Sequential endoscopic findings and histological changes of N-nitrosomethylbenzylamine-induced esophageal carcinogenesis in rats

TETSUYA BADEN[1], KEIGO YAMAMICHI[1], TAKU MICHIURA[1], AIRO TSUBURA[2] and KOSHIRO HIOKI[1]

Second Departments of [1]Surgery and [2]Pathology, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan

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Abstract. We performed a sequential endoscopic examination of esophageal carcinogenesis induced by N-nitrosomethylbenzylamine (NMBA) in F344 rats. The endoscopic findings were consistent with the histological changes observed in the specimens obtained by a biopsy and/or an autopsy. Seven-week-old male F344 rats received a weekly subcutaneous injection of 0.5 mg/kg NMBA for 15 weeks. The first endoscopic change that was detected was redness of the musosa due to the dilatation of the submucosal blood vessels. Subsequently, the mucosal redness became obscure, and we observed a focal loss of the visible blood vessel network due to hyperkeratosis, followed by the appearance of plaque-like elevated lesions due to acanthosis. Then, smooth and irregular polyps appeared as a result of the development of papilloma without or with dysplastic potential, respectively. Finally, rough elevation appeared as a result of carcinoma *in situ* and invasive squamous cell carcinoma. The present endoscopic findings correlated closely with the histological changes, indicating that sequential fiberscopic examination may be useful for monitoring esophageal carcinogenesis.

Introduction

Squamous cell carcinoma of the esophagus accounts for a significant number of cancer deaths, but its incidence and mortality rate vary widely around the world (1). The geographic variation in human esophageal carcinoma suggests that environmental factors contribute to its development. It has been suggested that nitroso compounds are a factor in the etiology of human esophageal carcinoma (2). Esophageal tumors have been produced experimentally in several animal species by administering various nitroso derivatives (3-7). Several of these nitroso compounds, including N-nitrosomethylbenzylamine (NMBA), are potent inducers of esophageal cancer in rats (8-10). Several investigators have used NMBA to induce esophageal cancer in rats, using various routes and dosing regimens (9). The advantage of NMBA-induced esophageal carcinogenesis is that multiple exophytic tumors develop randomly along the entire length of the esophagus (11). However, NMBA-induced esophageal carcinomas in rats rarely metastasize.

Sequential endoscopic fiberscope examination may contribute to the early detection of human esophageal cancer and may thus help in decreasing its mortality rate. Moreover, minimally invasive endoscopic methods may have great advantages in the treatment of early esophageal cancers. Animals, including pigs (12,13) and sheep (14), have been used to evaluate the usefulness of endoscopic examination for the development of therapeutic strategies for esophageal cancer. Sequential endoscopic observation of esophageal carcinogenesis has been performed using several animal species. However, the endoscopic findings must be correlated with the histological changes before endoscopic fiberscopes can be reliably used for the detection and treatment of esophageal cancers. Squamous cell carcinoma has been induced in dogs by administering N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG) (6,15,16), and has been induced in rabbits by administering N-amyl-methyl-nitrosamine (16,17). These models are useful for studying the carcinogenic process with an endoscopy and a biopsy (17), which can be easily performed with animals in the size range of rabbits and dogs. There have been no reports of studies in which an endoscopy and a biopsy have been performed to examine esophageal carcinogenesis in small laboratory animals such as rats. In the present study, we performed a sequential endoscopic observation of esophageal carcinoma in rats, and assessed the correlation between the endoscopic findings and histological changes.

Materials and methods

Rats. Five-week-old male F344/Jcl rats were purchased from CLEA Japan (Osaka). The rats were housed 3 per cage in the animal room at 22±2˚C and 60±10% relative humidity, with a 12-h light/dark cycle.

