Enhancement by Methionine Enkephalin of Colon Carcinogenesis Induced by Azoxymethane

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ABSTRACT

The effect of the opioid receptor agonist methionine enkephalin (Met-enkephalin) and the opioid receptor antagonist naloxone on colon carcinogenesis induced by azoxymethane was investigated in Wistar rats. Rats received weekly i.p. injections of 7.4 mg/kg of body weight of azoxymethane and injections of Met-enkephalin (50 µg/kg of body weight), naloxone (2 mg/kg of body weight), or Met-enkephalin (50 µg/kg of body weight) plus naloxone (2 mg/kg of body weight) once every 2 days. In wk 40, the group treated with Met-enkephalin had a significantly increased incidence of colonic tumors. A combination of Met-enkephalin and naloxone attenuated the enhancing effect by Met-enkephalin on the development of colonic tumors. Administration of naloxone alone had no influence on colonic tumorigenesis. During and after administration of the carcinogen, the bromodeoxyuridine-labeling indices of the colon mucosa and/or cancers were significantly increased in rats treated with Met-enkephalin. However, a combination of Met-enkephalin and naloxone significantly decreased the labeling indices of the colon mucosa and/or cancers. These findings indicate that Met-enkephalin enhanced colon carcinogenesis and that naloxone attenuated this enhancement. Because naloxone is an opioid receptor antagonist, these findings also indicate that the enhancing effect of Met-enkephalin on colon carcinogenesis may be mediated through opioid receptors.

INTRODUCTION

Opioid peptides, enkephalins, and endorphins, originally isolated from the brain, have been detected immunocytochemically and radioimmunologically in the digestive system and shown to be localized in the nerve fibers of the myenteric plexus (1). Although the physiological role of endogenous opiates is still unclear, recent investigations demonstrate the involvement of endogenous peptides in regulation of tumor growth (2). The results were conflicting: opioid peptides were found to exert either a stimulatory (3-5) or an inhibitory (6) effect on tumor growth, depending on the experimental conditions. However, they suggested that Met-enkephalin might affect colon carcinogenesis. To test this possibility, we examined the effect of Met-enkephalin on the development of colonic tumors induced by AOM in Wistar rats.

MATERIALS AND METHODS

Animals. One hundred 7-wk-old male Wistar rats were purchased from SLC, Japan (Shizuoka, Japan). The animals were housed in suspended, wire-bottomed metal cages in animal quarters with controlled temperature (21-22°C), humidity (30 to 50%), and light (12-h cycle), and they had free access to tap water and regular chow pellets (Oriental Yeast, Tokyo, Japan).

Experimental Design. The animals were randomly divided into four groups of 25 rats each and were given ten weekly s.c. injections of 7.4 mg/kg of body weight of AOM (Sigma Chemical, St. Louis, MO) in 0.9% NaCl solution. From the start of administration of the carcinogen, the rats also received the following injections on alternate days until the end of the experiment: Group 1, olive oil, 1 ml/kg of body weight; Group 2, Met-enkephalin, 50 µg/kg of body weight; Group 3, naloxone, 2 mg/kg of body weight; and Group 4, Met-enkephalin, 50 µg/kg of body weight plus naloxone, 2 mg/kg of body weight.

Met-enkephalin (Sigma), naloxone (Sigma), and Met-enkephalin plus naloxone were injected s.c. as suspensions in a volume of 1 ml/kg of body weight of olive oil, between 2 and 3 p.m. each day, various sites being chosen for injections. Group 1 received injections of equal amounts of the vehicle (olive oil) only.

Histological Observations. Five rats in each group were sacrificed in experimental wk 8 for determination of the labeling index of the colon mucosa. Other animals were killed if they became moribund, and all survivors were sacrificed at the end of wk 40. All the animals sacrificed during the study were necropsied, and the internal organs were carefully examined. The body weight and colon length were measured. The large intestine was opened, pinned flat on a cork mat, and fixed with buffered picric acid-formaldehyde solution (7). The fixed colon was cut into five segments of equal length, which are referred to hereafter as Part 1 (adjacent to the anal orifice) to Part 5 (adjacent to the cecum). Tumor-bearing areas and areas suspected of having lesions were excised and embedded in paraffin. Semiserial sections 5 µm thick were cut to expose the central part of the tumor or the stalk, when present, and were stained with hematoxylin and eosin. In addition to tumors, flat mucosa from each segment of the fixed colon with no visible tumors was cut into two strips 3 mm wide, which were embedded in paraffin. Thin sections were prepared and were inspected microscopically for tumor foci. All sections were examined without knowledge of which group they were from.

