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Early Alterations of Apoptosis and Cell Proliferation in Azoxymethane-initiated Rat Colonic Epithelium

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Alterations of apoptosis and cell proliferation in the colonic epithelium of rats after exposure to azoxymethane (AOM) were estimated by means of the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method, measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation, immunohistochemical staining for proliferating cell nuclear antigen (PCNA), and counting of mitotic cells. F344 male rats were given a single s.c. injection of AOM (15 mg/kg body weight) at 6 week of age, and killed 4 h, 8 h, 3 days, and 7 days after the AOM treatment. At 4 h after the treatment, many damaged cells were already observed in the colonic epithelium, and they were positive by TUNEL staining. At 8 h, the number of TUNEL-positive cells was largest. The reduction of DNA synthesis in the colonic epithelium, confirmed by BrdU incorporation, was not distinct in comparison with the mitotic inhibition. There was no remarkable change in PCNA labeling index, except that strong expression of PCNA was detected in many damaged cells. On the 3rd day, the appearance of cell death became infrequent and an increase of cell proliferation occurred. On the 7th day, the expression of TUNEL and the cell proliferation biomarkers were at almost normal levels. These findings suggest that AOM induces apoptosis, which is associated with synchronous inhibition of mitosis. The data also indicate that PCNA immunostaining does not reflect the true proliferation state in the early phase after AOM exposure, probably due to the occurrence of cell cycle arrest or DNA repair.

Key words: Apoptosis — Cell proliferation — Azoxymethane — Colon — Rat

Apoptosis is considered to play a vital role in various physiological processes, including embryogenesis, and has implications for carcinogenesis and cancer therapy.[1–3] In experimental large bowel carcinogenesis using animal models, dying cells appear in the target sites soon after exposure to a carcinogen.[4–6] Classically, early appearance of nuclear abnormalities was described as pyknotic or karyorrhectic nuclei with fragmented bodies and nuclear condensation. These findings are currently interpreted as a part of the morphological characteristics of apoptotic cell death. Ijiri[7] and Potten *et al.*[8] first reported that dying cells in intestines of mice induced by 1,2-dimethylhydrazine (DMH) or methylazoxymethanol (MAM) acetate, showed the histological characteristics of apoptosis. However, little is yet known about the biochemical features, such as DNA fragmentation, of carcinogen-induced cell death.

The mechanisms of cancer initiation and induction of cell death by the colonic carcinogens DMH, MAM, and azoxymethane (AOM) have been studied.[9–13] Activated metabolites of these carcinogens exhibit alkylating activity, and formation of O^6-methylguanine in colon DNA is associated with tumor initiation.[14, 15] Some of the damaged cells are saved by intrinsic repair systems, whereas some are defective in repair, resulting in elimination by apoptosis or the acquisition of potential, though in only a few of the surviving cells, to give rise to cancer.[16, 17]

Cell proliferation in cancer tissue has been investigated extensively because it may be a predictor of prognosis.[18] Clonal expansion of preneoplastic lesions presumably depends upon a highly proliferative nature.[19, 20] In the early stage of experimental tumorigenesis, it is known that inhibition of nucleic acid synthesis and mitosis are related to the occurrence of carcinogen-induced cell death,[12] and these alterations may reflect induction of cell cycle arrest after DNA damage.[16, 21] Among methods for measurement of cell proliferation, proliferating cell nuclear antigen (PCNA) staining was expected to be useful in this study because PCNA has a dual role in DNA replication and in DNA excision repair; transient overexpression of PCNA without accompanying cell proliferation is observed soon after ultraviolet irradiation.[22–24] Therefore, carcinogen treatment should modify PCNA expression.

The present study was conducted to examine concomitantly the alterations of apoptosis, cell proliferation and morphology of the colonic epithelium of rats in the initiation phase of AOM-induced large bowel tumorigenesis. Apoptosis was evaluated by using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method[25] and cell proliferation by using immunohistochemical BrdU label-

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ing analysis, immunostaining of PCNA, and mitotic counting in the same colonic epithelium.

