Review article

Inducement of canine gastric cancer by N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)

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Abstract:

Achievements in the experimental inducement of canine gastric cancer in Japan are reviewed. Canine gastric cancer was initially induced experimentally by the administration of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in drinking water. However, intestinal sarcoma caused death before the gastric cancer was detected. By giving an N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)-soaked diet to dogs, we specifically induced gastric cancer and followed the process of carcinogenesis by X-ray, endoscopy, and biopsy. The initial macroscopic findings were areas of discoloration, with erosion or mucosal unevenness which became visible from approximately the 9th to 10th experimental months in mongrel dogs and around the 13th to 17th months in beagle dogs. The protruded type of cancer occurred in mongrel dogs early in the experimental period, while the less protruded but more ulcerated gastric cancer occurred in beagle dogs later. Histological examination of the autopsied stomachs 277, 395, 546 and 813 day in mongrel dogs and 804, 946, and 1591 day in beagle dogs revealed well differentiated, poorly differentiated, and undifferentiated adenocarcinoma. In animals with a lengthy survival (more than 600 days from the start of the experiment), metastases to lymph nodes, and, rarely, to liver, lung, bones, and other organs, were observed, as well as peritonitis carcinomatosa. Heterotransplantation of gastric cancer to nude mice succeeded to the fifth passage. Development of scirrhous cancer was induced in dogs by the combined use of ENNG and subcutaneous injection of gastrin, indicating that the experimentally induced canine gastric cancer resembled human gastric cancer. Although the induction of canine gastric cancer takes 2–3 years and the macroscopic type and time period differs among individual dogs, our results in the experimental induction of canine gastric cancer should be useful in various areas of research that require an animal model, such

as genetic alteration during carcinogenesis, modulation of carcinogenesis, and prevention of gastric cancer.

Key words: dog, gastric cancer, experiment, ENNG

Introduction

One of the central achievements in gastric cancer research in the past 30 years has been the experimental induction of gastric cancer. For some time, the experimental induction of gastric cancer was considered to be a task of extreme difficulty, as the gastric mucosa is covered by mucus which would prevent the action of orally administered carcinogens [1]. However, Sugimura and Fujimura [2] and Sugimura et al. [3] induced adenocarcinoma in the glandular stomach of rats (in 1967) with a solution of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). MNNG is a potent mutagen, as shown in microbiology, and a carcinogen, as reported by Druckrey et al. [4], Schoental [5], and Sugimura et al. [6] in 1966. The induction of gastric cancer by MNNG has been confirmed by many researchers and has resulted in many new findings in relation to gastric carcinogenesis, such as the establishment of a cell culture line of rat gastric cancer [7], determination of a lower incidence of gastric cancer in female and castrated rats than in intact male rats [8] and identification of the inhibitory effect of calcium chloride on gastric carcinogenesis [9]. Gastric cancer of the glandular stomach (induced by Sugimura et al.) in living rats was detected on radiographs by our group [10]. In 1971, Sugimura et al. [11] and Shimosato et al. [12] succeeded in inducing gastric cancer in dogs. In 1973, we reported well advanced cancer formation in the stomach of dogs, without lesions in other regions of the gastrointestinal tract, induced by giving them food soaked with N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG) and 2% Tween

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60 [13]. As gastric cancer growth can be followed in the dog by X-ray, endoscopy, and biopsy [14], follow-up studies of experimental gastric cancer rapidly increased. One of two subjects at the 37th Meeting of the Japanese Research Society for Gastric Cancer (organized by O. Abe, Keio University, 1982) was "Experimental gastric cancer."

In this article, developments in the induction of canine gastric cancer by the oral administration of nitroso-guanidines (NGs), in particular, ENNG, are reviewed.

Carcinogen and administration method

MNNG is stable in neutral aqueous solution [15]. In alkaline conditions, as in the duodenum, MNNG is degraded, yielding azoxymethane; once MNNG is absorbed into tissue, it is quickly converted to N-methyl-N'-nitrosoguanidine (MNG) [15]. MNG is a fairly inert compound, and is neither mutagenic nor carcinogenic. As MNNG administration to dogs in drinking water provided ad libitum produced large intestinal sarcomatous lesions before gastric cancer was induced [12], our group used a less reactive carcinogen, ENNG [16]. To promote stronger and longer-lasting contact of the carcinogen with the mucosa, the enforced ingestion of an ENNG-soaked diet was used. Our experimental results, indicate highly concentrated ENNG can be retained in the canine stomach.