Classification of Colon Tumors. Colon tumors induced by AOM were classified histologically into adenomas, CIS, and adenocarcinomas (8). The adenocarcinomas were further classified as either well-differentiated or mucinous carcinomas.

Measurement of Labeling Indices of Colon Mucosa and Colon Cancers. The labeling indices of colon mucosa and/or colon cancers were measured in wk 8 and 40 with an immunohistochemical analysis kit for assaying the incorporation of bromodeoxyuridine (Becton-Dickinson, Mountain View, CA) (9, 10), by the modified method described by Tada et al. (11). For this, the rats were fasted for 12 h and then received s.c. injections: Group 1, olive oil, 1 ml/kg of body weight; Group 2, Met-enkephalin, 50 µg/kg of body weight; Group 3, naloxone, 2 mg/kg of body weight; and Group 4, Met-enkephalin, 50 µg/kg of body weight plus naloxone, 2 mg/kg of body weight. One h later, the animals received an i.p. injection of bromodeoxyuridine of 20 mg/kg of body weight, and after another hour they were sacrificed with ether. The colon was removed and fixed in 70% ethanol for 4 h. From the start of administration of the carcinogen, the rats also received the following injections on alternate days until the end of wk 40. All the animals sacrificed during the study were necropsied, and the internal organs were carefully examined. The body weight and colon length were measured. The large intestine was opened, pinned flat on a cork mat, and fixed with buffered picric acid-formaldehyde solution (7). The fixed colon was cut into five segments of equal length, which are referred to hereafter as Part 1 (adjacent to the anal orifice) to Part 5 (adjacent to the cecum). Tumor-bearing areas and areas suspected of having lesions were excised and embedded in paraffin. Semiserial sections 5 µm thick were cut to expose the central part of the tumor or the stalk, when present, and were stained with hematoxylin and eosin. In addition to tumors, flat mucosa from each segment of the fixed colon with no visible tumors was cut into two strips 3 mm wide, which were embedded in paraffin. Thin sections were prepared and were inspected microscopically for tumor foci. All sections were examined without knowledge of which group they were from.

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: Met-enkephalin, methionine enkephalin; AOM, azoxymethane; CIS, carcinoma in situ; GABA, γ-amino-n-butyric acid.

785
counted. On the basis of these measurements, we derived the labeling index as the number of bromodeoxyuridine-labeled cells/the total number of cells within the zone of proliferation or colon cancer lesion.

**Statistical Analysis.** Data were analyzed by the $\chi^2$ test, Fisher’s exact probability test, or by one-way analysis of variance with Dunn’s multiple comparison (13-15). Data are given as the mean ± SE. Differences were regarded as significant when the calculated $P$ value was less than 0.05.

**RESULTS**

Incidence and Number of Colon Tumors. Animals that survived for more than 36 wk were included in the effective numbers because the first colon tumor was found in a rat in Group 1 that died in wk 36. Three, one, one, and one rat in Groups 1 to 4, respectively, were killed before wk 36 because they became moribund. No tumors were found in any of these animals, which were excluded from the effective numbers.

The incidence of colon tumors and their number per rat and the body weight in each group are summarized in Table 1. At wk 40, the animals that had received either Met-enkephalin or naloxone had slightly, but not significantly, lower body weights than did the olive oil-treated rats.

In Group 1 (olive oil only), colon tumors were found in 10 (59%) of 17 rats examined. In Group 2 (Met-enkephalin), the incidence of colon tumors was significantly higher than that in Group 1. However, concomitant administration of Met-enkephalin and naloxone (Group 4) significantly reduced the incidence of colon tumors as compared with that in Group 2. Administration of naloxone alone (Group 3) had little or no influence on the incidence of colon tumors. In Group 1, the average number of colon tumors per rat was 0.7 ± 0.2. Administration of Met-enkephalin (Group 2) slightly increased the number of colon tumors per rat, but the increase was not statistically significant.

Histological Types of Colon Tumors. The distribution of different histological types of colon tumors is summarized in Table 2. There was no significant difference in the distributions of adenomas, CIS, and adenocarcinomas in the four groups. However, Table 2 shows that, in Group 1 (olive oil), 22% of the adenocarcinomas were of the well-differentiated type, whereas in Group 2 (Met-enkephalin), well-differentiated adenocarcinomas were significantly more frequent than in Group 1. Combined administration of Met-enkephalin and naloxone decreased the incidence of well-differentiated adenocarcinomas, but the difference was not statistically significant.

