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Morphogenesis of Esophageal Carcinoma Induced by N-Methyl-N′-nitro-N-nitrosoguanidine in the House Musk Shrew, *Suncus murinus* (Insectivora)

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The histological changes occurring in the esophageal mucosa of shrews (*Suncus murinus*) after N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) treatment were investigated sequentially. Six-week-old female shrews were given a 50 μg/ml MNNG solution as drinking water for 30 weeks, and 5 selected at random were killed at 10 and 20 weeks of age, and thereafter at 5-week intervals until 45 weeks of age. Controls were killed at 45 weeks of age. The MNNG-induced esophageal lesion in shrews began from basal cell hyperplasia at 20 weeks of age, followed by dysplasia occurring at 25 weeks of age, then progressed toward intraepithelial carcinoma to invasive squamous cell carcinoma at 35 weeks of age. Apparent sequential dysplasia-carcinoma transition was seen. Papillomas were seen from 25 weeks of age but there was no evidence of papilloma-carcinoma sequence. Five MNNG-untreated shrews killed at the end of the experiment were free of esophageal tumors.

Key words: Esophageal carcinoma — Squamous cell carcinoma — MNNG — *Suncus murinus* — Insectivora

Esophageal cancer, despite advances in treatment, is still one of the most common fatal cancers worldwide.[1] The low survival rate is due to patients not visiting the hospital until the cancer is in a late stage. Therefore, little is known about the early stage of this carcinoma. Marked geographic variations suggest that environmental factors contribute to the etiology of this cancer.[2] Cases of esophageal cancer in humans and domestic animals geographically clustered in China suggests that nitrosamines and their precursors may be an important etiologic factor.[3] In other parts of the world, tobacco and alcohol consumption are commonly accepted as the major risk factors.[4] As it is known that minute quantities of nitrosamine are present in cigarette smoke and in alcoholic beverages,[5] nitroso compounds appear to play a major role in the development of esophageal cancer.

Esophageal carcinomas have been induced experimentally by nitrosamines and other nitroso compounds in rats, mice, rabbits, dogs and other species[6] by various routes.[6–8] Neoplastic transformation involves a series of consecutive stages that are progressively associated with acquiring a malignant condition. Esophageal carcinomas induced in experimental animals are useful tools for studying the development of those which occur in humans. However, the majority of previous studies on esophageal carcinogenesis have been related to the induction of tumors, and information regarding the histogenesis of squamous cell carcinoma of the esophagus is still incomplete. The esophageal epithelium of rats shows marked cornified changes that do not resemble those of humans, and they have a forestomach, which humans do not possess. The esophagus of dogs and monkeys is more similar to that of humans, but their long lifespan requires long-term studies. Thus, the development of a more suitable model is required.

One member of the class insectivora, the house musk shrew (*Suncus murinus*; family Soracidae) has been bred under laboratory conditions.[9] Phylogenetically, insectivora are considered to be the most primitive class of primates,[10] and may represent a suitable animal model from which findings may easily be extrapolated to humans. In this context, inductions of leukemia,[11] intestinal cancer,[12] musk gland tumor,[13] and pulmonary tumor[14] in these animals using a variety of chemical carcinogens have been reported. Using N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), a nitroso compound recognized as a potent gastric carcinogen, we recently succeeded in inducing esophageal squamous cell carcinomas selectively, regularly, and with a high incidence in shrews.[15] As in humans, the esophagus of these animals is lined with stratified non-cornified squamous epithelium, and they have no forestomach; thus, shrews may be useful for investigating the series of events leading to squamous cell carcinoma of the esophagus. However, to date, precise analysis of the early changes of the esophageal epithelium of shrews have not been carried out. Therefore, the aim of the present study was the sequential observation of serial changes leading to invasive squamous cell carcinoma induced by MNNG. In this report, we discuss the histogenesis of esophageal squamous cell carcinomas in shrews, and compare this with the stages of development of human esophageal cancer.