Initial methods and results

In our first experiment, using four mongrel dogs (three males and one female), 250 ml of solution containing 150 µg/ml of ENNG was mixed with a pellet diet and given to the animals twice daily for 8 months, except for Sundays. The average total dose of ENNG for the three male dogs was 14.2 g. Because the body weight of the female dog was 40% that of the male dogs, 120 ml of the ENNG solution was administered twice daily, and the total dose was 7.6 g. Adenocarcinoma induction was confirmed by biopsy on days 176, 213, and 317 in three dogs, and intramucosal cancer in the fourth dog was detected by microscopic examination of autopsied material on the 277th experimental day. Advanced cancer was found in three dogs on experimental days 395, 546, and 813, respectively [14].

In our second experiment, using the same method, but with four beagles, the average ENNG intake was 13.4 g [17]. One dog died of anesthetic-related causes at endoscopic examination on the 330th day. Gastric cancer was revealed in the other three dogs on the 399th, 414th, and 504th days, respectively, by gastric biopsy. Advanced gastric cancer with lymph node metastases was noted on the 804th, 946th, and 1591th days, respec-

tively. Other metastases in two of the beagles are described later.

Change in ENNG concentration and duration of ENNG feeding

When we changed the ENNG concentration to 200 µg/ml, with all other experimental conditions the same as for the experiments outlined above, esophageal cancer was induced in four beagles about 1 year before the induction of gastric cancer [18]. Esophageal cancer in dogs induced by modified methods of ENNG administration was also reported by other investigators [19–21].

Sunagawa et al. [22] investigated the effects of the duration of ENNG administration on histological tumor differentiation and found that a small dose of ENNG (3 months; total, 5.85 g) produced only undifferentiated adenocarcinomas in beagles, while differentiated adenocarcinomas developed with high doses (6 months; 11.7 g, and 9 months; 17.6 g). However, Sano et al. [23] reported that a small dose (4 months; 7.65 g) resulted in the development of differentiated adenocarcinomas in 7 of the 21 dogs and that antral conditions, antral pH, the number of G-cells, achlorhydria, and hypergastrin, were significantly associated with the development of differentiated adenocarcinomas.

Animals

In both mongrel dogs [11,24] and beagle dogs [25,26] experimental gastric cancer was first induced by MNNG in the late 1970s. Beagles, which are commonly used in pharmacological studies, were most frequently used in canine gastric cancer experiments throughout Japan. Since that time, our group succeeded in inducing gastric cancers in four mongrel dogs within a year with ENNG [14]; these animals were considered as spitz hybrids. After the success in inducing gastric cancer with ENNG in four beagles [17], we therefore used four pedigreed spitzes. However, gastric cancer was not induced by ENNG in these spitzes. Xiao et al. [27] succeeded in producing gastric cancers in three wolf-dogs by our method.

In three cynomolgus monkeys given ENNG for 10 months, intramucosal gastric cancer was found in the 109th experimental month at autopsy [28,29]; however gastric cancer was not induced in two rhesus monkeys given MNNG for 10 months [29].

We applied our method, using ENNG (8 mg/kg) for 15–17 months, to six miniature pigs and followed gastric tumor induction by X-ray and endoscopy from 3 years to 6 years and 10 months [30] after the beginning of the experiment (joint research project with the late

Fig. 1. Autopsied stomach of a miniature pig (no. 31) 3 years and 3 months after the beginning of the experiment using N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG) (8mg/kg) given for 15–17 months. There are multiple polypoid lesions of various sizes; histologically, these are predominantly hyperplastic polyps, with a few adenomas or adenocarcinomas limited to the intramucosa. On microscopic examination, minute gastric cancer foci (well differentiated adenocarcinoma on top of a polypoid lesion at the ventral wall of the fornix (Fig. 2) and well differentiated and two areas of poorly differentiated adenocarcinoma in the flat mucosa at the dorsal wall of the fornix (Fig. 3)