MATERIALS AND METHODS

Animals and treatment A total of 32 male Fischer 344 rats, 4 weeks old, obtained from Japan SLC Inc., Hamamatsu, were used for this experiment. AOM was purchased from Sigma Chemical Co., St. Louis, MO. Animals were housed two to five to a wire cage in the experimental room under controlled conditions of 23±2°C (SD), 50±10% humidity, and 12 h light/dark cycle. The animals were fed a basal diet CE-2 (Clea Japan, Inc., Tokyo) throughout the experiment.

Experimental procedure After quarantine for 2 week, 17 rats received a single s.c. injection of AOM (15 mg/kg body weight) at 9:00 a.m. Two of them were killed 4 h after the treatment and 5 rats each were killed at 8 h, and on the 3rd day and 7th day. Another group of 15 rats received a saline injection alone and these animals were killed in the same manner as the controls, except at 4 h. One hour prior to being killed, all rats were given an i.p. injection of BrdU (40 mg/kg body weight, Sigma Chemical Co.). At autopsy, the intestines were removed, cut longitudinally, cleaned, and inflated with 10% neutral buffered formalin. After fixation for 12 h, the rectum (1 cm from the anus) was removed, and the proximal and distal colons were separated. The colons were rolled and embedded in paraffin. Four serial sections were cut (4 μm in thickness), and subjected to hematoxylin and eosin (HE) staining for morphological examination and counting of mitotic cells, the TUNEL technique for apoptosis, and immunohistochemical stainings for calculating BrdU and PCNA labeling index.

Mitotic counting The sections stained with conventional HE were used for morphological examination and counting of mitotic cells. For counting of mitosis, 20 crypts having good shapes with clearly visible neck, lumen, and base were selected in each portion.[4]

TUNEL technique The TUNEL method was carried out as described previously.[25] After deparaffinization, sections were treated with proteinase K (20 μg/ml) in 10 m*M* Tris-HCl (pH 8.0) for 15 min at room temperature and washed in a distilled water for 10 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 5 min and then the sections were washed. After preincubation with TdT buffer (30 m*M* Tris-HCl pH 7.2, 140 m*M* sodium cacodylate, 1 m*M* cobalt chloride) for 5 min, the slices were immersed in 50 μl of TdT buffer containing 0.4 U/μl of TdT (Takara, Kyoto) and 40 μM biotinylated 16-dUTP (Boehringer Mannheim, Mannheim, Germany) and were incubated for 1 h at 37°C in a moist chamber. Before this incubation, the slices of the positive control were incubated with DNase I (1 μg/ml) in TdT buffer for 10 min. The reaction was discontinued by transferring the slices to TB buffer (300 m*M* NaCl, 30 m*M* sodium citrate) for 15 min. The slices were washed in a distilled water for 10 min, blocked in 2% bovine serum albumin in phosphate-buffered saline (PBS) for 10 min, rinsed in PBS for 10 min, and immersed in PBS with peroxidase-conjugated streptavidin (1/100 dilution; DAKO, Glostrup, Denmark) at 37°C for 30 min. They were washed with PBS for 10 min, then the peroxidase activity was visualized with H_2O_2/diaminobenzidine for 7 min and the sections were weakly counterstained with hematoxylin. For the determination of the TUNEL index (TI), 20 well-shaped crypts from each portion of one rat colon were divided down the long axis. The left side of each crypt was examined. Starting at the base of the colonic crypt, epithelial cells were numbered. The cells with a brownish nucleus were regarded as labeled. The positions of the labeled cells in each crypt column were recorded for evaluation of the incidence and distribution of the labeled cells in the colonic crypt. When several TUNEL-positive fragments were seen at the position of a particular cell, they were counted as one labeled cell. The labeling index was determined by calculating the ratio of the labeled cells in a half-crypt to the total number of epithelial cells in it. To evaluate the distribution of the TUNEL-labeled cells in the crypt column, the percentage of labeled cells was smoothed over 3 consecutive cell positions as described previously.[8, 26]

The BrdU and PCNA immunohistochemistry of colonic epithelium To assess the proliferative activity of colonic epithelium and the distribution of proliferative cells in the crypt, BrdU and PCNA immunohistochemistry was performed according to the methods described previously.[27, 28] The labeled streptavidin biotin method was carried out according to the manufacturer's instructions using LSAB KIT (DAKO). For PCNA, sections were incubated with primary antibodies against PCNA (1/100 dilution; Novocastra, Newcastle, UK) in 0.05 *M* Tris-HCl (pH 7.6), overnight at 4°C in a humidified chamber. Primary antibody against BrdU (DAKO) was used at 1/100 dilution in 0.05 *M* Tris-HCl (pH 7.6) and incubation was done for 2 h at room temperature. BrdU and PCNA labeling indices (BLI and PLI) and distribution were determined, as done in the case of TI.

