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Elevated Serum Growth Hormone Accelerates Gastric Tumorigenesis in F344 Rats after Treatment with *N*-Methyl-*N*-nitrosourea in Their Drinking Water

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We examined the effects of growth hormone on tumorigenesis in F344 rats treated with *N*-methyl-*N*-nitrosourea (MNU). Four-week-old male F344 rats were exposed to 100 ppm MNU in their drinking water for 15 weeks. Thereafter Group II animals received 100 μCi/100 g body weight of ^{131}I (radiothyroidectomy, Tx) injected i.p. and Group III rats were implanted with pituitary tumors (MtT) secreting growth hormone while Group I received no further treatment after MNU. Non-carcinogen control animals received MtT, Tx or no treatment. Animals were killed at 39 weeks after starting MNU administration. Gastric tumors were present in 13 of 31 (43%), 15 of 32 (47%) and 17 of 32 (53%) rats in Groups I to III, respectively. All tumors were of well-differentiated type. Spinal cord tumors appeared in 15 of 31 (47%) in Group I, 10 of 32 (32%) in Group II and 10 of 32 (32%) in Group III, most being malignant schwannomas. Thymic lymphomas also appeared in 10 of 31 (32%), 5 of 32 (16%) and 6 of 32 (19%) animals in Groups I to III, respectively. There were no significant differences among the groups. However, tumors in Group III developed significantly earlier than in Groups I or II. This was mainly due to gastric tumors, and cumulative incidence curves for spinal cord tumors or thymic lymphomas were similar in all groups. The results indicate that gastric tumors induced by MNU in F344 male rats are influenced by elevated levels of growth hormone.

Key words: MNU — Tumorigenesis — Rat — Growth hormone — Gastric tumor

N-Methyl-*N*-nitrosourea (MNU) is well known as a potent mutagen and direct-acting carcinogen producing tumors in several species in a variety of organs, including the central nervous system, intestine, kidney, stomach, mammary gland and skin.[1–10] Hirota *et al.*[11] reported that MNU in the drinking water selectively induces glandular stomach carcinomas at a high incidence in F344 rats. This was confirmed by Fujita *et al.*[12] and the MNU-F344 animal model has become established for investigations of gastric carcinogenesis. Recently, Shibutani *et al.*[13] reported induction of anaplastic astrocytomas and glioblastomas in adult F344 rats given MNU in their drinking water.

Growth hormone (GH) acts to increase both protein synthesis and blood sugar levels, thereby promoting growth.[14] Many authors have indicated that it may exert promoting effects on carcinogenesis due to stimulation of cell proliferation.[15–19] Accelerated growth of osteogenic sarcoma was found in patients with elevated levels of GH.[20] Female transgenic mice bearing human GH gene developed mammary carcinomas at 27–43 weeks.[21] The effect of GH on tumor growth is unknown. The present investigation was performed to determine whether GH can act as a tumor promoter in the male F344 rat MNU tumorigenesis model, when given subsequent to carcinogen withdrawal.

MATERIALS AND METHODS

Animals One hundred and fifty-one male F344/DuCrj rats (Charles River Japan Co., Ltd., Hino), four weeks old at commencement, were used in the present study. They were housed four or five to a polycarbonate cage and kept under constant conditions of temperature (24±2°C) and relative humidity (55±10%), with a 12 h light/12 h dark cycle. The animals were maintained under the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" established by Hiroshima University.

They were fed commercial diet MF (Oriental Yeast Co., Ltd., Tokyo) and were provided with normal tap water *ad libitum* except during the MNU treatment. The animals were maintained for 39 weeks from the beginning of the MNU treatment.

Chemicals MNU was purchased from Sigma Chemical Co., St Louis and dissolved in distilled water at a concentration of 100 ppm. Rats were given this solution in light-opaque bottles as their drinking water for 15 weeks. The MNU solution was changed at 3- to 4-day intervals.