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MATERIALS AND METHODS

Animals A total of 55 4-week-old female house musk shrews, Jic:SUN strain, were purchased from CLEA Japan Inc. (Osaka). The animals were housed in plastic cages, 3–5/per cage, with sterilized white pine chips as bedding. Room temperature was 22±2°C and relative humidity was 60±10%. They were fed a special pellet diet for shrews (CIEA-305; CLEA Japan Inc.) and had free access to water.

Chemicals and experimental procedures MNNG, purchased from Nacalai Tesque (Kyoto), was dissolved in deionized water at a concentration of 1 mg/ml as a stock solution and was kept at 4°C in the dark. Working solutions of 50 μg/ml, diluted with tap water just before use, were prepared every other day from the stock solution and given to the animals as drinking water. Shrews were divided at 6 weeks old, 50 into the experimental group and 5 into the control group. Experimental animals received MNNG for 30 weeks, then tap water until the end of the experiment. Control shrews received normal tap water throughout the experimental period. Five randomly selected MNNG-ingesting shrews were killed at 10 and 20 weeks of age, and thereafter at 5-week intervals until 45 weeks of age. Control shrews were killed at the termination of the experiment (45 weeks of age). Shrews were treated i.p. with 5-bromo-2′-deoxyuridine (BrdU: Sigma, St. Louis, MO; 20 mg/kg body weight) 1 h before being killed. The animals were then carefully autopsied: the esophagus was opened longitudinally and pinned onto a filter paper with the mucosa upward and fixed in Methacarn. After fixation and paraffin embedding, serial sections were cut across the full thickness of the wall. Other organs were examined grossly and microscopically after routine histological procedures and hematoxylin and eosin (HE) staining.

Histological criteria To avoid confusion in nomenclature, lesions were classified as normal, basal cell hyperplasia, papilloma, dysplasia, or carcinoma. The diagnosis of various grades of dysplasia was based on the degree of cellular atypia and structural alteration (mild, abnormalities confined to the lower third of the epithelium; moderate, abnormalities occupying up to the lower two thirds of the epithelium; severe, involving the upper third of the epithelium).[16] Carcinoma was distinguished by the depth of invasion (ep, intraepithelium; mm, up to the level of muscularis mucosae; sm, involving submucosa; mp, involving muscularis propria; a, into adventita).[17]

Immunohistochemistry Immunohistochemical staining was carried out according to the streptavidin-biotin (LSAB) method using the monoclonal antibody B44 (Becton Dickinson, San Jose, CA), which recognizes BrdU, and polyclonal anti-type IV collagen antiserum,[18] which recognizes basement membrane components. Deparaffinized slides were immersed in 0.01% Actinase (Kaken Pharm., Tokyo) in 0.01 *M* phosphate-buffered saline (PBS; pH 7.2) for 15 min at 37°C to allow visualization of type IV collagen.[19] Slides were incubated with 4 *N* HCl for 20 min followed by 0.05% Actinase for 1 min both at 37°C for BrdU labeling.[12] They were then reacted with the respective antibody for 1 h at room temperature. The kit manufacturer's protocol was followed for LSAB staining (Dako, Carpinteria, CA). Finally, 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemical, Osaka) was used as a chromogen, and the sections were counterstained with hematoxylin.

RESULTS

Fifteen animals died during the experiment. In some animals, tissues were not available due to cannibalism, and the mucosa of the esophagus in other animals was autolytic so that cellular details were difficult to evaluate. Thus, 15 animals were excluded from the analysis. Tumors were selectively evoked in the esophagus, and no other organ showed neoplastic change. The histological findings, incidence and time of detection of lesions in the esophagus of shrews treated with MNNG are shown in Table I.

Sequential studies on MNNG-treated shrews showed that basal cell hyperplasia appeared at 20 weeks of age, followed by dysplasias at 25 weeks of age, and carcino-

Table I. Sequential Changes in the Esophageal Epithelium of Female Shrews Treated with MNNG

