Jpn. J. Cancer Res. 83, 699–704, July 1992

Reversibility of Carcinogen-induced Rat Forestomach Basal Cell Hyperplasia Is Due to Squamous Cell Differentiation

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The mechanisms of reversibility of basal cell hyperplasia in the rat forestomach were investigated. Male F344 rats were given an initial single gastric intubation of N-methyl-N′-nitro-N-nitorosoguanidine and then received 2% butylated hydroxyanisole in the diet from the third week to the 26th week. Rats were killed at weeks 26 and 46 after return to basal diet and their forestomachs were removed. Bromouracil deoxyriboside (BUdR) was administered as a single i.p. injection 1 h before death or by osmotic minipump (120 μg/h) continuously for 7 days before death. Additional animals were maintained for 2 or 4 weeks after removal of osmotic minipumps to allow assessment of the fate of proliferating populations. In each case BUdR-labeled cells were demonstrated by immunohistochemistry. At week 26, hyperplastic changes were more pronounced than at week 46. Squamous cells above basal cell hyperplasias were strongly labeled even 4 weeks after cessation of continuous BUdR administration, in clear contrast to those in normal-appearing epithelium. Three-dimensional reconstruction of persisting basal cell hyperplasias showed almost all basal cells limited to a thin sheet in direct contact with the squamous cell layer, occasional separate islands demonstrating differentiation to squamous cells and formation of epidermal cysts. The results thus showed that the mechanism of reversibility of basal cell hyperplasia involves differentiation of basal cells to squamous cells.

Key words: Reversibility — Forestomach — Hyperplasia — Rat

Cell proliferation is thought to play an essential role in carcinogenesis.[1] Butylated hydroxyanisole (BHA),[4] which does not possess mutagenic activity[2–4] can induce squamous cell carcinomas in the forestomach of rats[5–7] and hamsters.[7] Uracil also has no genotoxicity[8] and induces transitional cell carcinomas in the bladder of rats.[9] However, both BHA and uracil do produce severe hyperplastic lesions in the forestomach[5–7] and bladder,[9–11] respectively, from the early phase of treatment onwards. Hyperplasia caused by uracil is reversible.[10,11] BHA induces two kinds of hyperplasia,[12–15] one being composed of squamous cells and the other of basal cells. Squamous cell hyperplasia very quickly disappears after cessation of chemical insult, whereas the basal cell type persists far longer.[12–15] For this reason, basal cell hyperplasia has been considered a potential preneoplastic lesion.[12]

Recently we investigated in detail the fate of rat forestomach lesions induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and/or BHA[15] and reported marked differences in cell kinetics between squamous cell hyperplasias and basal cell lesions. In a squamous cell case a rapid decrease in mitotic cells and differentiation to keratotic cells, which were sloughed off, were apparent. The situation with basal cell hyperplasias was not so clear. Theoretically, their reversibility could occur due to differentiation to squamous cells, or by apoptosis,[16–18] and replacement by surrounding cells after simple cell death. In the present sequential study, we therefore assessed the differentiating fate of basal cell hyperplasias after bromouracil deoxyriboside (BUdR) labeling. Three-dimensional reconstruction was also applied with the aid of an image analyzer.[19]

MATERIALS AND METHODS

Animals and treatment Male, 5-week-old F344 rats were purchased from Charles River Japan, Inc., Atsugi. The animals were housed in plastic cages in an air-conditioned room at 22±2°C with a 12-h light-dark cycle and the experiment was started after 1 week of acclimation. All animals were given MNNG (Aldrich Chemical Co., Inc., Milwaukee, WI) at a dose of 150 mg/kg of body weight by a single gastric intubation and then fed on diet (Oriental M; Oriental Yeast Co., Tokyo) containing 2% BHA (for additive use from Wako Pure Chemicals, Ltd., Osaka) from the beginning of the third week to the end of the 26th week. After this they were returned to a basal diet. Groups of rats were killed at the end of weeks 26 and 46.

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[4] The abbreviations used are: BHA, butylated hydroxyanisole; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; BUdR, bromouracil deoxyriboside.

