Diethylnitrosamine may induce esophageal dysplasia after local intramural administration

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Abstract

Purpose: To assess the possibility of promoting esophageal squamos cell carcinoma after direct administration of diethylnitrosamine (DEN) into the wall of the esophagus.

Material and methods: Adult male Wistar rats weighing 250-300 g were used in the studies. Via laparotomy, solution of DEN (at the volume of 0.1 ml) was injected directly into the esophageal wall. Animals were divided into 3 groups: CONTROL group – injected with saline, DEN1 group – injected with DEN 100 mg, DEN2 group – injected twice with DEN at the dose of 100 mg with 7 days interval (total dose of 200 mg).

Results: Microscopic evaluation after 180 days revealed signs of esophagitis in 20% and 30% subjects in DEN1 and DEN2 group respectively. In 30% of animals from DEN1 and 50% animals from DEN2 group, low-grade dysplasia was recognized. The difference between DEN2 and control animals was statistically significant with $p<0.03$. Neither high-grade dysplasia nor invasive carcinoma were found in both experimental groups. None of the liver specimens showed the evidence of pathology.

Conclusions: These initial results may indicate the possibility of development of premalignant lesions after local administration of carcinogen into esophageal wall. Observed changes were limited exclusively to esophagus which became the "target organ" in this model.

Key words: diethylnitrosamine, esophageal cancer, intramural administration.

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Introduction

In 1961 nitrosamines were first recognised as the most potent carcinogens responsible for the induction of esophageal squamous cell carcinoma (SCC) [1]. Since that time several experimental studies as well as researches on human subjects have confirmed the crucial role of nitrosamines in carcinogenic processes in the esophagus. Several nitrosamines, including N-nitrosomethylbenzylamine (NMBA), have been isolated and identified in the diets and gastric juice collected from subjects in regions of high incidence of esophageal SCC. The detection of O^6-methylguanine in the DNA of normal esophageal tissue taken from esophageal cancer patients further indicates to the role of methylating nitrosamines in the development of esophageal cancer [2,3]. Contaminated food often contains nitrates, nitrites and secondary and tertiary amines, which act as precursors for nitrosamine formation *in vivo*. Under acidic conditions *N*-nitroso compounds can easily be formed in the stomach by the reaction of nitrites and amines [4]. Diethylonitrosamine (DEN) is one of the most powerful nitrosamines for experimentally induced esophageal cancer [5]. Systemic administration: oral (p.o.), intraperitoneal (i.p.), subcutaneous (s.c.) of DEN can also result in tumorigenesis in the liver and tracheobronchial tree. Experimental procedures include long-term, repeated administration of low doses of carcinogen, and usually require prolonged observation until malignancy occur. The purpose of the present study was to assess the possibility of promoting esophageal SCC in rats after direct administration of DEN into the wall of the esophagus.

Material and methods

Animals and experimental conditions

The study was conducted in accordance with the Declaration of Helsinki and the Guiding Principles for the Care and Use of Laboratory Animals, approved by Lublin University Ethical Committee (approval 23/2000). Adult male Wistar rats weighing 250-300 g were used in the studies. Animals were

housed in standard, laboratory conditions, with an alternating 12-h light–dark cycle, and room temperature maintained at 22±1°C. All animals had free access to rodent chow and water. Groups of five animals were allocated to plastic cages covered with a metal grid and bedded with sawdust. The cages were cleaned every two days.

Experimental solution

Diethylonitrosamine (DEN) was supplied by Sigma Chemicals, St. Louis, MO, USA (cat. no. 0756, $C_4H_{10}N_2O$, density 950 mg/ml, molecular weight 102.1).

Surgical procedure

Before surgery, the animals were starved overnight and water was discontinued on the morning of surgery. Rats were anasthetized with an intraperitoneal injection of xylazine hydrochloride (12 mg/kg), and ketamine (75 mg/kg). Laparotomy was performed via short (2.5-3 cm) upper midline abdominal incision. Abdominal part of the esophagus was identified and isolated. Then solution of DEN (at the volume of 0.1 ml) was injected directly into the esophageal wall. Water was permitted when animals awoke and food was provided on the next day.

Experimental groups

A total of 30 animals were randomly divided into 3 experimental groups. According to the individual regimens, they were submitted to: group 1-10 animals injected with 0.1 ml of 0.9% NaCl solution (CONTROL), group 2-10 animals injected with DEN at the single dose of 100 mg into the esophageal wall (DEN1), group 3-10 animals injected twice with DEN at the dose of 100 mg with 7 days interval (total dose of 200 mg) (DEN2).