Statistical analysis TI, BLI, PLI, and the number of cells lining a half-crypt were compared by a two-sample *t*-test with Welch's correction. A difference was considered statistically significant when the *P* value was 0.05 or less.

RESULTS

Histological observation and counting of mitotic cells The histological alteration induced by AOM treatment and the results of mitotic cell counting are shown in

Tables I and II and Fig. 1. At 4 h after the treatment, damaged cells with typical morphology of apoptotic cell death were already apparent.[29, 30] Cell shrinkage with chromatin condensation and eosinophilic cytoplasm were present in these cells. Some cells showed crescent-shaped chromatins at the nuclear margin and other cells possessed scattered dots of various sizes. Basically these changes of the colonic epithelium were seen within the lower half of the crypt column.

At 8 h after the carcinogen treatment, the number of damaged cells was increased. Some fragmented bodies were extruded from the epithelium to the sack of the crypt, and others were incorporated into neighboring healthy epithelial cells by phagocytosis. A significant decrease in the number of mitotic cells was seen in both colons.

On the 3rd day after the treatment, damaged cells were infrequent. Some cells were enlarged and/or irregular in shape, and others had large nuclei and nucleoli. The shape of the crypt was irregular. Focal fibrosis extended into the interstitial space of the colon and infiltration of the inflammatory cells was seen around the crypts. The number of cells lining the crypt in the distal colon was significantly decreased in comparison with the respective controls. A significant increase in the number of mitotic cells in the proximal colon was seen.

On the 7th day after the treatment, the crypt column had essentially recovered its normal structure. In the interstitial area of the colonic mucosa, however, neutrophil infiltration was present, and in the proximal colon, crypt abscesses, with regenerating cells in the epithelium, were often recognized. The number of crypt cells in both portions was significantly increased in rats given carcinogen treatment, compared with that of control rats. The frequency of mitosis in the proximal colon was still higher than that in the control.

Table I. Alterations of Apoptosis and Cell Proliferation in the Colonic Crypt of the Proximal Portion during the Early Phase after AOM Exposure

Time killed	Treatment (no. of rats examined)	No. of cells /half-crypt	TUNEL index (%)	BrdU-labeling index (%)	PCNA-labeling index (%)	No. of mitoses /20 crypts[a)]
4 h	AOM (2)	37.1[b)]	3.8	8.1	21.4	6.8
8 h	AOM (5)	35.8±2.3[c, d)]	6.6±1.4[d)]	4.0±3.2	23.1±4.9	5.0±1.9[d)]
	Saline (5)	35.4±1.7	0.4±0.6	7.3±1.1	24.5±1.9	16.0±4.0
3 days	AOM (5)	33.7±2.4	1.2±0.8	14.3±5.6	32.6±7.5	16.6±1.9[d)]
	Saline (5)	36.0±1.3	0.9±0.5	9.3±1.1	24.3±1.8	13.8±1.1
7 days	AOM (5)	43.6±1.7[d)]	0.1±0.2	11.1±3.1	22.4±5.3	16.2±2.6[d)]
	Saline (5)	39.4±2.3	0.3±0.2	10.3±2.7	20.2±2.3	9.6±2.3

a) Mitotic cells in 20 colonic crypts were counted in each rat.
b) The data indicate mean values of the 2 examined rats.
c) The data indicate mean±SD.
d) Significantly different from that of the respective control group by Welch's test ($P<0.05$).