Colon Length and Labeling Indices of Colon Mucosa and Colon Cancers. Table 3 summarizes data on the colon length and bromodeoxyuridine-labeling indices of the colon mucosa and colon cancers in wk 8 and 40. At both times examined, there was no significant difference in the colon lengths in the four groups. Table 3 also shows that administration of Met-enkephalin (Group 2) led to a significant increase in the labeling indices of both parts of the colon mucosa and/or of colon cancers at both times examined than in Group 1 (olive oil). However, the concomitant use of Met-enkephalin and naloxone (Group 4) significantly reduced the labeling indices of colon mucosa and/or colon cancers, as compared with those in Group 2 (Met-enkephalin).

**DISCUSSION**

The data presented in this paper show that the opioid peptide Met-enkephalin enhanced colon carcinogenesis induced by AOM in Wistar rats. Treatment of rats with Met-enkephalin in depot form every other day from the start of the experiment led to a significant increase in the incidence of colon cancers in wk 40.

The mechanisms involved in this effect of Met-enkephalin are not fully understood, but five possible direct and indirect mechanisms may be considered. One possibility would involve an inhibitory effect on acetylcholine release in the central and peripheral nervous systems (16, 17). Vizi et al. (18) found that Met-enkephalin inhibited the release of[^1]C]acetycholine from the myenteric plexus in a dose-related fashion. Acetylcholine has been demonstrated to be capable of influencing cell division (19). Tutton (20) showed that cholinoreceptor stimulation, either by injection of carbachol or by inhibition of acetylcholinesterase, resulted in an increase in the mitotic rate in the crypts of Lieberkühn in rat jejunum. Gurkalo and Volkson (21) suggested that pharmacological compounds that enhanced the activity of the sympathetic nerves stimulated carcinogenesis, whereas those that enhanced cholinergic functions inhibited carcinogenesis. We recently found that the long-term administration of the parasympathomimetic compound neostigmine resulted in a significant decrease in the incidence and number per rat of colon tumors in wk 40 (22). Moreover, we also found that prolonged administration of parasympatholytic atropine resulted in a significant increase in the number of gastric cancers (23).

A second mechanism may involve an effect on the secretion of anterior pituitary hormones. Endogenous opioid peptides participated in control of anterior pituitary hormone function. Endogenous opioid peptides (24, 25) can increase pituitary prolactin and growth hormone secretion and reduce gonadotropin and thyrotropin secretion. Dorchester and Haist (26) found that the weight and secretin content of atrophic small intestine of hypophysectomized rats were increased by multiple injections of growth hormone, corticotropin, or anterior pituitary extracts. These hormones may affect cancer growth.

A third mechanism is an effect on GABA. Brennan et al. (27) showed that Met-enkephalin provided a very potent stimulus for the release of GABA from nerve endings, and that the stimulation of release by Met-enkephalin was not prevented by naloxone. The dose of naloxone used in this experiment is the upper range for specific opiate antagonism, and it is likely that
Jankovic and Marie (29, 30) found that Met-enkephalin was a potent immunosuppressor. Therefore, immunomodulation with opioid peptides may stimulate mammary tumor growth. Similarly, Kikuchi et al. (35) examined the effects of naloxone on gastric carcinogenesis. Recently, however, we examined the effects of GABA, the GABA $\alpha_1$ receptor agonist muscimol, and the GABAB receptor agonist baclofen on the development of gastric cancers induced by N-methyl-$N'$-nitro-$N'$-nitrosoguanidine, and found that GABA and baclofen, but not muscimol, inhibit gastric carcinogenesis (28).

A fourth mechanism is an immunomodulation with Met-enkephalin. Opioid peptides modulate both cellular and humoral immune function via an opiate receptor-mediated action. Jankovic and Marie (29, 30) found that Met-enkephalin was a potent immunosuppressor. Therefore, immunomodulation with enkephalin is also considered to be responsible for enhancement of colon carcinogenesis.

A fifth mechanism may involve an effect on cell proliferation. Opiate peptides are involved in the regulation of tumor growth (31, 32). Zagon and McLaughlin (31) examined the effect of Met-enkephalin on the growth of transplanted neuroblastoma and found that Met-enkephalin delayed the appearance of tumors and prolonged survival of the mice. Murog (33) demonstrated the antitumor effect of Met-enkephalin in C57BL/6J mice inoculated with B16-BL6 melanoma cells. Local s.c. tumor growth was inhibited by treatment with Met-enkephalin for 7 or 14 days. The antitumor effect of Met-enkephalin was inhibited by the administration of the opioid receptor antagonist naloxone. However, Aylsworth et al. (34) found that naloxone and naltrexone, both antagonists of endogenous opioid peptides, significantly inhibited growth of carcino-containing mammary cancers in rats, and they suggested that endogenous opioid peptides may stimulate mammary tumor growth. Similarly, Kikuchi et al. (35) examined the effects of naloxone on the growth of the human ovarian cancer cell line KF in vitro and in vivo, and they concluded that naloxone is a growth inhibitor of KF cells. These authors also found that naloxone inhibited cell proliferation and protein synthesis. Ilyinsky et al. (36) found that opioid peptides, including Met-enkephalin, exert a strong growth-promoting effect on nervous tissue in culture. The present study showed that administration of Met-enkephalin significantly increased the bromodeoxyuridine-labeling indices of the colon mucosa and cancers during and after treatment with the carcinogen AOM.