Correspondence to: Dr Airo Tsubura, Second Department of Pathology, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan
E-mail: tsubura@takii.kmu.ac.jp

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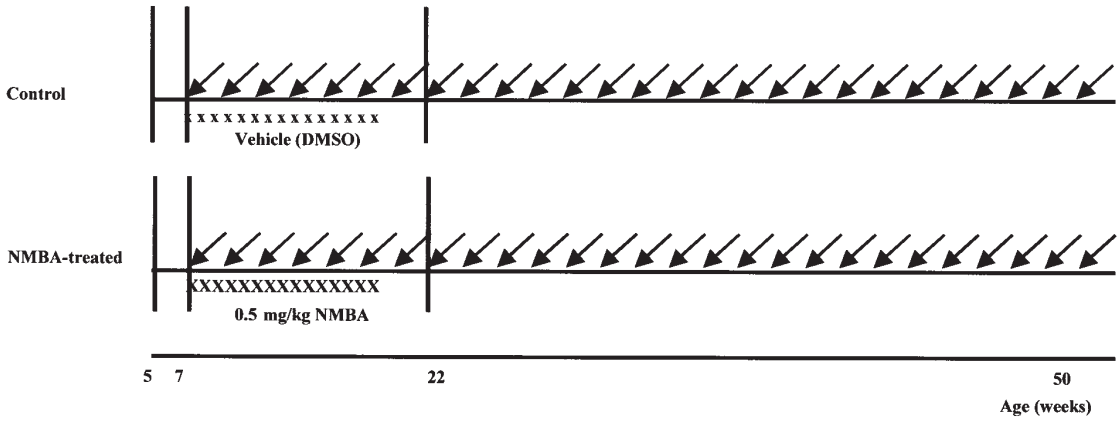

Figure 1. Schematic representation of the experimental protocol. Each large X indicates subcutaneous (s.c.) injection of 0.5 mg/kg/body weight NMBA, and each small x indicates an s.c. injection of 20% DMSO dissolved in distilled water (vehicle). Each arrow indicates a fiberscopic examination including an arbitrary biopsy or autopsy.

Experimental procedures. The rats were acclimatized to the animal facility for 2 weeks. Beginning at 7 weeks of age, 24 rats were given weekly subcutaneous (s.c.) injections of NMBA (Ash Stevens, Detroit, MI, USA; purity >99%, as determined by high-performance liquid chromatography) dissolved in 20% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA)/ water, at a dose of 0.5 mg/kg body weight, for 15 weeks. The controls were 4 rats that were given weekly s.c. injections of vehicle (20% DMSO) for 15 weeks, beginning at 7 weeks of age. The experiment was terminated when the rats reached 50 weeks of age. Hygienic conditions were maintained by cleaning the cages weekly, changing the shavings weekly, and changing the water daily. Throughout the experiment, the rats were fed a diet of AIN-76A (Oriental Yeast, Chiba, Japan).

Endoscopic examinations. An endoscopic examination was first performed at 7 weeks of age, and was performed every other week thereafter. Fig. 1 shows the schedule of the s.c. injections (NMBA or vehicle) and the endoscopic examinations. The procedure used for the endoscopic examinations was based on a method described in a previous report (18). Briefly, after 24 h of fasting with free access to water, the rats were anesthetized with evaporated diethyl ether in a closed chamber. Then, the animals were placed in the supine position, and the extremities and incisors were fixed. An endoscopy was then performed using a fiberscope (Olympus CHF_{TYPE}CB30S, Tokyo, Japan), a lighting system (Olympus CLV-U20D), and a 3-CCD camera module and video box (Olympus OTV-SX). All images obtained from the endoscopy or the biopsies were observed on a CRT monitor (Sony PVM-1442Q, Tokyo, Japan), and were recorded using an S-VHS video recorder (Sony SLV-RX9) and a video-to-video printer (Sony CVP-P77). The fiberscope was inserted into the stomach, and 3 to 5 ml of air was supplied. The fiberscope was then drawn out slowly to examine the surface and compliance of the esophagus. If needed, the biopsies were performed using forceps via the channel of the fiberscope (diameter, 1.2 mm). The examination took <2 min. During the experimental period, individual lesions were repeatedly biopsied to assess disease progression, and randomly selected NMBA-treated rats were sacrificed and autopsied; esophageal tissues obtained from those biopsies and autopsies were histologically examined. The control rats were autopsied at the end of the study. All procedures involving animals were approved by the Animal Experimentation Committee at Kansai Medical University.