Table II. Alterations of Apoptosis and Cell Proliferation in the Colonic Crypt of the Distal Portion during the Early Phase after AOM Exposure

Time killed	Treatment (no. of rats examined)	No. of cells /half-crypt	TUNEL index (%)	BrdU-labeling index (%)	PCNA-labeling index (%)	No. of mitoses /20 crypts[a)]
4 h	AOM (2)	33.4[b)]	2.8	7.4	27.6	2.9
8 h	AOM (5)	32.4±1.8[c)]	7.8±1.1[d)]	4.5±3.7	28.5±6.4	0.6±0.9[d)]
	Saline (5)	35.0±1.8	0.0±0.0	7.9±1.1	30.7±6.8	12.4±2.1
3 days	AOM (5)	26.6±3.2[d)]	1.5±0.8[d)]	23.3±8.6[d)]	40.2±3.3[d)]	11.6±3.4
	Saline (5)	36.9±2.4	0.2±0.2	9.0±1.2	28.4±2.7	8.0±1.2
7 days	AOM (5)	42.8±1.1[d)]	0.0±0.1	10.1±2.7	22.0±6.6	9.0±2.5
	Saline (5)	37.6±2.5	0.3±0.3	9.5±3.1	28.7±8.6	8.8±3.2

a) Mitotic cells in 20 colonic crypts were counted in each rat.
b) The data indicate mean values of the 2 examined rats.
c) The data indicate mean±SD.
d) Significantly different from that of the respective control group by Welch's test ($P<0.05$).