H. Satoh et al. Sasaki Institute). Multiple prominent polypoid tumors were induced in five pigs except for a pig which died of anesthesia on days 459 and carcinomas were strongly suggested by biopsy on experimental days 618, 632, 632, 985, and 1044 in these five animals. At 3 years and 3 months, four extremely minute intramucosal cancer foci were found in one animal. Well differentiated adenocarcinoma in the anterior wall of the fornix, well differentiated adenocarcinoma in the dorsal wall of the fornix and two areas of poorly differentiated adenocarcinoma in the dorsal wall of the fornix were revealed under microscopic examination (Figs. 1–3) [31]. Similar findings in the other four pigs were detected at autopsy 4–6 years from the start of the

Fig. 2. Microphotograph of well differentiated adenocarcinoma on top of a polypoid lesion at the ventral wall of the fornix (miniature pig no. 31). H&E, ×400

Fig. 3. Microphotograph of poorly differentiated adenocarcinoma in the submucosa of flat mucosa between polypoid lesions at the dorsal wall of the fornix (miniature pig no. 31). H&E, ×200

experiment. It was concluded that monkeys and miniature pigs were more resistant to nitrosoguanidines than rats and dogs.

Follow-up studies of cancer production by X-ray, endoscopy and biopsy

Because of the similarity of size between the dog and the human stomach, we were able to carry out long-term observation of the growth of the experimental gastric cancers by double-contrast barium meal and endoscopic biopsy without killing the dogs [32]. Four to 8 weeks after the beginning of ENNG administration,

all mongrel and beagle dogs (thirty eight animals) showed diffuse hyperemia over the antrum and stomach body. Biopsied material taken from hyperemic mucosa revealed simple hyperplasia of foveolar cell glands. Eight months after the beginning of ENNG administration the mucosal hyperemia gradually faded and the gastric mucosa showed signs of atrophy, with little inflammation. Microscopic examination of the biopsied material at this stage confirmed that the mucosal atrophy was often accompanied by derangement of glandular tissue, mild pleomorphism, and cellular disorganization. Thereafter, mucosal atrophic changes were much more pronounced. Macroscopically, an area of discolouration with erosion or mucosal unevenness was endoscopically visible by about the 9th to 10th experimental months in mongrel dogs and around the 13th to 17th months in beagle dogs [32]. The histological diagnosis of biopsied specimens from these areas was either well or poorly differentiated adenocarcinoma. The protruded type of cancer occurred in mongrel dogs at an early stage (14 months), while the less protruded but more ulcerated gastric cancer occurred in beagle dogs later in the experimental period (27 months). Healing and relapse of an ulcer inside the cancer focus, similar to the malignant cycle of cancer in the human stomach (Murakami 1968) [48], was observed in one beagle [32]. Similar follow-up studies were performed by Fujita et al. [24], Sunagawa et al. [50], Saito et al. [33], Noguchi et al. [34], and Xiao et al. [27]. No definitive intestinalization of the gastric mucosa was detected in this series of canine experiments.

Two important factors revealed that the experimentally induced tumors were truly malignant: (1) formation of metastases and (2) successful transplantation.

Formation of metastases

In our five experiments, gastric cancers were confirmed in 33 of in our total 46 dogs (72%) over 11 years [35]. Three early deaths (accidents at endoscopy in two beagles and a filarial disease in a mongrel dog) were experienced, and 5 dogs were lost to follow-up 6 months from the beginning of the experiment because of my change of university.

Metastases to lymph nodes and 1 or to other organs were observed in 9 of 33 autopsied dogs with confirmed gastric cancer (Table 1). Metastases to distant as well as regional lymph nodes were found in 6 dogs. The lymphatic spread went beyond regional nodes to the tracheo-bronchial, cervical, mandibular, and axilla nodes, with the lesions varying in size. Metastases to the liver were recognized in 3 dogs. Peritonitis carcinomatosa with ascites occurred in 3 dogs [35]. In dog no. 8, Borrmann type III gastric cancer (Figs. 4, 5), metastases to the liver (Fig. 6), lungs, bones, subcutaneous tissues, and peritonitis carcinomatosa (Fig. 7) were found [17]. A microphotograph of the liver metastase is in beagle dog no. 7 is shown in Fig. 8. Metastases of canine experimental cancer to the regional lymph nodes were reported by Sasajima et al. [20], using ENNG and Saito et al. [36], using MNNG to the liver by Sasajima et al. [20], using ENNG, and to the lung by Fujita et al. [37], using MNNG.

