxylin and eosin (HE). Thin sections prepared from formalin-fixed and PLP-fixed tissues were stained by enzyme-immunostaining (LSAB kit, DAKO Japan, Kyoto) using monoclonal antibodies against proliferating cell nuclear antigen (PCNA; PC10, DAKO, Glostrup, Denmark) and mutated p53 (Ab-3, Oncogene Res. Prod., Cambridge, MA).

RESULTS

Formation of macroscopic colorectal tumors The formation of macroscopic colorectal tumors was dependent on the frequency of AOM administration and the duration after administration. Colorectal tumors were observed in a few *TCRδ*-gene knockout mice 5 months after 5 administrations, but not 3.5 months after 3 administrations. Additionally, tumors were observed only in *TCRδ*-gene knockout mice 5 months after 5 AOM administrations and the frequency of tumor-bearing mice and the number of tumors per mouse had increased at 7 and 9 months after the last administration (Table I). All tumors were 1–5 mm in diameter and projected into the mucosal cavity of the descending colon and rectum (Fig. 1B). In contrast, *TCRα*-gene knockout mice and wild-type C57BL/6N mice showed no macroscopic tumor formation by 9 months after AOM administration (Fig. 1A).

Morphological findings of colorectal tumors Morphological analysis of the colorectal tumors revealed well-differentiated adenocarcinoma. The tumor cells were similar in size and formed a villus-structure, with destruction of the normal configuration (Fig. 2, A and B). The large nuclei of the tumor cells were stained with anti-PCNA antibody more intensely than the nuclei of the neighboring non-malignant cells (Fig. 2C). The tumor cells were also stained by monoclonal antibody against mutated p53.

Formation of ACF Because ACF formation, which appears within a few weeks after AOM administration, is suggested to be the first step of colorectal carcinogenesis, ACF formation was examined in each group at 1 or 1.5 months after 1 to 4 AOM administrations (Table II). The resected colon was fixed with 10% formalin and stained with methylene blue. Using this method, the ACF were

Table I. Frequency of Macroscopic Colorectal Tumor-bearing Mice at 3.5–9 Months after AOM Administration

Months after AOM administration (rout, frequency)	C57BL/6N		*TCRα*-gene knockout mice		*TCRδ*-gene knockout mice	
	AOM (−)	AOM (+)	AOM (−)	AOM (+)	AOM (−)	AOM (+) (%)
3.5 (i.p., 3)	0/3	0/3	0/3	0/3	0/3	0/3 (0)
5 (i.p., 5)	0/5	0/5	0/5	0/5	0/4	1/6 (17)
7 (i.p., 5)	0/5	0/5	0/5	0/5	0/10	5/10 (50)
9 (i.p., 5)	0/5	ND	0/5	0/7	0/5	4/5 (80)

Each mouse group was given AOM (10 mg/kg) intraperitoneally (i.p.) once a week for 3 or 5 weeks. Formation of macroscopic tumors was compared at 3.5, 5, 7 and 9 months after the last administration between groups with AOM administration (AOM (+)) and without AOM administration (AOM (−)).
Fractions indicate the number of tumor-bearing mice in total number of mice examined. The percentages in parentheses show the frequency of tumor-bearing mice.
ND: not done.