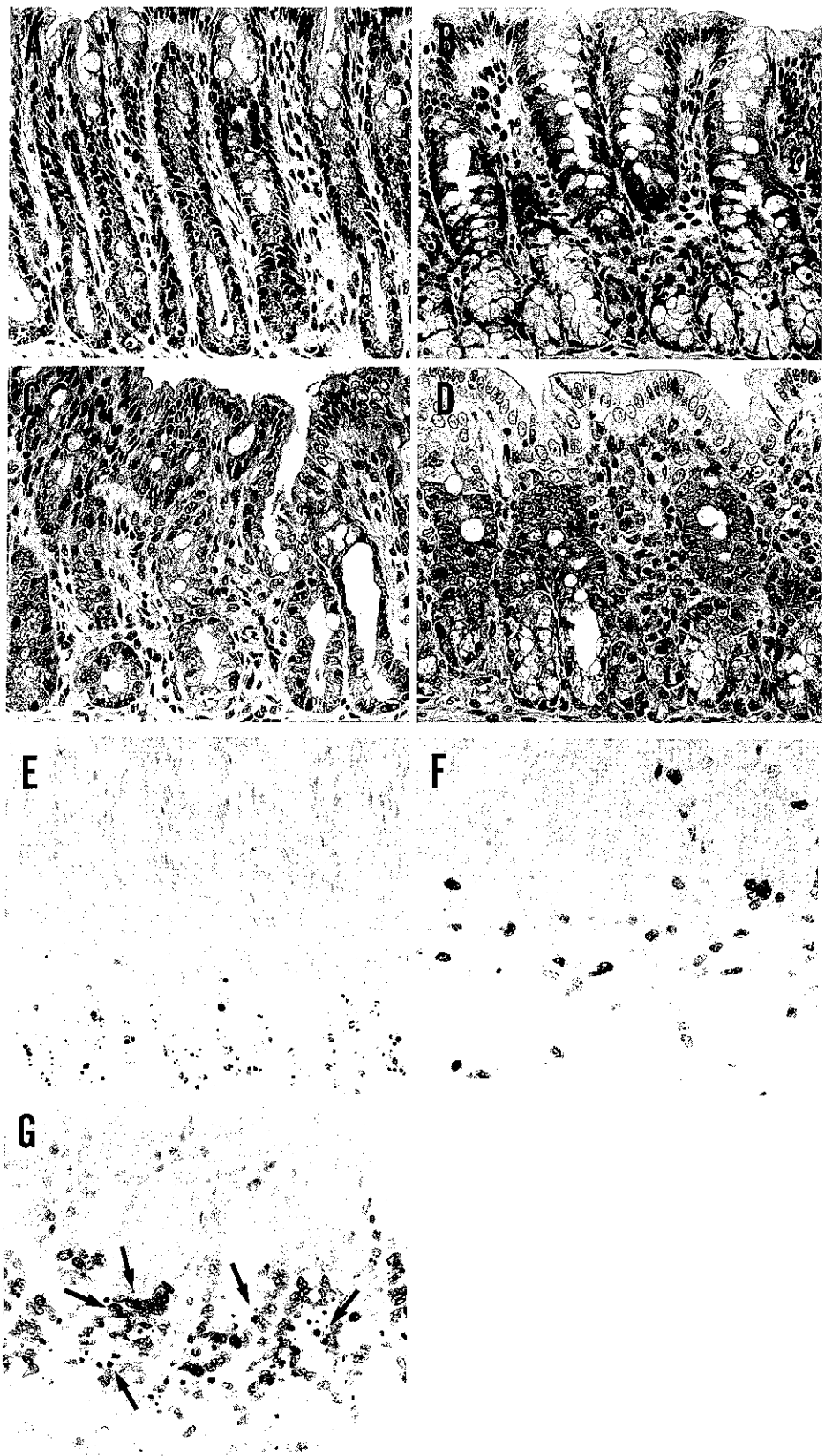

Fig. 1. Histology with HE staining, and BrdU and PCNA immunohistochemistry of the colonic crypt soon after AOM exposure. (A) Histology in the distal colon at 8 h after AOM treatment (HE staining, original magnification×80). Damaged cells with cell shrinkage or condensed chromatin are recognized in the lower half of the crypt. (B) Histology in the proximal colon at 8 h after AOM treatment (HE, ×80). Many damaged cells are observed. Mucous-secreting goblet cells are concentrated at the bottom of the crypt. (C) Histology on the 3rd day in the distal colon (HE, ×80). Some cells were enlarged and/or irregular in shape, and others had large nuclei and nucleoli. The shape of the crypt was irregular. (D) Histology on the 3rd day in the proximal colon (HE, ×80). (E) TUNEL staining of the colonic mucosa in the distal colon at 8 h after treatment (×80). Dying cells exhibit TUNEL staining. A and E are serial sections. (F, G) Serial sections of the colonic crypt in the proximal portion at 8 h after treatment with BrdU (F) and PCNA (G) immunohistochemistry (× 100). The number of PCNA-labeled cells is greater than that of BrdU-labeled cells, and their distribution is wider. Damaged cells with apoptotic morphology have strongly positive PCNA immunostaining (arrows).

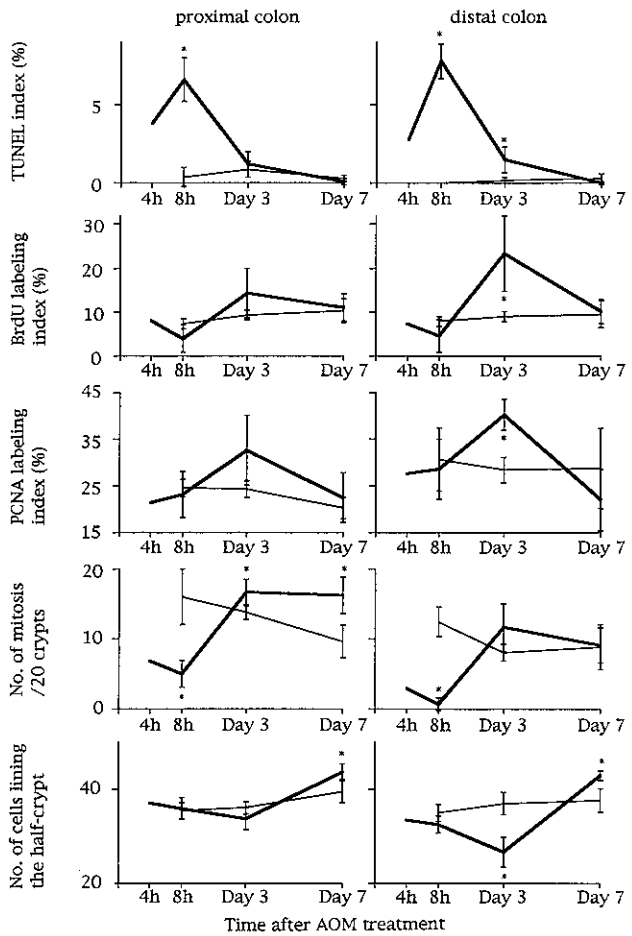

Fig. 2. The alterations of TUNEL index, biomarkers of cell proliferation and the number of cells lining the colonic crypt following AOM treatment. * Significantly different from the respective control at $P<0.05$. The bars indicate SD values.

