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Disturbance of the Cell Cycle with Colchicine Enhances the Growth Advantage of Diethylnitrosamine-initiated Hepatocytes in Rats

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The effect of cell cycle disturbance due to colchicine on the induction of enzyme-altered foci during liver regeneration in rats was studied. For initiation, diethylnitrosamine (DEN) at a dose of 10 mg/kg was injected intraperitoneally and partial hepatectomy (PH) was performed 4 h thereafter. Colchicine at doses of 0, 0.1, 0.25 and 0.5 mg/kg was injected intraperitoneally 1 and 3 days after the initiation, followed by application of selection pressure consisting of 2-acetylaminofluorene (AAF) and carbon tetrachloride (CCl_4) administration. As end point lesions, γ-glutamyltransferase (GGT)-positive enzyme-altered foci were assayed at week 5. There was no significant effect of colchicine on numbers of foci. However, a significant, dose-dependent increase in the area of GGT-positive lesions in the groups treated with colchicine was observed. Bromodeoxyuridine labeling indices were higher in foci induced in colchicine-treated rats than in the untreated rats. In a separate experiment, serum glutamic pyruvic transaminase was not increased significantly after DEN and colchicine treatment, and the mitotic index at 6 days after PH was increased in the liver of colchicine-treated rats. These results suggest that the cell cycle disturbance induced by colchicine causes more pronounced selective growth of cells initiated by DEN and colchicine, and this experimental model may be useful for analyzing the mechanisms underlying that growth advantage and the effects of cell cycle abnormalities in liver carcinogenesis.

Key words: Colchicine — Cell cycle — Carcinogenesis — Rat — Liver

Neoplasia is characterized by deregulated cell proliferation and phenotypic abnormalities that confer properties of invasion and metastasis. Recently, with the progress of research into gene abnormalities in various cancers and the mechanisms of cell proliferation in normal cells, it has been suggested that deregulation of the cell cycle may be a key event for acquisition of malignant potential during the development of cancer cells.[1–4] Indeed, several oncogenes and tumor suppressor genes have been found to participate directly in the cell cycle. For instance, cyclins, which are proteins regulating DNA synthesis and mitosis, have been implicated as oncogenes and the retinoblastoma tumor suppressor is a substrate of cyclin-dependent protein kinase.[3] However, the influence of abnormalities induced by exogenous deregulation of the cell cycle during carcinogenesis has not been elucidated in any detail.

Colchicine is well-known as a chemical which can induce a block of the cell cycle at the M-phase by inhibition of synthesis of microtubules.[5] Chemicals which inhibit mitosis can induce endoreduplicated or polyploid cells as a result of DNA synthesis without cell division.[6,7] In many cases, such endoreduplicated or polyploid cells then divide to give normal diploid cells, when the chemical is removed from the culture medium. However, it has been reported that cells treated with mitotic-blocking chemicals have a strong propensity for gene amplification under some experimental conditions, as for instance with colcemid-treated BALB/3T3 cells following a stepwise-increase selection protocol in methotrexate.[8] In the present study, the effects of a disturbance of mitotic checkpoint control by colchicine on the initiation phase of rat hepatocarcinogenesis were investigated.

Male Fischer 344 rats, 6 weeks old (Nihon SLC, Shizuoka), were used. They were housed in wire cages in an air-conditioned room at 22°C and 60% humidity under a daily cycle of alternating 12 h periods of light and dark. The animals were maintained on a commercial stock diet, Oriental MF (Oriental Yeast Co. Ltd., Tokyo), and given water *ad libitum.*

DEN[3] (Wako Chemical Co. Ltd., Kyoto) and colchicine (Sigma, St. Louis, MO) were dissolved in 0.9%

[3] Abbreviations: DEN, diethylnitrosamine; AAF, 2-acetylaminofluorene; CCl_4, carbon tetrachloride; GGT, γ-glutamyltransferase; PH, partial hepatectomy; BrdU, bromodeoxyuridine.

NaCl solution. AAF (Tokyo Kasei Co. Ltd., Tokyo) was mixed into powder diet at a dose of 0.02%. CCl_4 (Nacalai Tesque, Kyoto) was diluted 1:1 with corn oil.