Age (weeks)	No. of animals	Basal cell hyperplasia	Dysplasia			Squamous cell carcinoma					Papilloma
			mild	moderate	severe	ep	mm	sm	mp	a	
10	5	—	—	—	—	—	—	—	—	—	—
20	5	1	—	—	—	—	—	—	—	—	—
25	5	3	2	—	—	—	—	—	—	—	4
30	5	1	—	2	2	—	—	—	—	—	2
35	5	—	—	2	—	—	1	2	—	—	5
40	5	—	—	1	—	—	1	3	—	—	5
45	5	—	—	—	—	—	1	1	2	1	5

mas at 35 weeks of age, whereas the esophageal epithelium of 10-week-old shrews remained normal, the same as that of control shrews killed at termination of the experiment. In the normal esophagus, there was a well-oriented squamous epithelium containing a single row of basal cells located along the border between the epithelium and submucosa (Fig. 1a). BrdU labeling was sporadic and restricted to basal cells (Fig. 1b). Usually, the epithelial-stromal border was straight, and the expression of type IV collagen was characterized by a thick, continuous and linear staining pattern (Fig. 1c). At 20 weeks of age, basal cells characterized by the scant basophilic cytoplasm showed thickening in 1 animal (basal cell hyperplasia), but remained normal in the other 4. At 25 weeks of age, 3 animals showed basal cell hyperplasia and 2 revealed mild dysplasia. At 30 weeks of age, 1 showed basal cell hyperplasia, 2 showed moderate dysplasia (Fig. 2a) and the other 2 revealed severe dysplasia (Fig. 3a). Epithelial proliferation into the underlying tissue resulted in the formation of fairly long epithelial buds. There was