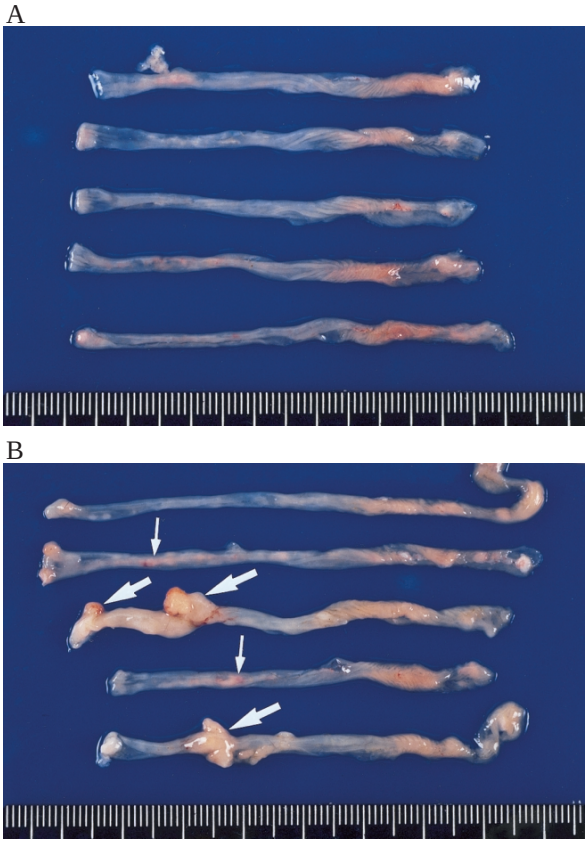

Fig. 1. Macroscopic tumor formation on the mucosal side of colorectal tissue. Colorectal tissues were resected from *TCRα*-gene knockout mice and *TCRδ*-gene knockout mice 9 months after AOM administration. Macroscopic tumors were not observed on colorectal tissues from *TCRα*-gene knockout mice (A), but were observed on 4 in 5 colorectal tissues from *TCRδ*-gene knockout mice (B). Large tumors (shown by large arrows) were observed in 2 tissues and small tumors (shown by small arrows) were detected in 2 other tissues.

clearly observed under a microscope at low magnification (Fig. 3). The ACF were also observed mostly in the mucosa of the descending colon and rectum. A few ACF were observed in some *TCRδ*-gene knockout mice 1.5 months after AOM administration and the frequency of ACF-bearing mice and the number of ACF per mouse increased with increasing frequency of AOM administration. A total of 70%, 70% and 100% of the *TCRδ*-gene knockout mice bore 1 to 16 ACF one month after 2, 3 and 4 AOM administrations, respectively. The number of ACF in *TCRδ*-gene knockout mice was highest at 1 month after AOM administration and slightly decreased thereafter (data not shown). ACF were also observed in wild-type mice and *TCRα*-gene knockout mice 1 month after 3–4 administrations, but the frequency of ACF-bearing mice