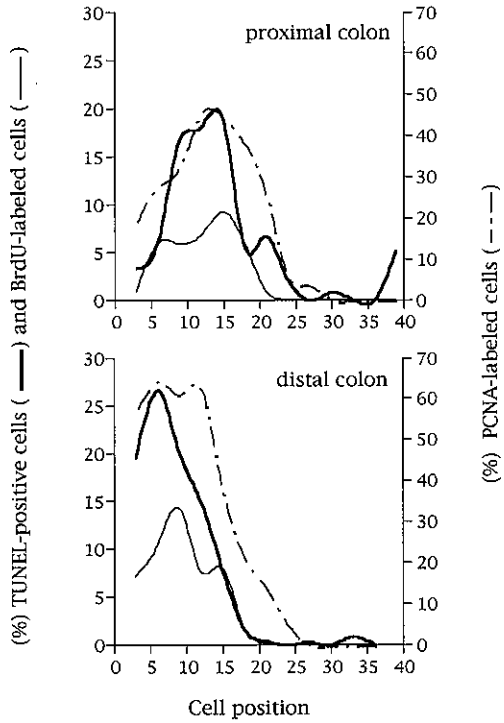

Fig. 3. Distribution of positive cells for BrdU incorporation (——), PCNA immunohistochemistry (– – –), and TUNEL staining (——) in crypts of the proximal and distal colon at 8 h after AOM treatment. Starting at the base of the colonic crypt, the epithelial cells were numbered. The position of the labeled cells was recorded in terms of their positional number. The percentage was smoothed over 3 consecutive cell positions and is presented as a smoothed curve.

Incidence and distribution of apoptosis detected by TUNEL staining At 8 h following AOM treatment, nuclei of damaged cells in the colon showed strong TUNEL staining (Fig. 1). Weak TUNEL staining was also seen in the cytoplasm. Quantitative data on TUNEL staining in the colonic epithelium are shown in Tables I and II and Fig. 2. At 4 h after the carcinogen exposure, approximately 1.4 positive cells per half crypt in the proximal colon and approximately 0.9 cells in the distal colon were already seen. In 8 h, there were approximately 2.4 positive cells in the proximal colon and 2.5 in the distal colon. Without carcinogen treatment, few TUNEL-positive cells were found in the colonic crypt. On the 3rd day, there were approximately 0.4 positive cells per half crypt of the carcinogen-exposed rats. On the 7th day, the incidence was at almost the same level as that of the negative control rats. The distribution of TUNEL-positive cells in the colonic crypt column at 8 h is shown in Fig. 3. Most TUNEL-positive cells were within the lower half of the crypt in the distal colon. In the proximal portion, the frequency of apoptosis at the base of the crypt was low, and the area within which TUNEL-positive cells were seen was wider than that of the distal colon. Therefore, the peak position of TUNEL-positive cells in the crypts of the proximal colon was generally higher than in the crypts of the distal colon.

BrdU labeling index The data for BLI are shown in Tables I and II and Figs. 2 and 3. At 8 h, the values of BLI were rather small, although there was no statistically significant difference from the controls. Most BrdU-positive cells were present within the lower half of the crypt, where the TUNEL-positive cells were located. On the 3rd day, BLI in the distal colon was significantly greater than that of the respective control.

PCNA-positive nuclei The results of PCNA immunostaining are shown in Tables I and II and Figs. 2 and 3.

As compared with the respective control groups, there was no significant alteration of PLI in the proximal or distal colon at 8 h following carcinogen treatment. Interestingly, many cells with morphologically apoptotic features exhibited strong staining reactivity of PCNA (Fig. 1). On the 3rd day, PLI in the distal colon was significantly higher than that of the corresponding control. The distribution of PCNA-immunopositive cells in both colons at 8 h was slightly wider than that of BrdU, and the number of the PCNA-labeled cells in a half crypt was greater than that of BrdU-labeled cells.