Endoscopic nomenclature. The endoscopically observed esophageal lesions were classified as follows: i) Redness of the mucosa (esophageal mucosa exhibited diffuse redness or patches of redness), ii) loss of the visible blood vessel network (a white-spotted area in the mucosa blocked the observation of the blood vessel network), iii) plaque (a slightly elevated white disc-like lesion with a well-defined border and a dull smooth surface), iv) smooth polyp (a round or oval polypoid lesion with a smooth surface), v) irregular polyp (a polypoid lesion with an irregular surface), vi) rough elevation (a protruding lesion with an uneven and erosive surface).

Tissue handling. Specimens of esophageal tissue obtained from the biopsies or autopsies were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with H&E.

Histological nomenclature. The histological changes were classified as follows: i) Blood vessel dilation (dilated blood vessels in the submucosa), ii) hyperkeratosis (hyperkeratosis without clear thickening of the squamous cell layer), iii) acanthosis (local thickening of the squamous cell layer with hyperkeratosis), iv) papilloma (local hyperkeratosis and hyperplasia of the squamous cell layer, with proliferation of the connective tissue papillae), v) dysplastic papilloma (squamous cells acquire atypical cytological features and abnormal polarity), vi) carcinoma *in situ* (CIS; malignant epithelial cells, without stromal invasion), vii) invasive carcinoma (malignant epithelial cells, with stromal invasion). The surface of CIS and invasive squamous cell carcinomas often exhibits irregular erosion and/or ulceration.

Results

An endoscopic examination was successfully performed on all the animals, and no handling problems occurred during the procedure. Anesthesia was well tolerated, and no major

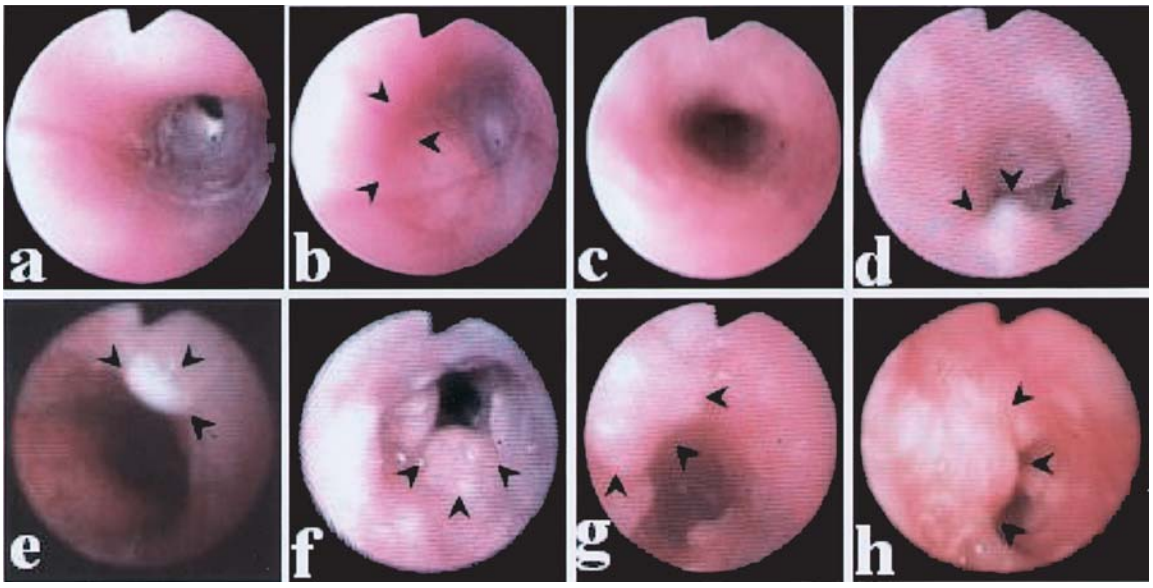

Figure 2. Representative endoscopic findings in F344 rats treated with or without NMBA. (a) Normal-appearing musosa. (b) Redness of the mucosa (between the arrowheads). (c) Loss of visible vessel network. (d) Plaque. Note the localized white elevation (between the arrowheads). (e) Smooth polyp. Note the greater elevation of the lesion, compared with the plaque (between the arrowheads). (f) Irregular polyp. The elevated lesion between the arrowheads is irregular. (g and h) Rough elevation. Lesions are more widely extended than polyps, and the degree of surface irregularity is greater in (h) than in (g). (a) Untreated control rat esophagus. (b-h) NMBA-treated rat esophagus).