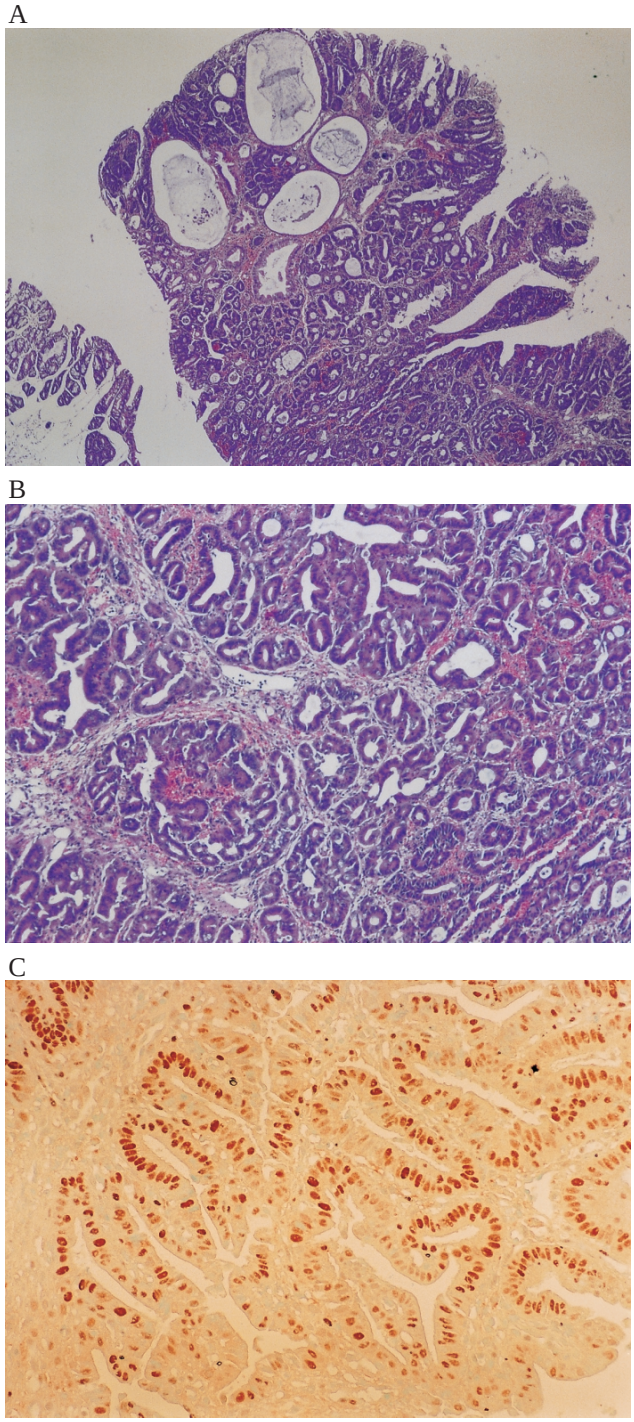

Fig. 2. Morphological analysis of AOM-induced colorectal adenocarcinoma in *TCRδ*-gene knockout mice. Tumor tissue was obtained from a *TCRδ*-gene knockout mouse 9 months after AOM administration and thin sections of formalin-fixed tissue were stained with hematoxylin-eosin (A, ×10 and B, ×25) and anti-PCNA antibody (C, ×50).

Table II. Relationship between the Frequency of ACF-bearing Mice and the Frequency of AOM Administration

Months after AOM administration (rout, frequency)	C57BL/6N		*TCRα*-gene knockout mice		*TCRδ*-gene knockout mice	
	AOM (−)	AOM (+)	AOM (−)	AOM (+)	AOM (−)	AOM (+)
1.5 (i.p., 1)	0/5	0/5	0/5	0/5	0/5	3/16 (0.7, 1–5)
1 (i.p., 2)	ND	ND	0/5	1/4 (0.3, 1)	0/5	7/10 (1.8, 1–8)
1 (i.p., 3)	0/5	1/6 (0.5, 3)	0/6	3/5 (3.8, 3–9)	0/10	7/10 (3.1, 1–12)
1 (i.p., 4)	0/5	4/4 (5.0. 2–9)	0/5	ND	0/7	6/6 (9.2, 2–16)

Each mouse group was given AOM (10 mg/kg) intraperitoneally once a week for 1–4 weeks. Formation of ACF was compared 1.5 or 1 month after the last administration between groups with AOM administration (AOM (+)) and without AOM administration (AOM (−)).
Fractions indicate the number of ACF-bearing mice in total number of mice treated. The numbers in parentheses show the mean number of ACF per group and the number of ACF on each ACF-bearing mouse.
ND: not done.

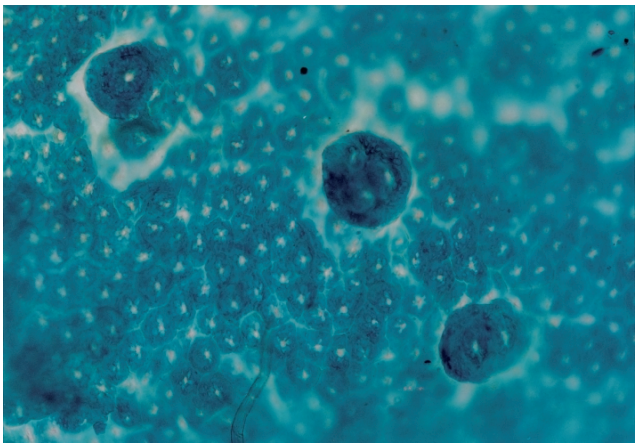

Fig. 3. Aberrant crypt foci in colorectal mucosa of a *TCRδ*-gene knockout mouse. Colorectal tissue was obtained from a *TCRδ*-gene knockout mouse 1 month after AOM administration and was fixed with 10% formalin. The surface of the tissue was stained with 0.2% methylene blue and the mucosal surface was observed under a microscope at low magnification (×25).

and the number of ACF per mouse were much lower than in *TCRδ*-gene knockout mice (Table II).

DISCUSSION

In the present study, the formation of colorectal adenocarcinoma was observed only in *TCRδ*-gene knockout mice (γδ T cell-deficient mice) and more ACF were detected in these mice. The results suggest that γδ T cells might play a role in the suppression of colorectal adenocarcinoma formation and progression.