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Inoculation of MtT cells Pituitary tumor (MtT) cells which secreted only GH were established from MtT/84 by Inoue *et al.*[22] When the cultured cells reached a density of about 5×10^7 per 10 cm dish, the cells were harvested with 0.02% trypsin and counted with a hemocytometer. Cells in suspension (10^6 cells) were mixed with an equal volume of 50% brain homogenate (brains were obtained from syngeneic adult rats and homogenized in DEM/F2 mixed medium) and the mixture was inoculated into the inguinal fat pads at a volume of 0.06 ml/site.[23, 24] Three months after the cell inoculation, tumor tissue was removed and the new cells were introduced into other inguinal fat pad sites.

GH levels The radioimmunoassay for GH was carried out with NIDDK (National Institute of Diabetes and Digestive and Kidney Disease, Bethesda) reagents, in accordance with recommended methods. Iodination was performed by the lactoperoxidase method ($Na^{125}I$, Amersham). The second antibody, anti-rabbit immunoglobulin, was a gift from the Institute of Endocrinology, Gunma University, Takasaki.

Radiothyroidectomy (Tx) Animals were maintained on a low iodine diet and distilled water for 2 weeks before receiving ^{131}I at 100 μCi/100 g body weight, injected i.p. The animsls were thereafter maintained in the radiation facility.

Experimental design Animals were divided into 6 groups. In Group I, MNU was given alone. In Group II, animals were thyroidectomized with ^{131}I after the MNU treatment. In Group III, 10^6 MtT cells were implanted into inguinal fat pads after MNU. Animals of Groups IV to VI received the same treatment as those in Groups I to III, respectively, except MNU. All had free access to food and water throughout.

Examination of animals All animals were regularly observed and killed when paralysis appeared in one or two legs or at the termination of the experiment 39 weeks after the start of MNU treatment. At the time of necropsy, serum samples were collected from sub-groups to determine GH values. The body, liver and other major organs were weighed and prepared for histopathologic studies. Scars were opened and animals were observed for brain and pituitary lesions. The backbone were placed in fixative for more than 24 h and the spinal cord was opened. Each stomach was cut open along the greater curvature, stretched out, pinned on a board with the mucosal surface facing upward and washed several times with physiological saline before gross examination and fixation in 10% neutral formalin. Strips of stomach were cut perpendicularly to the mucosal surface, two strips being taken at the lesser curvature and four at the greater curvature. The strips were embedded in paraffin and serially sectioned at 3 μm. Sections from all tissues were routinely stained with hematoxylin and eosin, and with alcian-blue (AB)-periodic acid-Schiff (PAS). Additional sections were also stained for mucin with high-iron diamine(HID)-AB.

S-100 protein antibody was used to identify glial cells, sections being sequentially incubated with a biotin-labeled secondary antibody and alkaline phosphatase-conjugated streptavidin complex using new fuchsin as the chromogenic substrate (Dako Labs kit alkaline phosphatase system 40, K0628, Dako Co., Corpintia).

Tumors were classified as: 1) atypical hyperplasia of mucosa if proliferation of atypical glands was observed or; 2) adenocarcinoma if atypical glands had proliferated and invaded all the layers of the gastric wall.[25]

Intestinal metaplasias were categorized using the following histological criteria[25–27]: type A, gastric mucosa with goblet cells which were positive for AB-PAS and HID; type B, intestinal-type crypts without Paneth cells; type C, intestinal metaplasia with Paneth cells (alkaline phosphatase-positive foci). Using these criteria, the numbers of intestinal metaplastic crypts on the same slide were counted separately for both the pyloric glands and the fundic glands in a double-blind fashion. Metaplastic crypts within 5 crypts from the pyloric ring were not scored.

For quantitative analysis, the heights of stomach mucosae were examined with the aid of a color image analyzer (Model CIA 102, Olympus Co., Tokyo), allowing measurement on a color TV monitor. Heights of pyloric and fundic glands without lesions were recorded.

Statistical analysis The significance of differences in numerical data was evaluated by use of the chi-squared and Student's t tests by fitting linear calibration lines and by the generalized Wilcoxon test for Kaplan Meier survival rates.