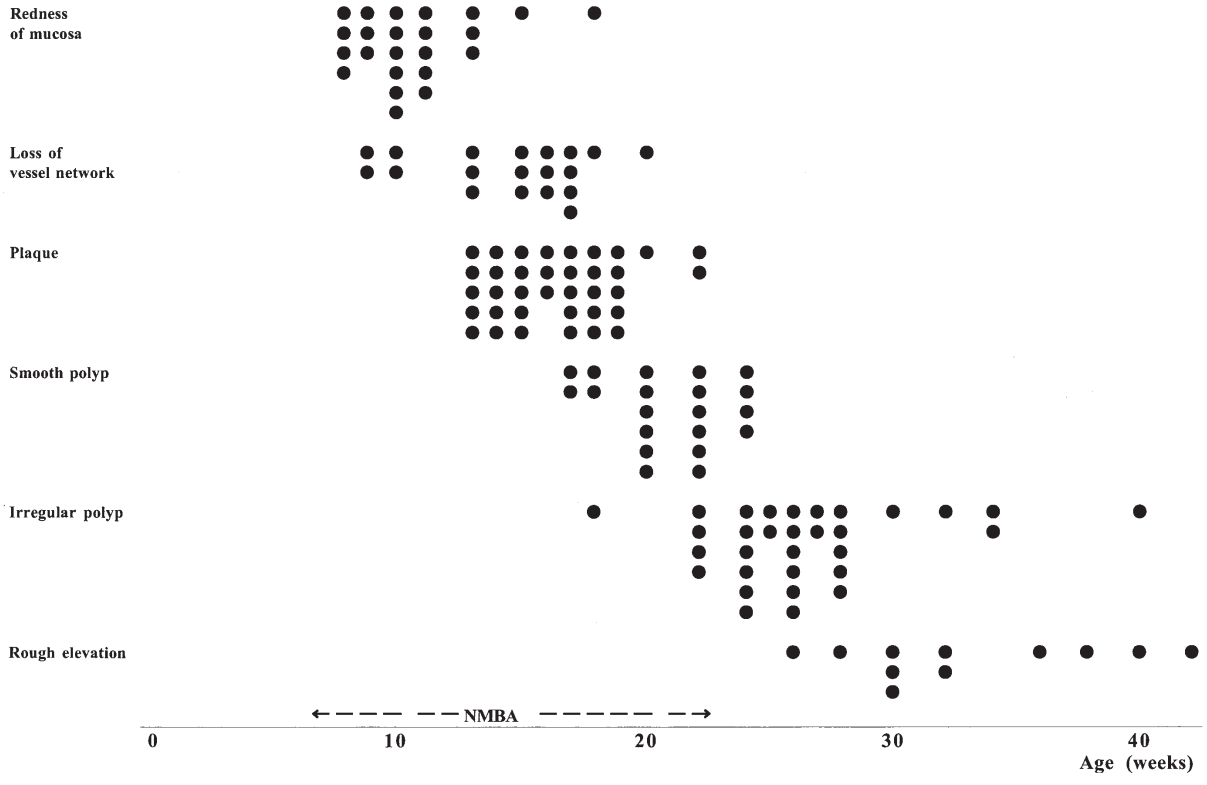

Figure 3. Summary of the sequential endoscopic findings of the esophagus of F344 rats treated with NMBA.