Two experiments were performed in this study (Fig. 1). In experiment 1, the effects of colchicine on the induction of GGT-positive foci in rat liver initiated with DEN were investigated. Rats were divided into 6 groups. Groups 1–4 received DEN intraperitoneally at a dose of 10 mg/kg body weight followed by PH, by the method of Higgins and Anderson,[9] 4 h after DEN and then colchicine at doses of 0, 0.1, 0.25 or 0.5 mg/kg was injected intraperitoneally 1 and 3 days after DEN treatment. Groups 5 and 6 received saline instead of DEN, followed by PH 4 h after the saline injection and colchicine twice at doses of 0 or 0.5 mg/kg. All rats were subjected to the selection procedure consisting of AAF mixed in stock diet at a concentration of 0.02% and CCl_4 diluted 1:1 with corn oil as described by Cayama *et al.*[10] and were killed under ether anesthesia 5 weeks after the beginning of the experiment. BrdU (Sigma) at a dose of 20 mg/kg was given intraperitoneally 2 h before killing. The livers were removed, weighed and fixed in ice-cold ethanol. Ethanol-fixed tissues were embedded in paraffin and cut serially at 5 μm thickness. For histological examination, tissue sections were routinely stained with hematoxylin and eosin (H-E). Histochemically, GGT activity was demonstrated by the method of Rutenberg *et al.*[11] The number and size of GGT-positive foci were analyzed with an image-analyzing system, HTB-c995 (Hamamatsu Television Co. Ltd., Shizuoka), connected to a Desktop Computer System-45 (Hewlett-Packard Co.). BrdU labeling was detected histochemically by means of the avidin-biotin-peroxidase complex (ABC) method.[12] BrdU labeling indices of GGT-positive foci were estimated for the rats receiving DEN without colchicine and with 0.5 mg/kg of colchicine. Ten foci were selected randomly per rat and BrdU-positive nuclei were counted in 100 hepatocytes per focus. All data were statistically analyzed using the t test. The effects of colchicine on the hepatocyte cell cycle induced by PH were studied in experiment 2 (Fig. 2). Rats received 10 mg/kg of DEN followed by PH 4 h after DEN, and were divided into 3 groups. Groups 1–3 received 0, 0.25, and 0.5 mg/kg of colchicine 1 and 3 days after DEN and animals were killed under ether anesthesia 4, 5, and 6 days after the beginning of the experiment. Blood was taken from the aorta and the livers were removed and fixed in ice-cold ethanol. Serum glutamic pyruvic transaminase (GPT) values were measured and liver sections processed for H-E staining and histological examination.

The results of experiment 1 are summarized in Table I. Final body weights showed a tendency for growth retardation of rats from groups 2 to 4 as compared to group 1. However, relative liver weights showed a tendency of increase in rats from groups 3 and 4 as compared to group 1. Macroscopically, the liver surfaces in animals treated with DEN and colchicine showed a nodular appearance.

Representative liver sections stained for GGT in experiment 1 are shown in Fig. 2. The mean numbers of GGT-positive foci did not significantly differ between rats from groups 1 to 4. However, the average size of the