RESULTS

Body, liver, kidney, spleen and adrenal weights in Group III and also body, liver and kidney weights in Group I were significantly heavier than those of Group II. Liver, kidney and spleen weights in Group V, and body, kidney, testis and spleen weights in Groups IV were significantly decreased as compared to those in Group VI. Relative weights of liver and kidney in Group I and liver, kidney, testis and spleen in Group III were significantly increased as compared to Group II (Table I). Liver, kidney, testis and spleen weights in Group IV and V were significantly different from those in Group V.

The first sarcoma and first gastric atypical hyperplasia had both appeared by 54 days from the beginning of MNU treatment. Paralysis of both legs was observed at 106 days in Group I and a thymic lymphoma was detected at 122 days in Group II. Incidences of total tumors were 94, 84 and 97% in Groups I to III, respec-

Table I. Mean Survival and Body and Relative Organ Weights (mean±SD values)

Group	Treatment	Effective number of rats	Mean survival days	Body (g)	Liver	Kidneys	Testes	Spleen	Adrenals
I	MNU	31	206±58	289±49[a]	28.8±8.7[a]	7.8±3.4[a]	9.5±1.1	3.6±5.7	1.9±1.0
II	MNU+Tx	32	232±39	258±38	22.5±6.5	5.7±1.3	9.9±1.1	1.7±1.7	2.3±2.7
III	MNU+MtT	32	204±44	308±61[a]	39.1±8.7[a]	7.4±1.4[a]	9.1±1.1[a]	2.6±1.4[c]	3.1±1.1
IV	Control	10	287	432±44[b]	26.2±0.8[b]	5.6±1.0[b]	7.1±0.8	1.6±0.1[b]	1.1±1.5
V	Tx	26	274±28	367±32[b]	24.1±1.6[b]	4.4±0.3[b, c]	8.4±1.0[a]	1.2±0.2[b]	1.1±0.8
VI	MtT	12	282±3	456±46	41.0±4.4	7.3±1.0	7.0±0.9	2.4±0.3	2.1±0.4

a) Significantly different from MNU+Tx ($P<0.01$).
b) Significantly different from MtT ($P<0.01$).
c) Significantly different from MNU+Tx ($P<0.05$).

Table II. Tumor Incidences

Group	Treatment	Effective number of rats	Tumors (%)						
			Total	Gastric	Spinal cord	Brain	Thymic lymphoma	Sarcoma	Other
I	MNU	31	29 (94)	13 (43)	15 (47)	1 (3)	10 (32)	3 (10)	8 (26)[a]
II	MNU+Tx	32	27 (84)	15 (47)	10 (32)	0	5 (16)	2 (6)	2 (6)[b]
III	MNU+MtT	32	31 (97)	17 (53)	10 (32)	1 (3)	6 (19)	7 (22)	5 (16)[c]
IV	Control	10	0	0	0	0	0	0	0
V	Tx	26	0	0	0	0	0	0	0
VI	MtT	12	0	0	0	0	0	0	0

a) Three thyroid tumors, 2 prostate tumors, 2 kidney tumors, and 1 lung, tumor.
b) One prostate tumor and 1 kidney tumor.
c) Two prostate tumors, 1 kidney tumors, 1 pituitary tumor, 1 bladder tumor.

Table III. Tumor Multiplicity

Group	Treatment	Animal number	Number of tumors per animal					
			0	1	2	3	4	5
I	MNU	31	2 (6)	14 (47)	8 (27)	4 (13)	2 (6)	1 (3)
II	MNU+Tx	32	6 (19)	12 (38)	11 (34)	1 (3)	2 (6)	0
III	MNU+MtT	32	1 (3)[a]	10 (31)	10 (31)	8 (25)[b]	3 (9)	0
IV	Control	10	0	0	0	0	0	0
V	Tx	26	0	0	0	0	0	0
VI	MtT	12	0	0	0	0	0	0

a) Significantly different from MNU+Tx ($P<0.05$).
b) Significantly different from MNU+Tx ($P<0.01$).

tively, at the 39-week time-point. Gastric tumors, spinal cord tumors, thymic lymphomas and sarcomas were the predominant lesions in MNU-treated groups (Table II). Other tumors developed in the thyroid, prostate, kidney, lung, pituitary, and bladder. The number of rats bearing 3 or more different tumors in Group III was significantly increased as compared to that for Group II ($P<0.01$, Table III). Although the incidences of tumors were not significantly different among the MNU-treated groups, cumulative incidence curves of both total tumors ($P<0.01$; Fig. 1a) and gastric tumors ($P<0.05$; Fig. 1b) indicated significantly earlier appearance in Group III than in Group I. There were no differences in cumulative incidence curves for spinal cord tumors (Fig. 1c) or thymic lymphomas (Fig. 1d).