complications were observed. The representative endoscopic findings are sequentially shown in Fig. 2. The control esophagus is shown in Fig. 2a for comparison. In the NMBA-treated rats, redness of the mucosa was the first change in appearance (Fig. 2b). Then, the redness disappeared, and we observed a focal or diffuse loss of the visible blood vessel network (Fig. 2c). This was followed by the appearance of a white plaque-shaped localized elevation (Fig. 2d). The plaque increased in height to form a round or oval smooth polyp (Fig. 2e). The surface of the plaque then became irregularly shaped, forming an irregular polyp (Fig. 2f). Finally, these polyps became lesions with an erosive surface (Fig. 2g), and their surface irregularity progressed (Fig. 2h). Fig. 3 summarizes the sequential findings of the endoscopic

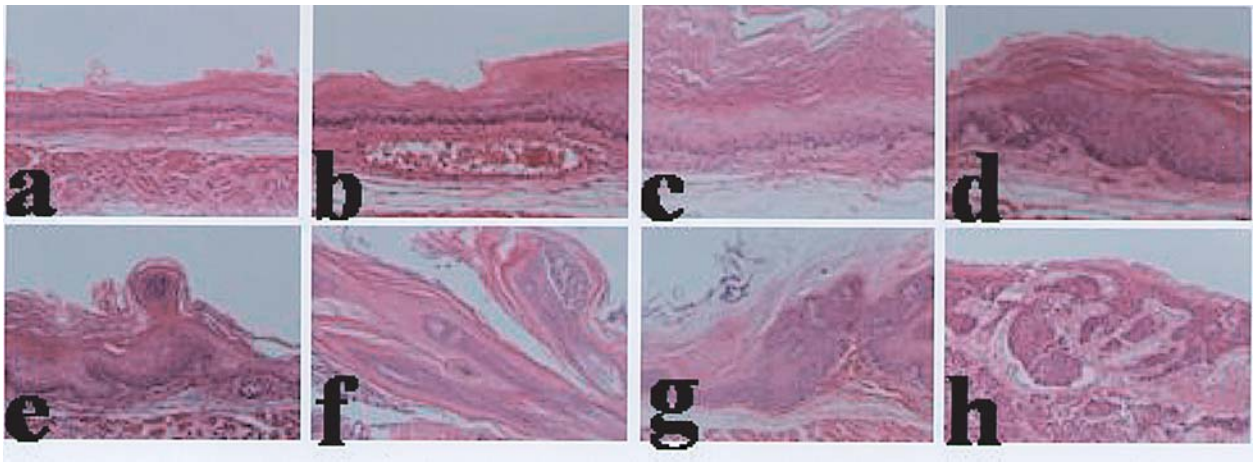

Figure 4. Representative histological findings of F344 rats treated with NMBA. (a) Normal esophagus. (b) Submucosal blood vessel dilatation. (c) Hyperkeratosis. (d) Acanthosis. (e) Papilloma without cellular atypia. (f) Dysplastic papilloma composed of atypical epithelial cells. (g) CIS (carcinoma *in situ*). (h) Invasive carcinoma. Note that the cancer cell nests are invading below the basement membrane.