For quantitative analysis of cellular proliferation of forestomach epithelial cells, BUdR (Sigma Chemical Co., St. Louis, MO) was administered to groups of 3 or 4 rats at weeks 26 and 46 by single i.p. injection 1 h before death, or using an Alzet Model 2001 osmotic minipump (Alza Corporation, Palo Alto, CA) continuously for 7 days prior to death. The osmotic minipumps, delivering 120 μg of BUdR/h, were implanted s.c. into their backs. Furthermore, at both time points, 3 or 4 rats were killed 2 or 4 weeks after the 7-day continuous labeling period (at week 28 or 30, respectively). Excised stomachs were cut into about 8 strips. Some tissues were fixed in 10% buffered formalin solution for routine staining with hematoxylin-eosin (HE), or in ice-cold acetone for immunohistochemical demonstration of BUdR.

Immunohistochemistry The avidin-biotin-peroxidase complex method[20] was used to demonstrate binding of antibody against BUdR as previously described.[15] For squamous cell lesions, which were characterized depending on previously described criteria,[15] all squamous cells above the basal layer were counted. Three or more lesions from each category comprising more than two hundred cells, covering 1 mm of basement membrane, were selected at random from each rat and labeling indices were assessed under $\times$200 or $\times$400 magnification. For basal cell hyperplasias, almost all lesions, consisting of 140–773 cells, were counted for labeled cells, under the $\times$400 view.

Structural analysis For three-dimensional reconstruction of typical hyperplastic lesions, paraffin-embedded forestomach was serially sectioned (3.5 μm thick) and every 2nd section was assessed with the aid of a computer graphics system (Spicca Color Image Processor, Nippon Avionics Co., Ltd., Tokyo) and a three-dimensional imaging analysis program (TRI, Ratoc System Engineering Co., Ltd., Tokyo) for 5–30 sections. Squamous and basal cell layers were considered separately on the screen.

RESULTS

BUdR labeling Table I shows the labeling indices for each epithelial lesion after a single i.p. injection of BUdR. At week 26, no obvious differences were observed between normal-appearing squamous cell epithelium and mild or severe hyperplasia. However, the labeling index of basal cell hyperplasia was significantly lower. At week 46, squamous cell hyperplasias had generally disappeared and neoplastic lesions such as squamous cell carcinomas and papillomas were found. Since a BUdR labeling value could not be reliably estimated for squamous cell hyperplasias, only normal looking epithelium and basal cell lesions were assessed. In both cases indices were lower than at week 26. Moreover, the numbers of labeled squamous cells per 1 mm of basement membrane in squamous lesions at week 26 depended directly on epithelium thickness. The numbers of labeled cells in normal-looking squamous epithelium, mild-squamous cell hyperplasia and severe-squamous cell hyperplasia were 24.6$\pm$10.9 cells/mm, 93.4$\pm$42.2 cells/mm, and 184.7$\pm$53.2 cells/mm, respectively.

After continuous BUdR administration for 7 days, the entire squamous epithelium was labeled with the exception of a few cells in the basal layer. Only approximately 30% of cells in basal cell hyperplasias were labeled at random (Fig. 1).

Table I. Labeling Indices of Normal Squamous Epithelium and Forestomach Lesions after a Single BUdR Injection

	Labeling indices (%)	
	week 26 (n=3)	week 46 (n=3)
Squamous cell		
Normal-looking squamous epithelium	9.4$\pm$2.8	3.4$\pm$2.7[b]
Mild SCH	13.0$\pm$5.2	ND
Severe SCH	13.3$\pm$2.3	ND
Basal cell hyperplasia	5.6$\pm$3.6[a]	1.0$\pm$1.2[c]

SCH, squamous cell hyperplasia; ND, not detected.
Significantly different from all squamous cell values at week 26 *a*) $P<0.05$.
Significantly different from the same lesion value at week 26 *b*) $P<0.001$, *c*) $P<0.05$.