Gastric tumors Gastric tumors appeared in 43%, 47% and 53% of animals in Groups I to III, respectively, without any significantly intra-group difference. Morpho-

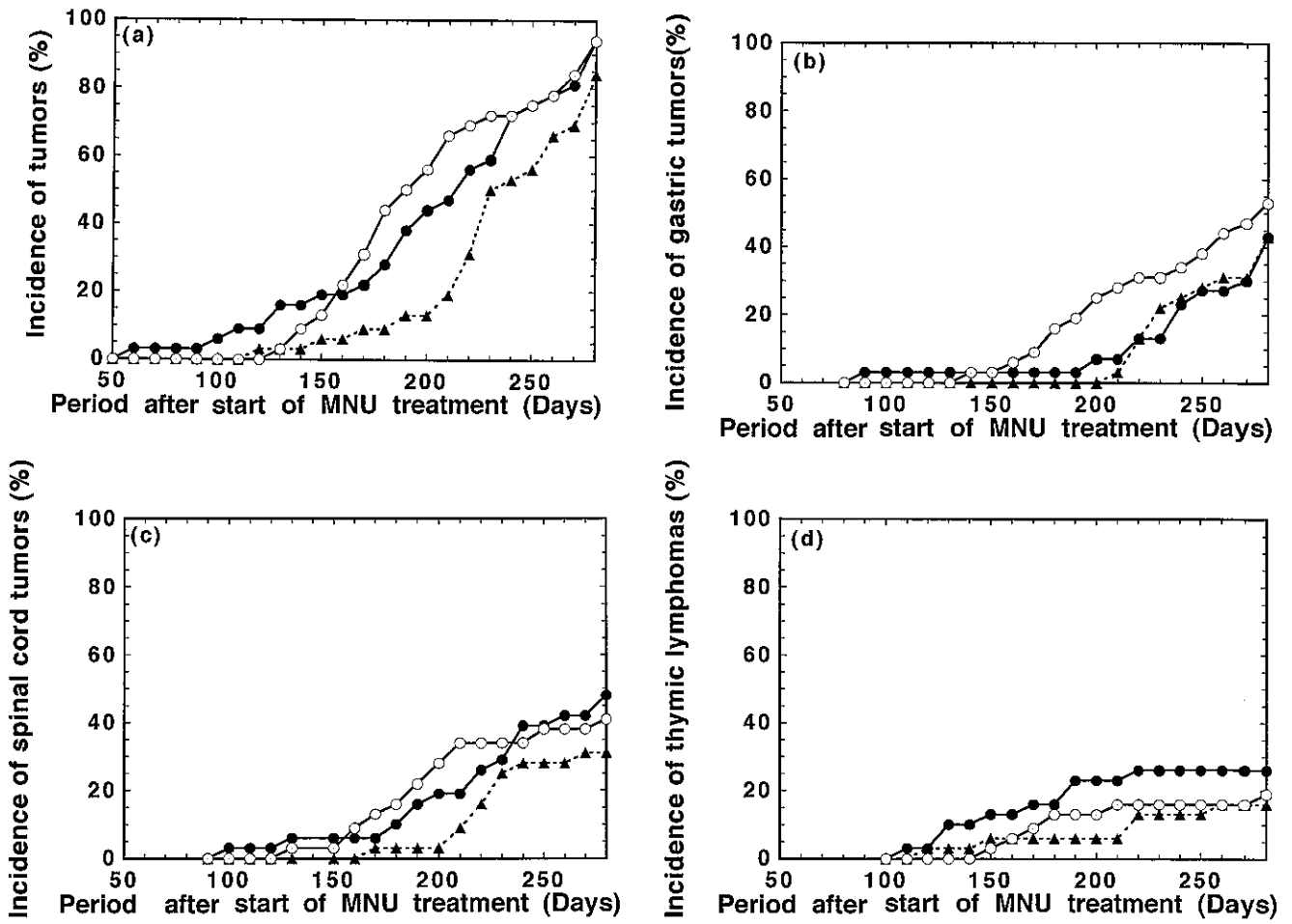

Fig. 1. Cumulative incidences for total tumors (a), gastric tumors (b), spinal cord tumors (c), thymic lymphomas (d). ●, Group I; ○, Group II; ▲, Group III.

Table IV. Height (μm) of Gastric Mucosae

Group	Treatment	Fundus	Pylorus
I	MNU	$652\pm73^{a,b)}$	$275\pm65^{a,b)}$
II	MNU+Tx	$641\pm66^{a,b,c)}$	$283\pm55^{a,b)}$
III	MNU+MtT	$730\pm76^{a)}$	$381\pm50^{a)}$
IV	Control	$763\pm110^{a,b,c)}$	$185\pm17^{a,b,c,d)}$
V	Tx	$711\pm56^{a,b)}$	$172\pm19^{a,b,c,d)}$
VI	MtT	860 ± 83	228 ± 33

a) Significantly different from MtT ($P<0.01$).
b) Significantly different from MNU+MtT ($P<0.01$).
c) Significantly different from MNU ($P<0.01$).
d) Significantly different from MNU+Tx ($P<0.01$).

logically, most tumors were well-differentiated, with a few moderate or poorly differentiated types. Larger-sized lesions exhibited both gastric mucosal and intestinal components. Most of them were adenocarcinomas.

Spinal cord tumors All animals with paralysis had spinal cord tumors. Incidences of these were 47%, 32% and 32% in Groups I to III. Morphologically, most of the spinal cord tumors were malignant schwannomas, positive for S-100 protein. Mitoses were frequently observed and growth rates were very rapid. Spinal cord tumors were located around the lumbar but not cervical or thoracic segments.