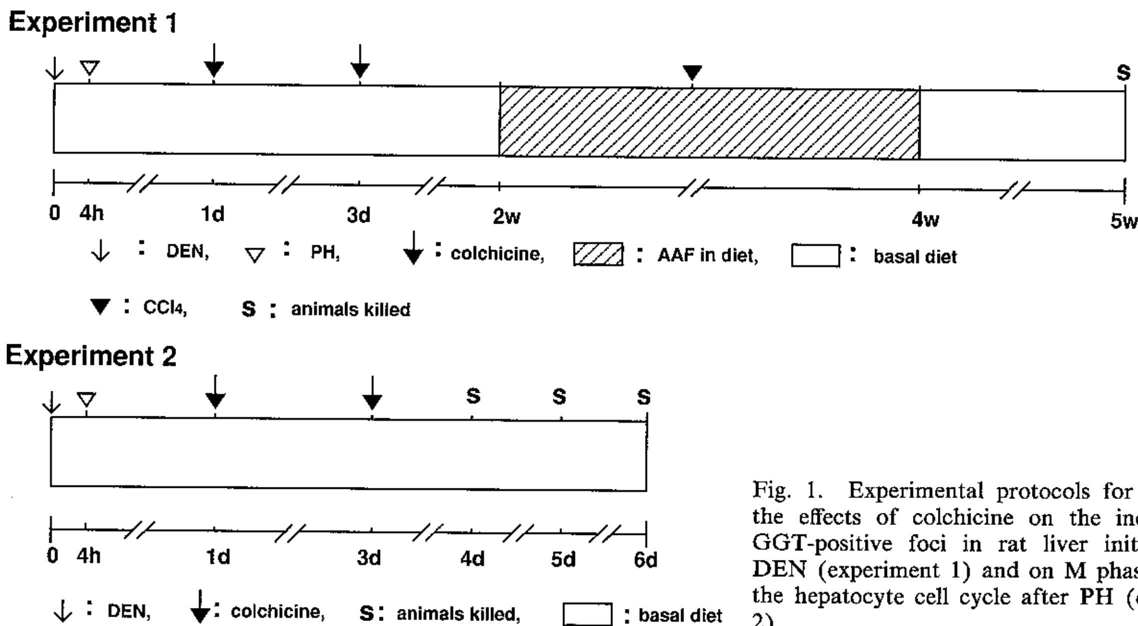

Fig. 1. Experimental protocols for studies of the effects of colchicine on the induction of GGT-positive foci in rat liver initiated with DEN (experiment 1) and on M phase block of the hepatocyte cell cycle after PH (experiment 2).

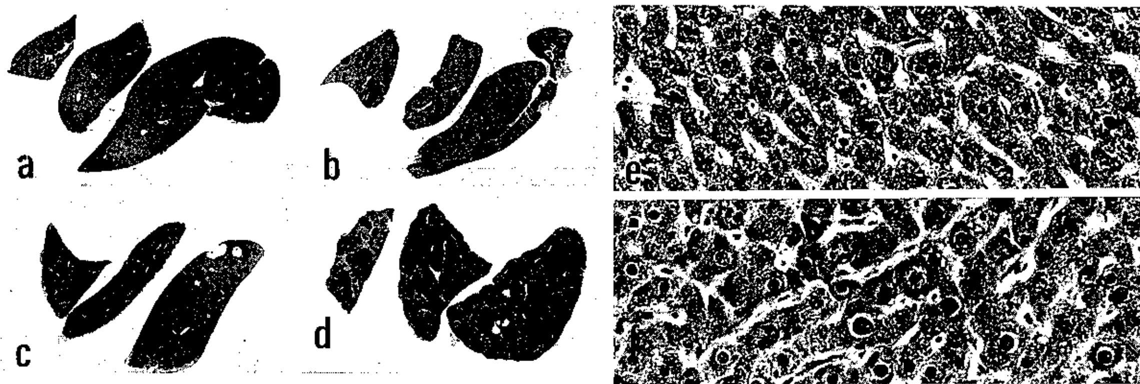

Fig. 2. Left column; Representative liver sections of GGT-positive foci induced by DEN with colchicine (GGT staining): a, liver from group 1 (DEN+saline); b, liver from group 2 (DEN+0.1 mg/kg of colchicine); c, liver from group 3 (DEN+0.25 mg/kg of colchicine); d, liver from group 4 (DEN+0.5 mg/kg of colchicine). Right column; Representative histology of liver 6 days after the beginning of the experiment (H-E staining, ×400): e, rat treated with DEN alone; f, rat treated with DEN and 0.5 mg/kg of colchicine. Mitotic figures are observed in the latter and hepatocyte nuclei are bigger than in the control liver.

Table I. Numbers, Sizes and BrdU Labeling Indices of GGT-positive Foci in the Liver of Rats Treated with or without DEN and Colchicine

Group	DEN	Colchicine (mg/kg)	Effective number	Body weight (g)		Relative liver weight (%)	GGT-positive foci			
				Initial	Final		No./cm^2	Average size (mm^2×10^{-1})	% area occupied by foci	BrdU labeling index (%)
1	+	0	8	131±8.2	197±12.0	3.57±0.14	20.8±7.0	3.6±4.7	7.3±4.7	12.4±4.85
2	+	0.1	10	129±3.9	183±7.9*	3.54±0.30	25.4±5.8	7.3±5.0	18.6±7.4**	ND
3	+	0.25	11	128±6.0	176±14.6**	3.88±0.33*	29.2±6.3	10.7±8.8*	31.2±11.9**	ND
4	+	0.5	11	131±6.0	178±14.6**	4.62±0.73**	28.6±7.5	15.4±14.6**	44.9±10.5**	42.3±19.1**
5	−	0	11	129±5.2	201±14.3	3.50±0.51	0.83±0.54	5.4±4.2	0.43±0.46	ND
6	−	0.5	9	123±4.2	184±22.3	3.64±0.46	0.59±0.47	4.3±4.2	0.26±0.21	ND

* $P<0.05$ as compared with group 1.
** $P<0.01$ as compared with group 1.
ND: Not done.