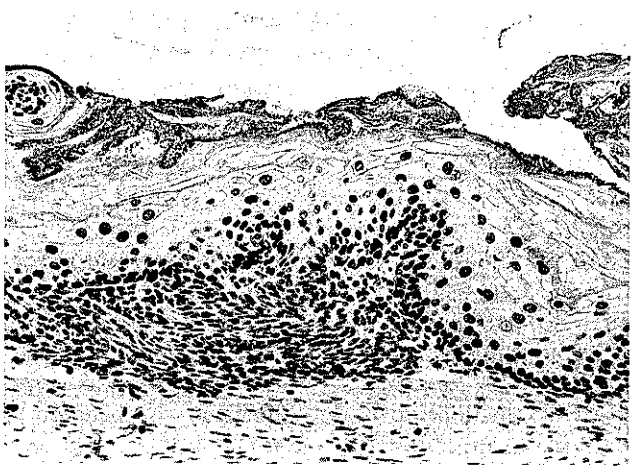

Fig. 1. Immunohistochemical staining of BUdR incorporation in squamous cell hyperplasia and basal cell hyperplasia of forestomach at week 26 after 7-day continuous BUdR administration. Almost all squamous cells are labeled, but only about 30% of the cells in the basal cell hyperplasia. $\times$200.

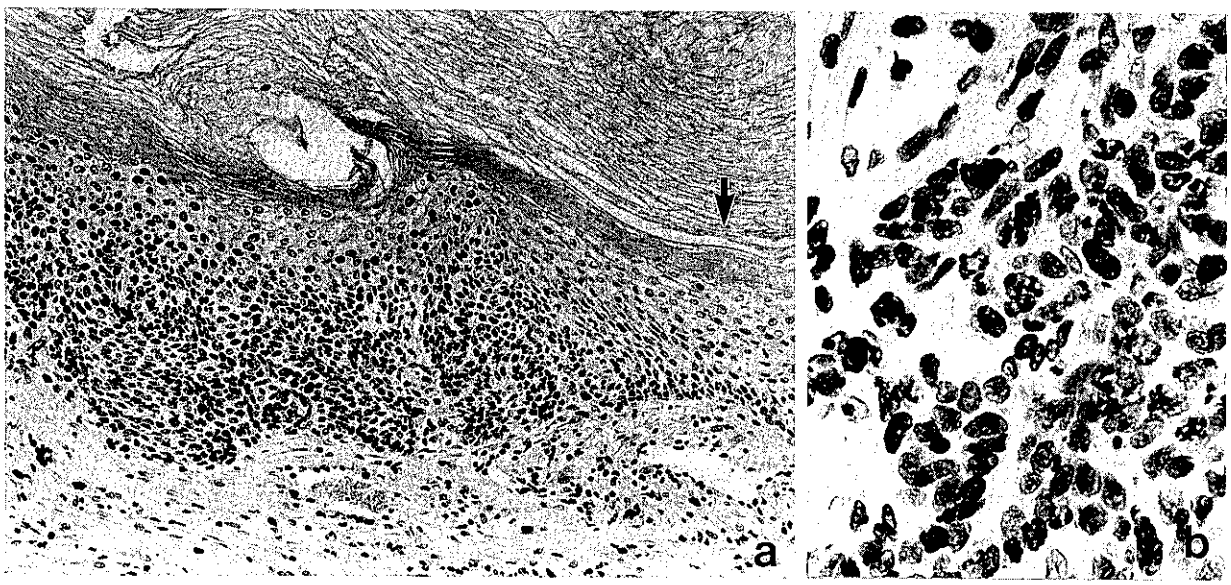

Fig. 2. (a) Four weeks after 7-day BUdR administration by minipump and withdrawal of BHA diet, the squamous cell layer above a basal cell hyperplasia is labeled strongly in comparison to the neighboring normal-appearing area (⬇) ×150. (b) Strong labeling is also evident within the basal cell hyperplasia ×400.