Thymic lymphomas Incidences of thymic lymphoma were 10 of 31 (32%), 5 of 32 (16%) and 6 of 32 (19%) in Groups I to III, respectively. Some had metastasized to the pancreas, spleen and liver.

Intestinal metaplasia In this experiment no intestinal metaplasias were observed in glandular stomachs without gastric tumors.

Gastric mucosal heights Heights of the fundic and pyloric glands were significantly increased in MtT groups compared to the remaining groups (Table IV).

Table V. Serum Growth Hormone Values

Group	Treatment	Number of animals	Growth hormone (ng/ml)
I	MNU	6	$37\pm15^{a,b)}$
II	MNU+Tx	7	$37\pm9^{a,b)}$
III	MNU+MtT	7	3818 ± 3616
IV	Control	7	$38\pm14^{a,b)}$
V	Tx	7	$37\pm6^{a,b)}$
VI	MtT	7	1669 ± 1213

a) Significantly different from MNU+MtT ($P<0.05$).
b) Significantly different from MtT ($P<0.05$).

GH in serum The size of the inoculated tumor was not measured. GH values in Group III and VI were significantly higher than those in Groups I and II, or in Groups IV and V, respectively (Table V).

DISCUSSION

The predominance of gastric and spinal cord tumors, and thymic lymphomas in the present experiment is in line with our findings for 8-week-old F344 rats treated with MNU in terms of histological types.[28] In Wistar rats, gastric tumors demonstrate cellular infiltration, fibrosis, myoblast-like cells, calcification or cartilage within the stroma[29] but these were not features in F344 lesions, indicating strain-specificity in the types of gastric tumors.