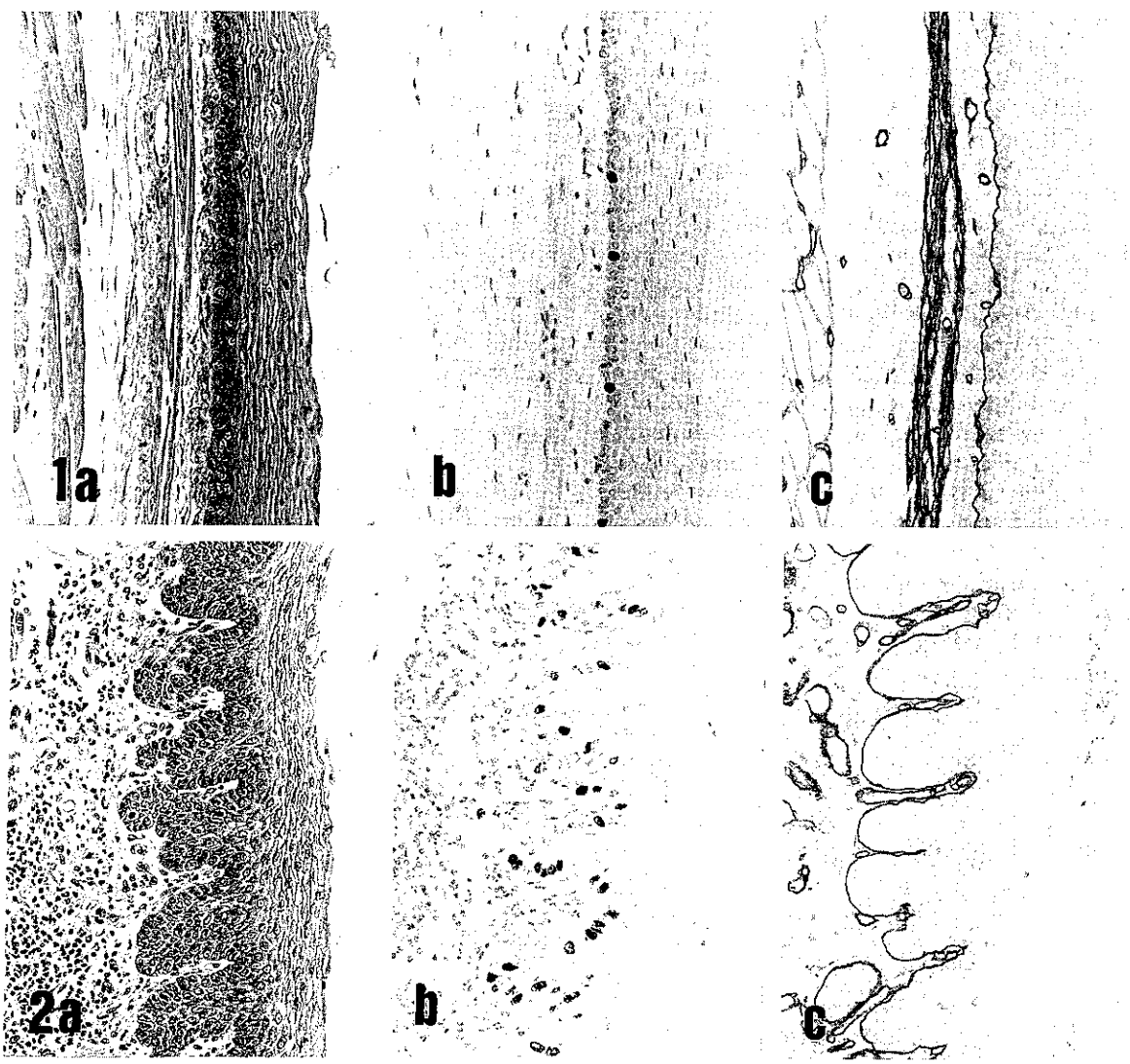
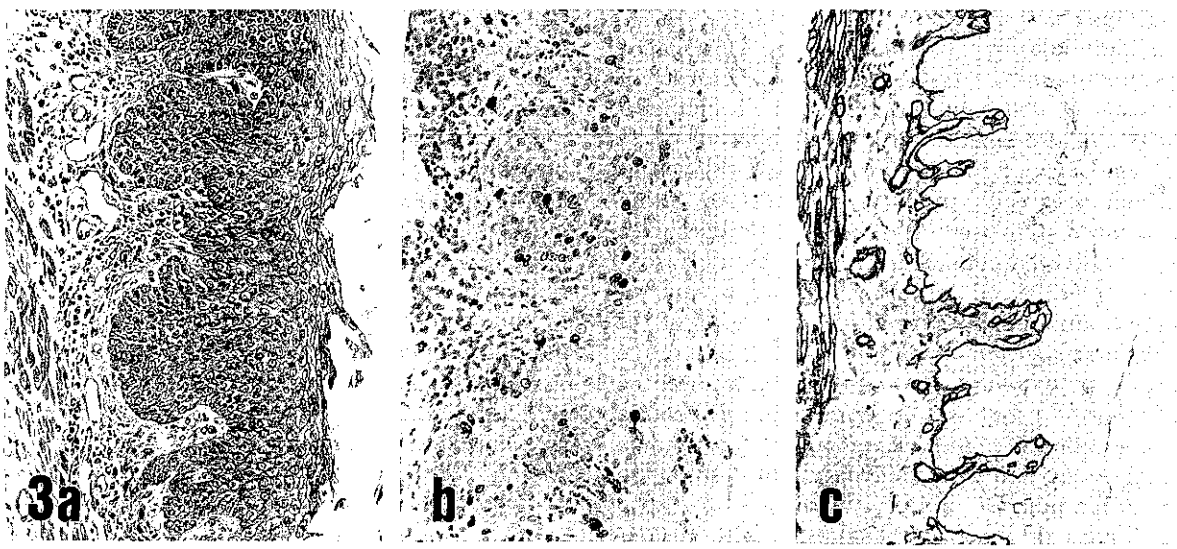


Fig. 1. Normal esophagus: (a) Mucosa consisting of non-keratinized stratified squamous epithelium. HE ×200. (b) Positive labeling was restricted to basal cells. BrdU ×200. (c) Continous and thick basement membrane was seen at the epithelium-stromal junction. Note blood vessels in the sub-epithelial mucosa and the muscularis mucosa were also stained. Type IV collagen ×200.

Fig. 2. Moderate dysplasia: (a) Elongation of the epithelial buds of atypical cells occupying one half of the epithelium can be seen. HE ×200. (b) Increased BrdU-labeling not restricted to basal cells. BrdU ×200. (c) Continuous basement membrane can be seen. Type IV collagen ×200.