Table II. Labeling Indices of Forestomach Epithelium after Removal of BUdR Minipumps at Week 26

	Labeling indices (%)		
	No removal	2 wk after removal	4 wk after removal
Squamous cell			
Normal-looking squamous epithelium	92.8±1.8	68.9±10.0	62.5±12.7
Squamous cells above basal cell hyperplasia	92.7±4.8	93.9±0.2[b]	86.5±3.1[c]
Basal cell hyperplasia	29.2±4.8[a]	70.5±19.7	83.9±4.1[c]

Significantly different from the respective value for normal-looking squamous epithelium: *a*) $P<0.001$, *b*) $P<0.01$, *c*) $P<0.05$.

Two weeks after 7-day continuous BUdR administration and BHA gavage, BUdR binding in individual cells was decreased, by mitosis, with dot-shaped staining.

Four weeks after 7-day continuous administration, BUdR binding per cell was further decreased to various degrees. The intensity of labeling in squamous cells was weaker than that in basal cells (Fig. 2). For the majority of squamous cells, detection of BUdR antibody binding was difficult. Table II summarizes labeling indices for normal-looking squamous epithelium with and without underlying basal cell hyperplasia, at week 26 and 2 or 4 weeks after withdrawal of BUdR minipumps and BHA administration. Hyperplastic squamous cell and neoplastic lesions showed a wide range of labeling indices after the removal, indicating variation in growth rates. Labeling indices of normal-looking squamous epithelium were reduced gradually with a rapid decrease in intensity of staining. Labeling indices of squamous cells above basal cell hyperplasias showed significant differences from those of normal-looking squamous epithelium at 2 weeks and 4 weeks. That of basal cell hyperplasia was very much lower than that of squamous cells at first, but increased sequentially thereafter, so that at 4 weeks after minipump removal, a value in excess of that for normal epithelium was found. Squamous cells located immediately above basal cell hyperplasias were labeled most strongly, in spite of the surrounding squamous cells being extremely weakly labeled (Fig. 2).

Structural analysis A three-dimensional reconstruction and a corresponding HE section of a typical mild squamous cell with underlying basal cell hyperplasia observed at week 26 are shown in Fig. 3. The majority of persisting basal cell hyperplasias at week 46 were only a few cell layers thick. In one or two lesions in 5 of 6 rats protrusions into the submucosa were also observed (Fig. 4). In 3 of 6 rats single basal cell hyperplasias were associated with cyst formation in the submucosa with squamous differentiation and keratin deposits evident in the lumina (see Fig. 5).

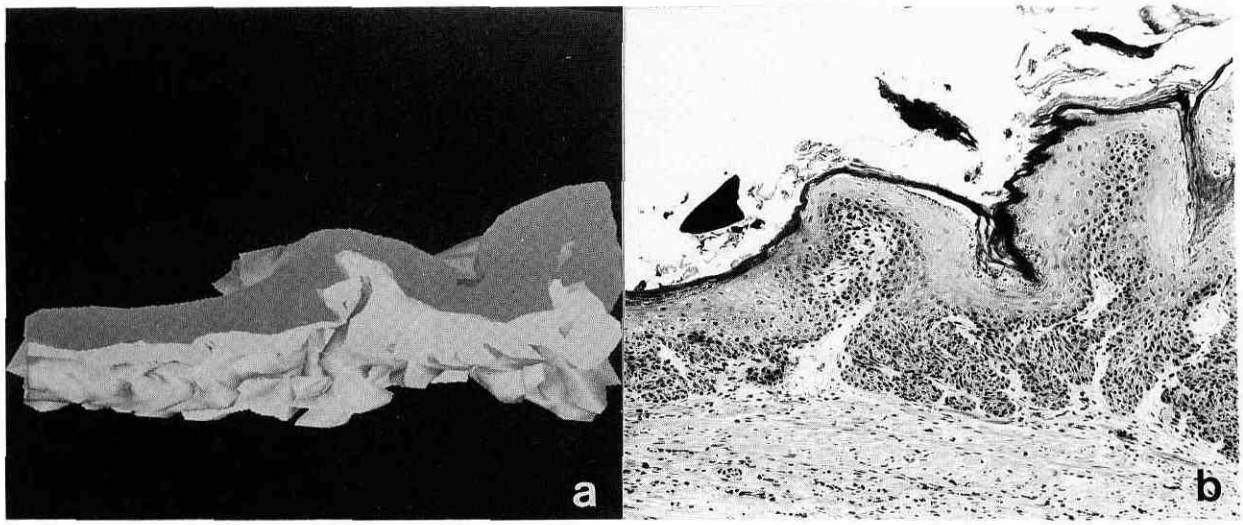

Fig. 3. Three-dimensional reconstruction of a typical mild squamous cell hyperplasia and basal cell hyperplasia (a) and a corresponding HE section (b). Darker and lighter masses indicate the squamous cell layer and basal cells, respectively. ×100.