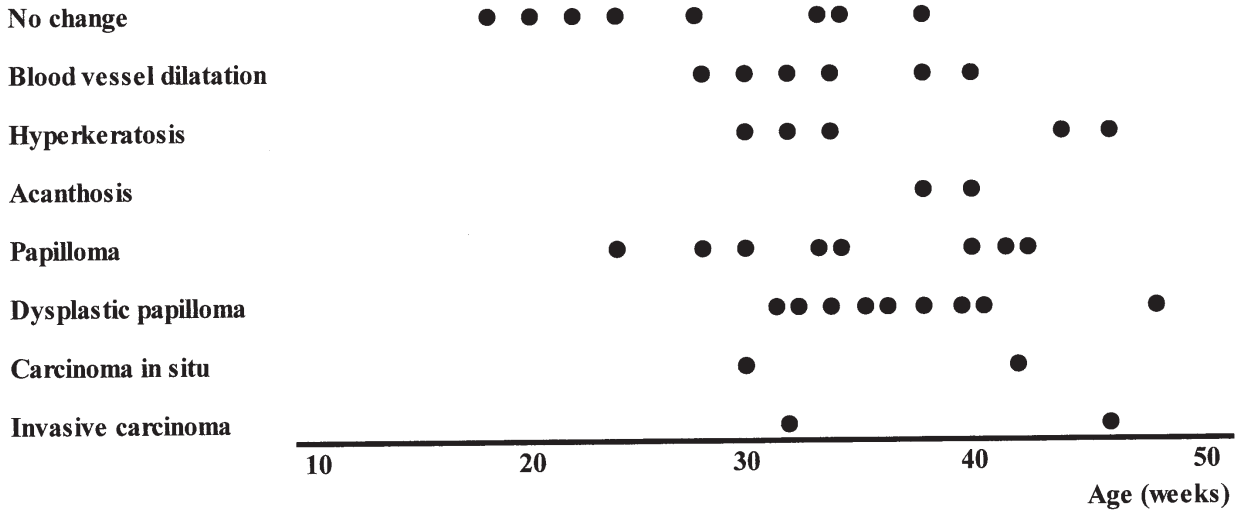

Figure 5. Summary of the sequential histological changes in F344 rats treated with NMBA.

examinations. No lesions were detected in any of the controls when they were examined at the end of the study (at 50 weeks of age).

In both the biopsy specimens and the autopsy sections, disease progressed with the passage of time. Therefore, the biopsy and autopsy results were combined. The representative histological findings are sequentially shown in Fig. 4. The histologically normal control mucosa is shown in Fig. 4a for comparison. The NMBA-induced changes began with submucosal blood vessel dilatation (Fig. 4b), followed by hyperkeratosis (Fig. 4c) and acanthosis (Fig. 4d). This progressed to papilloma without dysplasia (Fig. 4e), followed by papilloma with dysplastic potential (Fig. 4f). Finally, CIS appeared (Fig. 4g), followed by invasive squamous cell carcinoma (Fig. 4h). In all the controls sacrificed at the termination of the experiment, the entire esophagus was histologically normal. The histological changes, in relation to the time of sampling, are summarized in Fig. 5. In the NMBA-treated rats, mucosal epithelial cells tended to exhibit a time-dependent acquisition of the malignant potential.

Next, we compared the endoscopic findings with the histological changes (Fig. 6). Redness of the mucosa was due to submucosal blood vessel dilation with or without mild hyperkeratosis. Loss of the visible blood vessel network was due to hyperkeratosis with or without epithelial proliferative activity. Plaque formation was associated with acanthosis with or without epithelial projection. The formation of smooth and irregular polyps was associated with dysplastic papilloma. Rough elevation was associated with dysplastic polyps, CIS and/or invasive squamous cell carcinoma. The endoscopic findings were highly consistent with the histological findings.

Discussion

NMBA-induced rat esophageal cancer is a well-known model for the study of esophageal carcinogenesis. Varying the method of application of NMBA has produced marked differences in the incidence and multiplicity of esophageal cancer. Different durations of NMBA administration have produced different values of tumor incidence. The present