In the present case, total tumors and gastric tumors in the animals with elevated serum GH appeared earlier than in Group I. Previously we reported that prolactin lacks any promoting effect on MNNG tumorigenesis.[30] In this present experiment, body, liver and many organ weights were increased in animals receiving implanted MtT and blood GH levels were increased about 100 fold. GH acts to increase both protein synthesis and blood sugar level.[14] Administration of human GH increases body weight.[31,32] While malnutrition, in general, and dietary protein deprivation, in particular, result in failure to gain weight and skeletal growth.[33] Harel and Tannenbaum[33] and Thissen *et al.*[34] reported that nutritional protein sufficiency is essential for maintaining an adequate plasma GH concentration and especially, that GH was decreased by dietary protein restriction. Roebuck's group[35,36] have shown that reduced food intake resulted in fewer pancreatic cancers. Tx is known to decrease body and organ weights, and Takeuchi *et al.*[37] reported that Tx markedly reduces GH levels. However, in the present experiment, GH was not decreased by Tx and cumulative incidences of gastric tumors were similar to those after MNU treatment alone. Thus any drop in GH caused by Tx may be only temporary.

In this experiment DNA synthesis was not checked, but the gastric mucosal height was increased in animals receiving implanted MtT. Many authors have indicated that the enhancing effects on carcinogenesis may be related to stimulation of cell proliferation and elongation of the mucosa.[17-19] The present results are therefore in line with the concept that promoters of gastric carcinogenesis have the ability to increase cell proliferation.

Some effects of GH may be mediated by local production of insulin-like growth factor I (IGF-I).[14] Weights of kidney, spleen and thymus are increased by recombinant human IGF-I administration[31,32] and Roy *et al.*[24] reported that both serum GH and IGF-I levels became elevated in association with MtT. In the present experiment, kidney and spleen weights were similarly increased and further studies are clearly warranted to clarify whether IGF-I might have played a role in the observed acceleration of gastric tumorigenesis. Furthermore, it is unknown whether the effect of GH on tumor growth is a direct effect via GH receptor, or an indirect effect due to increased food intake.

The susceptibility of the developing nervous system to the carcinogenic effects of alkylating nitrosamines (*N*-ethyl-*N*-nitrosourea, ENU and MNU) is not uniform.[1,2,19,38] Druckery *et al.* reported that gliomas in the cerebrum and schwannomas in the spinal cord are induced in rats by repeated injections of MNU in young adults.[2] Naito *et al.*[38] reported that the susceptibility of the spinal cord is high in neonatal and one-week-old rats, but decreases at later ages. The peripheral nervous system is highly susceptible only during the neonatal period. Naito *et al.*[39] demonstrated that subpinal cells have a high labeling index in neonates and in rats on day 8 after birth but this declines abruptly thereafter with the index at day 29 being about 1%. They suggested that these subpinal cells in the spinal cord are target cells for ENU carcinogenesis in rats. The pattern of tumor development in the central peripheral nervous system depends greatly on the age of the rats and the shift of tumorigenesis with different ages has been found to be closely related to the total number of target cells in the central nervous system.[2,38,39] We have found that 5-bromodeoxyuridine incorporation is still observed in the spinal cord and brain of 4-week-old F344 rats (manuscript in preparation) and thus target cells would have been expected to exist in the animals used in the present experiment. This would explain the induction of spinal cord tumors. Recently, Shibutani *et al.*[13] reported that anaplastic astrocytomas and glioblastomas can be induced in 11-week-old F344 rats given 100 or 200 ppm MNU in their drinking water for 42 weeks. Differences between the experiments by Shibutani and ourselves include the age (11-week-old and 4-week-old, respectively), duration of MNU (42 weeks and 15 weeks, respectively), source

of MNU (Nacalai Tesque and Sigma, respectively) and the breeding place (Atsugi and Hino, respectively). Lack of promotion by GH would be in line with the fully differentiated status of the nervous system.

In the present experiment, thymic lymphomas also appeared but were not significantly influenced by GH. The latent time of thymic lymphoma was shorter compared to that of other tumors.[40,41] Torosian and Donoway suggested that GH selectively supports host growth of different organs,[42] but the underlying cause remains to be elucidated.

In conclusion, MNU targets many organs in young rats and some of the initiated lesions appeared to be susceptible to a promoting action of elevated serum GH.

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