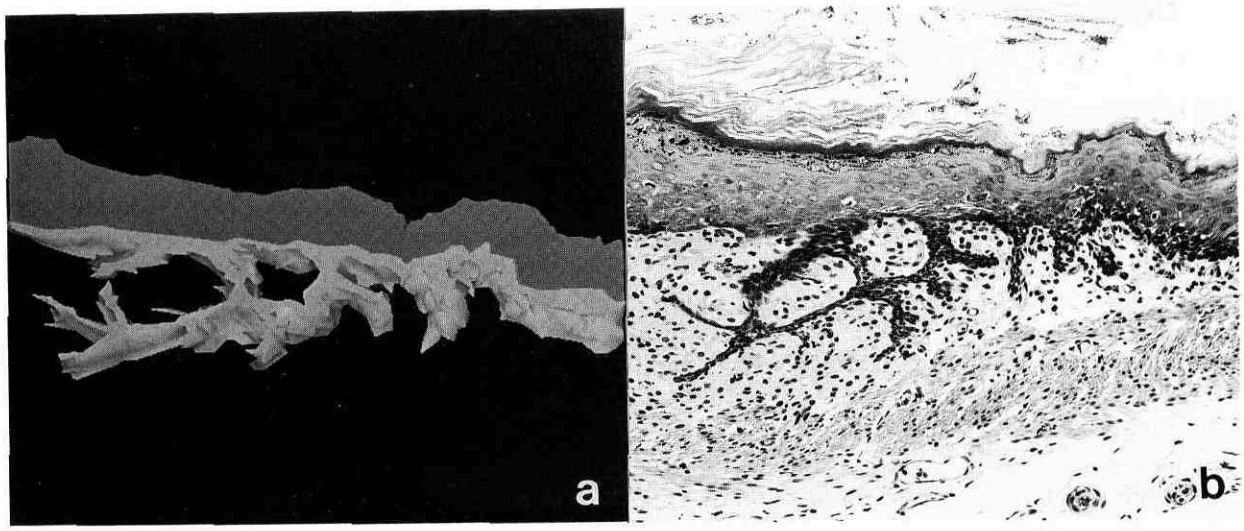

Fig. 4. Three-dimensional reconstruction of a basal cell hyperplasia (a) and a corresponding HE section (b) remaining at week 46. Protrusions from the thin basal cell layer into the underlying submucosa are evident. ×150.

DISCUSSION

In line with earlier reports[12-15] the present study revealed two kinds of hyperplasia to exist in the forestomach of MNNG-BHA treated rats, i.e., squamous cell hyperplasia and basal cell hyperplasia. We previously demonstrated that labeled cells located in the basal layers of squamous cell hyperplasia move to the surface, become hyperkeratotic and fall off.[15] Reversibility in the squamous case was thus due to this cell loss and the rapid decrease in the number of proliferating cells.

The first possibility regarding reversibility of basal cell hyperplasia is also decrease of proliferation. In fact, decrease in the labeling index of such lesions after withdrawal of BHA treatment was noted in our previous report[15] as well as the present study.