foci increased dose-dependently in groups 2 to 4, being approximately 4-fold greater than in group 1. Histological examination demonstrated that the increase of focus size was due to an increase in cell number per focus. Colchicine alone, at a dose of 0.5 mg/kg, did not induce GGT-positive foci, since the induction of small numbers of GGT-positive foci in rats from groups 5 and 6 appeared to be solely dependent upon the selection procedure with AAF and CCl_4. The BrdU labeling index was increased 3.5-fold in foci in the livers of rats from group 4 as compared to the group 1 value. In the background liver, BrdU labeling indices showed almost the same values in both groups. Histologically, nuclear chromatin of hepatocytes in foci induced by DEN and colchicine demonstrated a coarse granular pattern, in contrast to the fine granular appearance of those induced by DEN alone.

In experiment 2, serum GPT values were not significantly different among the groups. Mitotic indices were increased significantly in the livers of rats from groups 2 and 3 as compared to group 1 (Table II). Mitotic figures in the liver from group 3 still remained on day 6 after PH, and in the livers of rats from group 3 killed 6 days after PH, larger hepatocyte nuclei than in group 1 were observed (Fig. 2).

The present experiments demonstrated that colchicine given after DEN and PH enhanced the growth advantage of initiated cells without influencing their number. It is clear from the mitotic figures remaining at 6 days after PH that colchicine induced an M-phase block in regenerating liver. Colchicine did not cause marked liver necrosis under the present experimental conditions. It has been suggested that polyploid cells are characterized by genomic instability and such cells might exist in the liver

Table II. Serum GPT Values and Hepatocyte Mitotic Indices in Rats Given DEN with or without Colchicine

Group	Colchicine (mg/kg)	Serum GPT value at days after DEN administration			Mitotic index[a] (%)
		4	5	6	
1	0	70.8±22.3	43.0±13.5	107.8±137.8	0.2±0.5
2	0.25	73.8±21.8	54.0±15.2	40.7±6.2	2.6±1.1**
3	0.5	164.8±95.3	55.4±11.1	88.5±32.9	4.8±2.4**

a) Mitotic indices were estimated at day 6 of the experiment.
* $P<0.05$ as compared with group 1.
** $P<0.01$ as compared with group 1.

of rats given colchicine. In such colcemid-induced cells, gene amplification can easily be induced by serial stepwise methotrexate selection.[8] In fission yeast, mutant strains with disorders in replication checkpoints have been established and some have a propensity for lethal aneuploidy and replication abnormalities due to loss or overdose of chromosomes in daughter cells.[13, 14] In our study, an increased number of polyploid cells might be produced in liver after hepatectomy and colchicine treatment. This would be consistent with the histological results and the mitotic block known to be induced with colchicine. However, other mechanisms whereby colchicine could have caused large foci to arise must be considered. One possibility is that hepatocytes initiated with DEN escaped its effects because of chemical resistance,[10] and thus could take advantage of any liver necrosis due to colchicine. However, BrdU labeling indices of GGT-positive foci were different between rats treated with or without colchicine. Moreover, from the serum examination results, it is clear that colchicine does not cause marked liver necrosis. Colchicine itself does not have a carcinogenic effect in terms of induction of GGT-positive foci. The mechanism of increased BrdU index in colchicine-treated GGT-positive foci is not obvious. One possibility is that gene alteration induced with DEN is enhanced during M-phase block by colchicine and the growth advantage of colchicine-treated GGT-positive foci is therefore enhanced.

In human cancer cells, cell cycle disturbances are key mechanisms in genomic instability and mutation of *p53* gene or *p16* gene is closely related to G1 checkpoint disturbance.[4, 15] The most important function of the *p53* gene, which commonly displays abnormalities in human cancers, is regulation of the cell cycle at G1 by induction of cyclin-dependent kinase inhibitory protein, waf1/Cip1/p21.[2, 15] Moreover, it was reported that MTS1, which is lost in various human carcinomas at high incidence, encodes the cdk inhibitory protein, p16, that binds specifically to cdk4/cyclin D.[4, 16] The *p53* gene also regulates a mitotic-spindle checkpoint[17] and it has been suggested that *p53* abnormalities can result in an altered cell cycle and gene amplification in transformed fibroblasts.[18, 19] Although mechanisms involved in enhancement of the growth advantage of enzyme-altered foci induced by M-phase block *in vivo* remain to be elucidated, the present result is the first demonstration that cell cycle disturbance affects carcinogenesis *in vivo*. The further evolution of foci induced by DEN and/or colchicine into hepatocellular carcinomas is being followed in a long-term experiment.

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