Malignant hemangioendothelioma in the stomach, with metastases to liver, pancreas, diaphragm, and omentum were also recognized in our canine experiment [35]. Intestinal sarcoma produced in an MNNG experiment frequently metastasized to the liver and/or the peritoneum [12].

Fig. 4. Autopsied stomach of a beagle dog (no. 8) 1591 days from the beginning of the experiment in which ENNG-soaked food was given for 8 months at a concentration of 150 μ g/ml. Deeply ulcerated carcinoma of the subcardia (Borrmann type III) and two polypoid lesions of the antrum are seen (arrows "a, b"). The carcinomatous invasion was widely spread from the cardia to the duodenal bulb from the submucosa to the subserosa. The large polypoid tumor (a) with umbilical ulceration consisted of leiomyoma and adenocarcinoma. The small tumor (b) was poorly differentiated adenocarcinoma that invaded the submucosa

Table 1. Metastatic cases of canine gastric cancers induced by ENNG

Dog no.	Experimental period (days) from day 1 of administration of ENNG	Administration period (months)	Location of tumors	Macroscopic type	Histologic type ^b	Metastasis	
						Lymph node	Organs
4	813	8	C ^a (Fornix) Fornix A (Antrum) Antrum	Borrmann I IIa + IIc Borrmann I Borrmann I	Tub Tub Tub-por Tub	(+)	
6	804	8	C (Corpus) A (Antrum)	Borrmann I IIb	Por Sig	(+)	
7	946	8	M (Angle) Angle A (Antrum) Antrum	Borrmann III Borrmann I Borrmann IIa Borrmann IIb	Tub Ud Tub Sig	(+)	Liver
8	1591	8	C (Corpus) A (Antrum)	Borrmann III Borrmann IIa	Por Por	(+)	Liver, lung, heart, bone, subcutaneous, peritonitis carcinomatosa
19	844	12	C (Corpus) M (Angle)	Borrmann I Borrmann IIa	Tub-sig Tub	(+)	
23	665	12	C (Corpus) M (Antrum)	Borrmann IIa Borrmann II	Tub Tub	(+)	Peritonitis carcinomatosa
39	1255	8	M (Angle) A (Antrum) A (Antrum)	Borrmann II Borrmann IIa Borrmann IIa	Tub Tub Por	(-)	Peritonitis carcinomatosa
47	688	10	A (Antrum) Antrum	Borrmann II Borrmann IIa	Tub Tub	(-)	Liver
48	1668	10	C (Fornix) A (Antrum)	Multiple polypoid lesions	Malignant hemangio-endothelioma	(-)	Liver, diaphragma, omentum, spleen

ENNG, N-ethyl-N'-nitro-N-nitrosoguanidine

^a According to the Japanese classification of gastric carcinoma [44], three portions of the stomach are denoted: C, Upper third; M, middle third; A, Lower third^b Histological Type: tub, tubular adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet-ring cell carcinoma; ud, undifferentiated carcinoma **Fig. 5.** Microphotograph of the Borrmann type III in beagle dog no. 8. Carcinoma cells continuously fill the lymphatic vessels in the muscularis mucosa. H&E, ×20

The relation between the macroscopic and histological types of 23 cancerous lesions in 8 dogs with metastases is shown in Table 2. Macroscopic and histological findings that were similar to those in human gastric cancers were confirmed (e.g.; 49). Other investigators have also reported that the histological type of canine gastric cancers resembled that of human gastric cancers [20,36,38].