A second question is whether the cells composing basal cell hyperplasia die *in situ* by the so-called process of

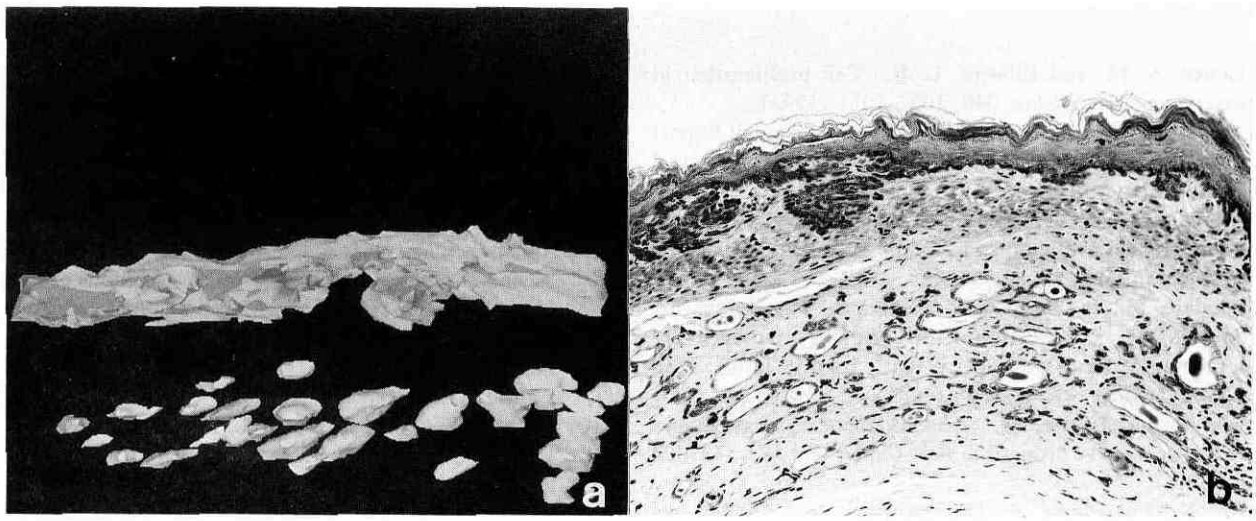

Fig. 5. Three-dimensional reconstruction of a basal cell hyperplasia (a) and a corresponding HE section (b) remaining at week 46. Note cyst formation in the submucosa with keratin deposits in the lumina. ×150.

apoptosis,[16-18] or whether they differentiate into other cells. Morphological evidence of cell death was scarce in the present study in spite of careful observation. The life span of a squamous cell is less than 7 days and with a 2- or 4-week interval after minipump removal BUdR concentration in individual squamous cells was therefore rapidly reduced. In addition to cell loss at the surface the participation of unlabeled basal cells in subsequent cell divisions would explain the observed drop in percentage of labeled cells. On the other hand, because of the long life span of basal cell[15] and with low proliferation, labeling was increased even after minipump removal. The fact that the density of labeled squamous cells above basal cell hyperplasias was higher than that in normal epithelium 2 or 4 weeks after cessation of BUdR exposure was a clear indication that basal cells had differentiated to form squamous cells.

If reversibility of basal cell hyperplasia is due to squamous cell differentiation, a physical connection with the surface squamous cell layer may not always be necessary. Thus the present study has provided evidence that well advanced basal cell hyperplasia can also exhibit the same process at a distance. Namely, when basal cells located near the squamous layer differentiate to squamous cells and disappear quickly, basal cells located in the deep submucosa may remain with no connection to the surface epithelium. These remnants of basal cell hyperplasia may still differentiate to squamous cells, with formation of cyst-like islands which include keratin masses. Indeed, three-dimensional reconstruction of such cystic lesions in the submucosa did not reveal any connection to the surface squamous layer. Such squamous cysts were therefore considered to be differentiated from islands of remaining basal cell hyperplasias.

Carcinogenesis is a multistep process[21,22] that results from the development of a variety of defects in the control of differentiation and proliferation.[23] The mechanisms underlying cell differentiation are very complicated and not well understood, but, as shown in the present study, a decrease of cell proliferation activity may facilitate cell differentiation in some cases. Induction of cell differentiation with lowered proliferation may lead to regression of tumorous lesions.

In conclusion, the present results and particularly the high labeling index in squamous cells above basal cell hyperplasias and cystic lesions could be explained by differentiation from basal to squamous cells.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare of Japan.

(Received February 22, 1992/Accepted April 28, 1992)

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