Collection of organs

180 days after the procedure animals were killed by ethyl ether inhalation in the glass chamber. Esophagus and stomach were dissected as a single block and fixed by the ends on the cork slide.

Macroscopic and microscopic analysis

The organ block was opened longitudinally on its anterior surface. Esophageal length (cm), and widest diameter (mm) were measured. The mucosa was stained with toluidine blue and assessed in order to reveal the presence of pathological lesions. For histopathological analysis the organs were fixed in 10% buffered formalin, sectioned transversely into about 2 mm-wide serial slices (9-12/case), processed routinely and embedded in paraffin blocks. Then, 5 μm sections were stained with hematoxylin and eosin (H&E) and examined in a blinded fashion by the pathologist. Examination included identification of the following findings: normal histology, esophagitis, low-grade dysplasia, high-grade dysplasia, invasive carcinoma. All histopathological changes were classified according to the reports of Robbins and Cotran [6] and Kruel et al. [7]. Normal esophagus: one wing of basal cells located between the border of the epithelium and submucosa, with egg-shaped or round nuclei; a corneal tract that occupies one-third to one-half of the epithelium; muscularis mucosae not defined; some rudimentary cells present; lamina, if present, usually contains few and sparse mononuclear inflammatory cells. Esophagitis: the epithelial papillae project one-third to two-thirds of the epithelial thickness and/or inflammatory infiltrate in the lower third of the epithelial thickness. Low-grade dysplasia: alterations of basal cell layers to one-third of the epithelial thickness, with pleomorphic nuclei; mitotic nuclei visible in lower third of the epithelium, outside the basal layer. High-grade dysplasia: dysplastic alterations occur throughout the epithelial thickness which is composed of markedly abnormal cells; nuclear polarity is lost; cytoplasmatic maturation occurs in most parts of the epithelial layer. Invasive carcinoma: submucosal invasion by squamous neoplastic epithelium, containing cells exhibiting a markedly increased nucleus to cytoplasm ratio and with keratinized cytoplasm, and large, grossly granulated and hyperchromatic nuclei, irregularly distributed; malignant cells are encountered below the lamina, with unquestionable stromal invasion.

Statistical analysis

All quantitative variables were compared with analysis of variance. Fisher's exact probability test was applied to assess qualitative variables. P values lower than 0.05 were considered as statistically significant.

Results

Macroscopic analysis

In the group of 30 animals included in the study, no losses were noted within the observation period. No significant differences in the esophageal lenght and widest diameter between all groups were noted. In 1 subject from control group, 1 from DEN 1, and 3 from DEN 2 group macroscopic signs of esophagitis (erythema, mucosal breaks) were observed. The differences in the prevalence were not statistically significant. In both experimental groups grossly visible esophageal tumors were not observed in the area injected with DEN (*Tab. 1*).

Microscopic analysis

In control group histopathological examination revealed normal esophageal wall (*Fig. 1*) in all subjects exept one in which mild esophagitis was found. Similary, signs of esophagitis were found in 2 (20%) and 3 (30%) subjects in DEN1 and DEN2 group respectively. In 3 (30%) animals from DEN1 and 5 (50%) animals from DEN2 group, low-grade dysplasia was recognized (*Fig. 2*). The difference between DEN2 and control animals was statistically significant with $p<0.03$. Neither high-grade dysplasia nor invasive carcinoma were found in both experimental groups (*Tab. 2*). None of the liver specimens showed the evidence of pathology.

Discussion

Nitrosamines as promotors of carcinogenesis have been investigated for almost 6-7 last decades. Their possible mechanism of action in inducing cancer development is DNA alkylation, as has been shown by Harris et al. in human tissues [8]. Most of the nitrosamines administered systemically

Table 1. Macroscopic analysis.

	CONTROL	DEN1	DEN2	P (ANOVA)
Esophageal length (cm)	7.5±0.17	7.63±0.21	7.63±0.23	n.s.
Widest diameter (mm)	5.5±0.84	6.3±0.94	5.6±0.84	n.s.
	CONTROL	DEN 1	DEN 2	P (Fisher's Exact Test)
Inflammation (%)	10	10	30	n.s.
Tumors (%)	0	0	0	n.s.

Table 2. Microscopic analysis.