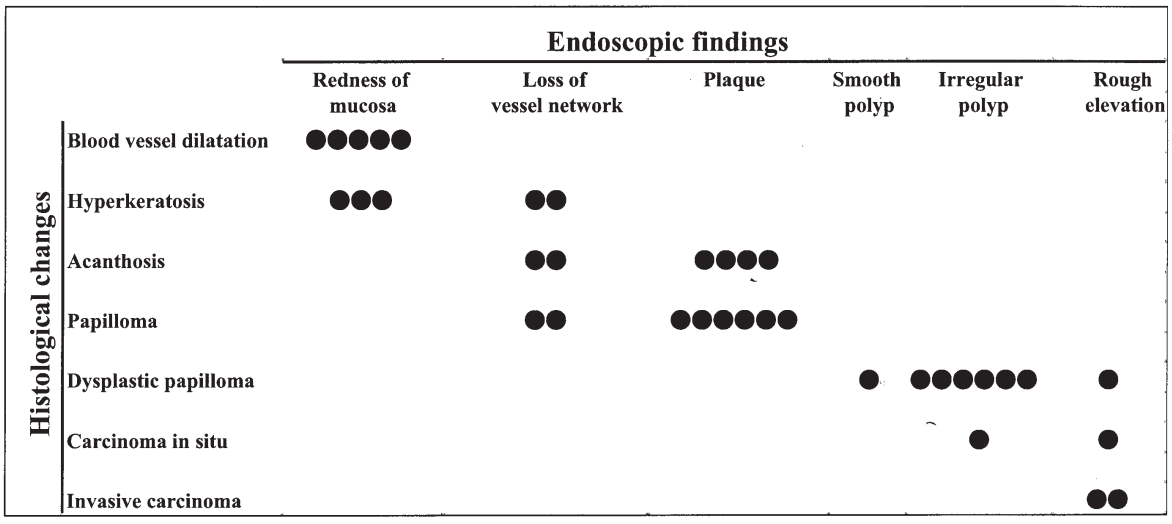

Figure 6. A comparison between the endoscopic findings and the histological changes.

weekly dose of NMBA was the same as in a previous study (9), but the present cumulative NMBA dosage was 3x as great as the previous dosage, and we therefore expected more tumors to form, which indeed was the case. In the present study, all detected tumors were located in the esophagus (i.e., no carcinoma was detected in the forestomach or oral cavity), and no remote metastasis was observed; sequential histological follow-up showed that the NMBA-induced esophageal carcinogenesis had a papilloma-carcinoma sequence. In the sequential biopsies of the lesions, a histological examination showed that the lesions gradually acquired malignancy.

Sasaki *et al* (17) investigated ENNG-induced esophageal tumors in dogs using a flexible fiberscope, and performed biopsies every 5 weeks; the endoscopic findings were highly consistent with the histological findings. However, endoscopic examination of small animals can involve technical difficulties. Hull *et al* (19) examined colon carcinogenesis in rats using a pediatric flexible bronchoscope. They performed a contrast barium enema, but they did not perform sequential endoscopic examination. Using a non-flexible miniarthroscope, Taylor *et al* (18) performed a sequential endoscopy and a biopsy of an intact rat stomach but not of gastric carcinogenesis. Fukawa *et al* (20) and Mori *et al* (21) sequentially investigated gastric ulcers in rats using a non-flexible side-view endoscope. However, there has been no reported time-course endoscopic study of rat esophageal carcinogenesis.

In the present study, we successfully conducted fiberscopic examinations and biopsies every other week using a flexible strait-view endoscope, and sequentially followed NMBA-induced esophageal carcinogenesis in rats. The endoscopic findings revealed the gradual progression of carcinogenesis. First, we observed a redness of the esophageal lumen. Subsequently, the superficial vessel network disappeared. Then, we observed the formation of plaques, which progressed to smooth polyps, irregular polyps, and rough elevation, in that order. A comparison of the endoscopic findings with the histology revealed the following relationships: The redness was due to submucosal blood vessel dilation. The loss of the visible blood vessel network was due to hyperkeratosis with or without epithelial proliferation. The plaque formation was due to acanthosis or papilloma without dysplasia. The formation of smooth and irregular polyps was associated with papilloma with dysplastic potential. The rough elevation was associated with the development of carcinoma.

The malignant potential of esophageal mucosa can be assessed immunophenotypically using several markers including keratins (22,23) and p63 (24,25). Immunophenotypic differentiation between the stages of esophageal carcinogenesis may more precisely correlate with the endoscopic findings than does histology. However, in the present study, there were clear relationships between the endoscopically classified lesions and the histologically categorized changes. Thus, with the present technique, we were able to monitor the time-course of esophageal carcinogenesis in rats, because the lesions preceding cancer could be clearly monitored by endoscopy.

In conclusion, using the present endoscopic criteria, the development of NMBA-induced esophageal carcinogenesis can be followed using minimally invasive procedures. We believe that this technique is useful for examining the esophagus, and that it can be used to plan the treatment of early esophageal cancer. Moreover, this technique can be used to monitor other sites in the intraluminal organs of rats. The investigation of cancer progression and treatment in small animals can provide information that is useful for the diagnosis and treatment of human carcinogenesis.

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