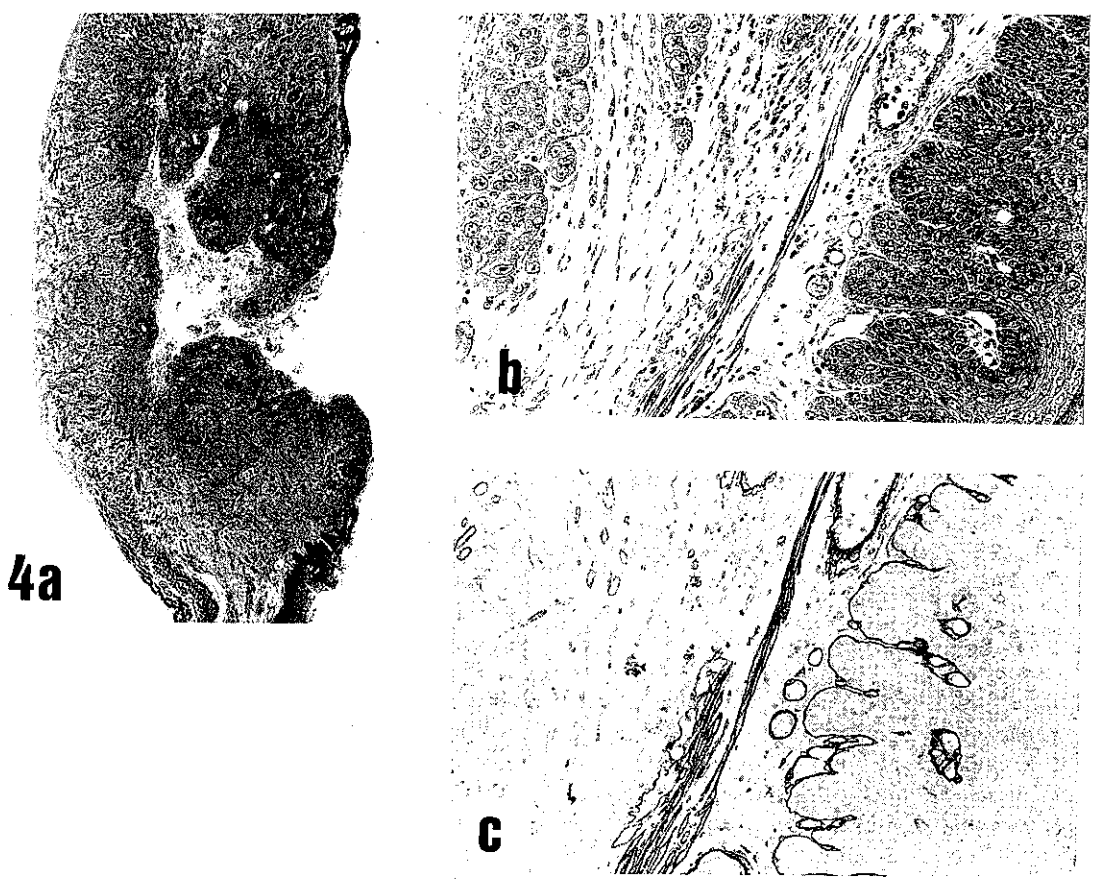


Fig. 3. Severe dysplasia: (a) Atypical cells involving the upper third of the epithelium can be seen. HE ×200. (b) Expansion of the proliferation zone can be seen. BrdU ×200. (c) Continuous basement membrane is preserved. Type IV collagen ×200.

Fig. 4. Invasive squamous cell carcinoma: (a) Depth of invasion reached the adventitia at 45 weeks of age. Ulcerative type. HE ×20. (b) Higher magnification of the invasive margin of Fig. 4a. HE ×200. (c) Lack of type IV collagen staining can be seen in the marked invasive margin of the tumor cell nest. Same area as Fig. 4b. Type IV collagen ×200.