DISCUSSION

In the present study, we investigated apoptosis and cell proliferation simultaneously in the colonic mucosa of AOM-exposed rats by using different biomarkers. Damaged cells were prominent in the crypts at 8 h after AOM treatment. In these cells, the nuclei showed positive TUNEL staining, which is characteristic of apoptosis. In spite of the inhibition of mitosis in the colonic epithelium, neither a significant reduction of DNA synthesis confirmed by BLI nor a change of PCNA expression was apparent at 8 h after AOM injection. Then, the number of TUNEL-positive cells in the crypts gradually decreased with a corresponding increase of cell proliferation activity.

It has been reported that inhibition of mitosis and DNA synthesis occurs after carcinogen exposure.[4-6] In this study, the rapid inhibition of mitosis following AOM treatment was confirmed, particularly in the distal colon, but a significant decrease of BLI was not observed at 8 h. These results suggest that a strong reduction of DNA synthesis does not appear in response to a single AOM exposure *in vivo*. One hypothesis regarding the AOM-induced cytological event is that the apoptosis induced by AOM is preceded by G1 arrest and occurs during the G1 phase.[26, 31] It is speculated that DNA damage is repaired during G1 arrest to prevent the damaged cells from surviving or developing to neoplasms.[16] However, in this study, AOM did not induce complete G1 arrest. This may support the existence of an apoptotic pathway which does not require G1 arrest.[32]

With regard to the distribution in the colonic crypt, the TUNEL-positive cells were located in the same regions where BrdU and PCNA-labeled cells were seen, suggesting that AOM-damaged cells are actually in proliferating states. At the bottom of the crypts in the proximal colon, the numbers of TUNEL, BrdU and PCNA-positive cells were small. It has been reported that mucous-secreting goblet cells are concentrated at the base of the crypt in the proximal colon, whereas they exist uniformly throughout the epithelium in the distal colon.[10, 33] The results indicate that these cells do not have potent activity for proliferation and are not sensitive to AOM.

It seems that more epithelial cells are eliminated in the distal colon than in the proximal colon following the carcinogen exposure. Consequently, the expression of cell proliferation biomarkers in the distal colon on the 3rd day was greater than that in the proximal colon. Histologically, the shape of the crypt in the distal portion on the 3rd day was more irregular than that in the proximal region, and the cryptal cells in the distal colon were more dyskaryotic. These results suggest that AOM treatment causes damage predominantly to the distal colon. Interestingly, we observed that AOM-induced aberrant crypt foci, regarded as putative preneoplastic lesions, occurred more frequently in the distal colon than in the proximal colon (unpublished data). This may explain why a single injection of AOM can cause colon tumors with the majority in the descending (distal) colon in male Fischer rats.[34]

Currently it is accepted that the most intense immunostaining of PCNA depicts the S-phase of the cell cycle and nonstaining cells represent quiescent (G0) cells.[23, 35, 36] We found a distinct difference between BLI and PLI in the same rat, suggesting that PCNA-positive cells may include not only cells in the S phase of the cell cycle, but also a certain number of cells in G1 and G2. At 8 h after AOM treatment, actively proliferating cells in the colonic epithelium were considered to be rare, with reduced BLI and mitosis. PLI, however, exhibited a similar level of immunoreactivity to that of the control. One possible explanation for this discrepancy is that AOM-induced damage may generate cell cycle arrest in the G1 and/or G2 phase. The preserved expression of PCNA in the arrested cells would be detectable immunohistochemically. Another possibility is that DNA excision repair may occur. Recently, a significant role of PCNA in DNA excision repair as well as in DNA replication has been emphasized.[22, 37, 38] In this study, many apoptotic bodies exhibited strong PCNA staining, suggesting that the expression of PCNA protein in the AOM-exposed cells continued until cell death. This is consistent with possible occurrence of DNA excision repair in cells which nevertheless fall into apoptotic death. Thus, owing to modification by cell cycle arrest or DNA repair, PCNA immunostaining does not reflect the actual proliferative state in the early phase after the appearance of a genotoxic event. Additional work is required to explore further the relationship between apoptosis, DNA repair, and cancer initiation.

There is accumulating evidence that components of the apoptotic pathway, such as Bcl-2, are associated with the pathogenesis of some types of malignancies.[39] Elucidation of carcinogen-induced apoptotic cell death is, therefore, important in connection with the mechanism of initiation in chemical carcinogenesis.

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