Transplantation of canine gastric cancer

Heterotransplantation of the gastric cancer induced in dogs by ENNG was attempted to two nude mice. Biopsied materials from Borrmann type III cancer in a beagle, which showed signet-ring cell carcinoma in the subcardiac region, were inoculated into the muscle of the hind leg of BALB/c-nu/nu nude mice. Serial transmission was obtained in two mice, and thus far there

Fig. 6. Macroscopic appearance of the liver in beagle dog no. 8. Two white round metastatic nodules were found (arrows)

Fig. 7. Signet-ring cells in peritonitis carcinomatosa ascites in beagle dog no. 8. Papanicolaou, ×40

Table 2. Relation between macroscopic location and histologic type of 23 cancerous lesions in eight dogs with experimental gastric cancers induced by ENNG, with metastases

	Tub	Por	Sig	Ud
C	○○○○ ○○	○○ □□		
M	□□ ○			○
A	○ ○○ □	○ ○○	△ □	

Circles, Protuded type; squares, depressed type; triangles, flat type; large symbols, >10mm; small symbols, <10mm

C, M, and A and tub, por, sig, ud (see Table 1) some animals had more than one type and location of metastases

Fig. 8. Microphotograph of liver metastasis in beagle dog no. 7. H&E, ×10

have been four to five passages [39]. Histological findings of the final passage, showed that most of the specimen was occupied by poorly differentiated adenocarcinoma; this was similar to human adenosquamous cell carcinoma (joint research project with the late K. Suzuki (et al.) Institute of Medical Science, University of Tokyo). Taguchi et al. [40] also reported transplantation of canine gastric cancer to nude mice.

Development of scirrhous cancer

With the combined use of ENNG and subcutaneous injection of gastrin [e.g. 500ml of 150μg/ml of ENNG solution per day for 8 months and further administration of ENNG was carried out at a concentration of 200μg/ml for 2 and half months after a month interval. Gastrin was administered at a dose of 5μg/kg twice a week for 4 months from the 5th experimental month and at a dose of 10μg/kg daily for the following 2 months], scirrhous carcinoma was induced in three of ten beagles. They were found to have annularly infiltrating advanced carcinoma with marked fibrous thickening of the antral wall, resulting in stenosis of the antrum (carcinoma scirrhosum), which resembled Borrmann's type IV carcinoma in the human stomach (Figs. 9, 10) [41,42]. However, the linitis plastica type of scirrhous cancer which is seen in the human stomach was not encountered in our experiment.

Therapeutic trials in canine gastric cancer and future expectations

To confirm on an experimental basis that the oral administration of anticancer agents has an effect on gastric

Fig. 9. Autopsied stomach of beagle dog no. 15 on the 610th experimental day of the combined use of ENNG and gastrin. There is scirrhous carcinoma in the pyloric antrum with marked thickening of the antral wall and narrowing of the pyloric antrum

Fig. 10. Histological section of the gastric antrum with scirrhous carcinoma, showing marked fibrous thickening of submucosa and invasion to the muscle layer (beagle dog no. 15). Azan-Mallory, $\times 10$

cancer, ^{14}C -5-fluorouracil (FU) was orally and intravenously administered to dogs with experimental gastric cancer [42].

In this study, the 5-FU level was higher in cancer tissues than in normal tissues, and the 5-FU level achieved through oral administration was higher than that achieved with intravenous administration. However, the therapeutic trials with 5-FU were not successful in the dogs with gastric cancer, because the animals could not tolerate even low doses of 5-FU. The therapeutic effects and dynamics of Tegafur Unacid (UFT) were studied using beagles with ENNG-induced gastric cancer [43]. One of ten beagles given 12 mg/kg per day of UFT for 45 days showed a Grade II effect according to the criteria of histopathological effects on cancer tissues in the Japanese classification of gastric carcinoma [44]. No effect was seen in the other nine dogs [43,45]. A similar study of canine experimental cancer treated with 1-N-hexyl-carbamoyl-5-fluorouracil (HCFU) was reported by Matsukura et al. [46], with similar results.

Conclusion

The induction of canine gastric cancer takes a long time and the time taken to induce various macroscopic types of cancer differs among individual dogs. In addition, it is necessary to house each dog in a fairly large metal cage. Therefore, recently, few experiments have been done to induce gastric cancer in dogs. However, as Sugimura [47] has pointed out "Studies on genetic alterations, especially during carcinogenic process, could be re-

searched on animal models as follow-up studies. Modulation of carcinogenesis using animal models could be especially valuable for studies on prevention" [47]. For such reasons, the results of canine experimental gastric cancer described above should be useful for new research in the future.

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