a clear association between dysplasias and increased BrdU labeling not restricted to the basal layer (Fig. 2b and 3b). Continuous linear staining of type IV collagen was preserved (Figs. 2c and 3c), and proliferation of vascular elements beneath the proliferating epithelial component was also labeled by type IV collagen. At 35 weeks of age, foci of intraepithelial cancer as well as invasive squamous cell carcinoma had evolved within highly advanced dysplasia. At this stage, lesions were multiple. Invasive carcinomas were seen as ulcerative, superficial or protruded lesions. In invasive cancers, malignant squamous cells invaded up to the level of the muscularlis mucosa (mm) in 1 animal and deeper to the submucosa (sm) in two. At 40 and 45 weeks of age, the depth of invasion progressed to the muscularis propria and to adventitia (Fig. 4a). Squamous cell carcinoma was strongly associated with some degree of dysplasia in the neighboring mucosa, and was surrounded by intraepithelial lesions that had epithelial buds with various degrees of cellullar atypia. Squamous cell carcinomas were mainly moderately differentiated, and rarely made keratin or keratin pearls. BrdU-labeled cells were not restricted to the periphery of the cancer cell nests. Usually, a continuous or partly discontinuous basement membrane was seen around nests of invasive carcinomas. However, marked invasive margins often lacked type IV collagen staining (Figs. 4b and 4c).

Progressing hyperplasia of the connective tissue papillae and proliferative changes of the epithelium led to the formation of papillomas. The earliest papillomas were first seen in 25-week-old shrews. Thereafter, they increased in size and number, and some demonstrated atypical features. Although atypical cells were seen, BrdU labeling was restricted to basal cells, and progression to carcinoma was not evident. No verrucous or papillary carcinoma was seen. Carcinomas did invade the esophageal wall, but spread of the cancer cells to the regional lymph nodes or distant organ metastasis was not found in any animal.

DISCUSSION

Identification of the morphogenesis of esophageal carcinoma in experimental animal may have a significant impact on the understanding of human esophageal carcinogenesis. In rats, induced esophageal carcinoma arises much more frequently via papillomas than via dysplasia.[20, 21] In hamsters, atypical foci of exophytic growth exist in the vicinity of carcinomas.[22] Non-papillary squamous cell carcinomas of the ulcerative, superficial and protruded types represent the overwhelming majority found in man, while verrucous or papillary carcinomas are rare.[23] In humans, frequent association of invasive carcinoma with dysplasia and/or intraepithelial carcinoma supports the hypothesis of a dysplasia-carcinoma sequence.[24] Therefore, the endophytic pathway of carcinoma development in experimental esophageal carcinogenesis is of interest since it shows stages of progression that are histologically comparable to those of the human counterpart. The spectrum of histological alteration of esophageal mucosa preceding carcinoma in animal models differs variously among species.[6, 23] Thus, the development of a suitable model is required for better understanding of the morphogenesis of human esophageal carcinoma.

The results of the present study confirmed that MNNG at 50 μg/ml in the drinking water selectively induced esophageal tumors in shrews, and the organotrophic response was similar to that in our previous report.[15] During the carcinogenic process, changes in the esophageal epithelium showed a gradual progression from basal cell proliferative change and the development of dysplasia at 25 weeks of age, to intraepithelial and invasive carcinoma at 35 weeks of age. Invasive cancer was always adjacent to intraepithelial lesions with varying degrees of severity. In addition to these lesions, papillomas were also found beginning from 25 weeks of age. Although invasive carcinomas showed exophytic and endophytic growth, exophytic tumors showed no apparent transition from papillomas, and no verrucous or papillary carcinomas were seen. In dogs, carcinomas arise abruptly from normal esophageal epithelium.[25, 26] In shrews however, no *de novo* carcinomas were found in our study. Therefore, basal cell hyperplasia and dysplasia may represent the main pathway leading to intraepithelial carcinoma and invasive carcinoma, which follows the same steps as human esophageal carcinoma.[24]

Reliable markers can define sequentially occurring events more clearly. Normal esophageal mucosa is composed of stratified non-keratinizing squamous epithelium, of which only the basal layer has a proliferating population of cells that synthesize DNA and have mitotic activity. As seen by BrdU labeling, the proliferating zone expanded as dysplasia progressed, accompanied with increased labeling index.

In conclusion, the present study in shrews has demonstrated a close association between the formation of dysplasia and progression toward invasive carcinoma, which showed stages of progression that were histologically comparable to those of the human counterpart. Therefore, this model may be useful for the better understanding of human esophageal carcinogenesis.

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