The difference between *TCRδ*-gene knockout mice and *TCRα*-gene knockout mice appeared not to be caused by the methods involved in preparing the knockout mice. Both knockout mice used in the study originated from the same inbred mouse strain, C57BL/6, the materials used to prepare the knockout mice were almost identical and the other subsets of T cells were left almost intact in both types of knockout mice.[10, 11] Many dietary carcinogens and pathogens which affect the formation and progression of colorectal adenocarcinoma have been reported.[13] In the present study, mice were given *ad libitum* access to the same lab chow and water, and were bred and maintained in the same isolator. Thus, the difference is not likely to be due to these factors.

The major cells affecting colorectal adenocarcinoma cells have been reported to be cytotoxic T lymphocytes, NK cells and activated macrophages. The major tumor-infiltrating cells of colorectal adenocarcinoma have been reported to be αβ T cells and activated macrophages.[14] In contrast, the function of γδ T cells in the formation of colorectal tumors has not been precisely analyzed. We reported that the frequency of γδ T cells that are present in colorectal adenocarcinoma tissues was lower than that in normal iIEL.[8] The results suggest that γδ T cells might also be related to the formation and progression of colorectal adenocarcinoma.

Recent reports demonstrated that γδ T cells play a role in repair of damaged intestinal epithelial cells[5, 6] and have the capacity to recognize certain antigens, such as special antigens on the surface of tumor cells and some superantigens, and that their cytotoxic activity is enhanced by TNFα.[15–19] Notably, it was reported that Vδ1-γδ T cells, which are predominant in the human intestinal mucosa, recognized the major histocompatibility antigen-related molecules, MICA and MICB, which are expressed on the surface of intestinal epithelial cells. The expression of the molecules was enhanced in colon tumor cells. Thus, Vδ1-γδ T cells recognize the colorectal tumor cells through MICA/MICB and show cytotoxic activity against the tumor cells.[9] Taken together, γδ T cells in iIEL appear to

eliminate aberrant intestinal epithelial cells, such as transformed cells and microbe-infected cells, and thus play an important role in immune surveillance at the intestinal mucosa to maintain the normal configuration of intestinal epithelium.

In contrast, Egawa *et al*. reported that γδ T cells isolated from mouse spleen cells secrete a factor which inhibits the activity of CTL and NK cells, and thus the γδ T cells enhance the formation of colorectal tumor.[20, 21] In the present study, γδ T cells in iIEL were suggested to act directly on aberrant intestinal cells rather than to modulate the activity of CTL and NK, because αβ T cell-deficient mice did not form any macroscopic tumor. The discrepancy may reflect the diversity of γδ T cells. The γδ T cells have been reported to express different Vγ regions according to their sites of localization. The γδ T cells which are present in intestinal intraepithelial spaces express exclusively Vγ7 or Vγ1, while those in circulation/lymph node/spleen express diverse Vγ, and the processes of differentiation and their functions have been suggested to be different.[22] In the connection with this, the difference of target tumors cells, AOM-induced intestinal adenocarcinoma cells in the present study and mammary carcinoma and hepatoma cells in their report, may be responsible for the discrepancy. Interestingly, human γδ T cells in peripheral blood of patients showed direct tumoricidal activity against glioblastoma cells.[23, 24] Further experiments are required to distinguish the functions of γδ T cells in intestinal mucosa and circulation/lymph node/spleen on colorectal adenocarcinoma formation.

The improper functioning of γδ T cells and their reduced frequency due to such factors as aging, malnutrition and chemical carcinogens, may disrupt their activity, resulting in the formation and progression of colorectal adenocarcinoma in humans. Alternatively, the transformed cells may secrete factors that negate the cytotoxic activity of γδ T cells.

In the present study, γδ T cells were also suggested to suppress the formation and progression of colorectal adenocarcinoma. They may influence the pathogenesis of colorectal adenocarcinoma and may also be useful for the prevention and immunotherapy of colorectal adenocarcinoma. However, further analysis is required to confirm this hypothesis.

ACKNOWLEDGMENTS

We would like to thank Dr. K. Morimura and Prof. S. Fukushima (Osaka City University Medical School) for their technical help in detecting ACF in intestinal mucosa. We thank M. Kubota and R. Kajita for their help in the maintenance of the mice. This study was supported in part by a Grant-in-Aid for Cancer Research from the Japanese Ministry of Health and Welfare.

(Received March 23, 2001/Revised May 17, 2001/Accepted May 25, 2001)

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