	CONTROL	DEN1	DEN2	P (Fisher's exact test)
				Control vs. DEN1 n.s.
Normal (%)	90	50	20	Control vs. DEN2 $p < 0.0055$
				DEN1 vs. DEN2 n.s.
Esophagitis (%)	10	20	30	n.s.
				Control vs. DEN1 n.s.
Low-grade dysplasia (%)	0	30	50	Control vs. DEN2 $p<0.03$
				DEN1 vs. DEN2 n.s.
High-grade dysplasia (%)	0	0	0	n.s.
Invasive carcinoma (%)	0	0	0	n.s.

Figure 1. Normal esophageal mucosa and submucosa in rat from control group (H&E, x200).

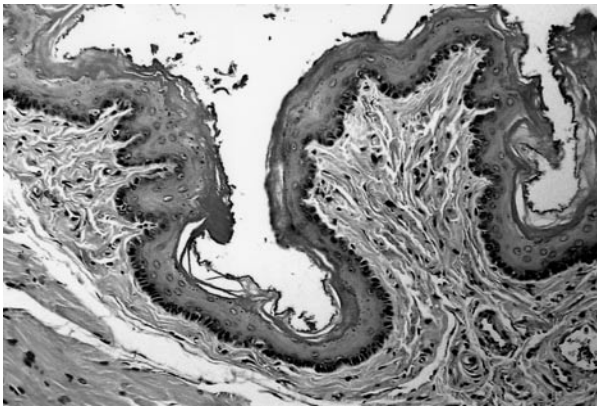
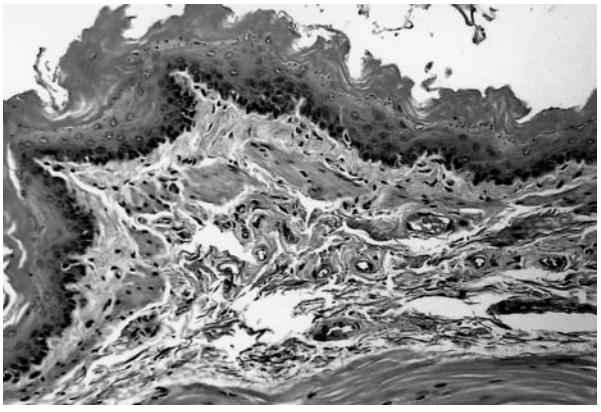


Figure 2. Esophageal squamous epithelial low-grade dysplasia in rat from DEN1 group (H&E, x200).



(p.o., s.c., i.p.) present specifity to particular organs e.g. liver, kidney lungs [9,10]. Thus, different nitrosamines are employed as the promotors of cancer in many, experimental models of carcinogenesis in several organs. DEN (compound used in the present study) produces esophageal and kidney tumors in rats in addition to tumors in the main target organ, the liver [11]. It is possbile that extrahepatic tumors need higher exposures to DEN, probably because low doses are subject to first pass clearance in the liver [12]. Most experimental procedures aiming the induction of esophageal cancer usually require administration of diluted carcinogen at low concentrations for long time to avoid or minimize the adverse tumors in other organs. Long-lasting observation periods of the experiments as well as their unrepeatable, inconsistent results make all those procedures complicated and unusable. Thus, the purpose of the present study was to assess the possibility of promoting esophageal SCC by delivering the specific, carcinogen directly to the esophageal wall. Such manner of administration would allow to obtain the direct action of the carcinogen on tissue of the esophagus, and to eliminate the adverse influence in other organs. Moreover, it would help to resolve the problem of poor diffusion (flux) of DEN into the esophagus that has been noted in Haorah research study [13]. DEN doses (100 mg and 200 mg in DEN1 and DEN2 group respectively) were established, as promoting the carinogenesis, basing on previous literature data. Given weekly i.p. as injections of 80 mg/kg for 10 weeks (total dose of 800 mg/kg), the incidence of esophageal tumors in BD6 rats was 35% [14]. Total dose of DEN per animal which has been administered in the present study in DEN2 group appeared to be equivalent. However, conditions and methods established in this study did not allow to induce either invasive carcinoma or even high-grade dysplasia, the incidence of low-grade dysplasia in esophageal specimens from DEN2 group reached 50%. These initial results may indicate the possibility of development of premalignant lesions after local administration of carcinogen into the esophagus. No pathological changes were noted in

liver. Observed changes were limited exclusively to esophagus which became the "target organ" in this model. Potential significance of these results seems to be very interesting in relation to future research. Strong esophageal cancer promotor e.g. DEN or NMBA, given directly into the esophagus, under another experimental conditions e.g. co-administered with ethanol, could result in esophageal tumors development with no other lesions. Obtaining the "pure" esophageal cancer model would allow to understand more about biology of this disease and investigate new methods of treatment or chemoprevention.

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