In this work, we found that prolonged administration of Met-enkephalin enhanced development of colon tumors, and that the opioid receptor antagonist naloxone inhibited the enhancing effect of Met-enkephalin. This finding indicates that the effect of Met-enkephalin may be mediated through opioid receptors.

REFERENCES

11. Tada, T., Kodama, T., Watanabe, S., Sato, Y., and Shimozato, Y. Cell kinetic effects of an explanation of treatments, see Table 1.
* Numbers in parentheses, percentage.
* Mean ± SE.
* Significantly different from the value for Group 1: (a) $P < 0.01$; (b) $P < 0.001$.
* Significantly different from the value for Group 2: (c) $P < 0.01$; (d) $P < 0.001$.

### Table 2 Histological types and depth of involvement of colon tumors in AOM-treated rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Total no.</th>
<th>Adenoma</th>
<th>Adenocarcinoma</th>
<th>Total no.</th>
<th>Well differentiated</th>
<th>Muscinous</th>
<th>Submucosal layer</th>
<th>Muscle layer or deeper</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Olive oil</td>
<td>12</td>
<td>3 (25)</td>
<td>0 (0)</td>
<td>9</td>
<td>2 (22)</td>
<td>7 (78)</td>
<td>2 (22)</td>
<td>7 (78)</td>
</tr>
<tr>
<td>2</td>
<td>Met-enkephalin</td>
<td>25</td>
<td>8 (32)</td>
<td>1 (4)</td>
<td>16 (64)</td>
<td>12 (75)</td>
<td>4 (22)</td>
<td>4 (25)</td>
<td>12 (75)</td>
</tr>
<tr>
<td>3</td>
<td>Naloxone</td>
<td>16</td>
<td>7 (44)</td>
<td>2 (12)</td>
<td>7 (44)</td>
<td>4 (57)</td>
<td>3 (43)</td>
<td>2 (29)</td>
<td>5 (71)</td>
</tr>
<tr>
<td>4</td>
<td>Met-enkephalin + naloxone</td>
<td>15</td>
<td>8 (53)</td>
<td>1 (7)</td>
<td>6 (40)</td>
<td>2 (33)</td>
<td>4 (67)</td>
<td>2 (33)</td>
<td>4 (67)</td>
</tr>
</tbody>
</table>

### Table 3 Colon length and labeling indices of colon mucosa and colon tumors

<table>
<thead>
<tr>
<th>Experimental wk</th>
<th>Group</th>
<th>Treatment</th>
<th>Colon length (cm)</th>
<th>Bromodeoxyuridine-labeling index</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1</td>
<td>Olive oil</td>
<td>25.5 ± 0.3$^a$</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Met-enkephalin</td>
<td>24.5 ± 0.3</td>
<td>0.42 ± 0.02 (b)$^c$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.43 ± 0.02 (a)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Naloxone</td>
<td>25.0 ± 0.4</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.33 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Met-enkephalin + naloxone</td>
<td>25.8 ± 0.3</td>
<td>0.27 ± 0.02 (d)$^c$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.31 ± 0.02 (d)</td>
</tr>
<tr>
<td>40</td>
<td>1</td>
<td>Olive oil</td>
<td>26.8 ± 0.3</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Met-enkephalin</td>
<td>26.4 ± 0.2</td>
<td>0.34 ± 0.01 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.40 ± 0.02 (b)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Naloxone</td>
<td>26.4 ± 0.3</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Met-enkephalin + naloxone</td>
<td>26.6 ± 0.2</td>
<td>0.21 ± 0.01 (d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.28 ± 0.01 (d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.30 ± 0.03 (c)</td>
</tr>
</tbody>
</table>

at the dose used, naloxone has a number of nonopiate actions. Naloxone has been reported to activate the central GABAergic system. Recently, we examined the effects of GABA, the GABA$\alpha_1$ receptor agonist muscimol, and the GABA$\alpha_2$ receptor agonist baclofen on the development of gastric cancers induced by N-methyl-$N'$-nitro-$N'$-nitrosoguanidine, and found that GABA and baclofen, but not muscimol, inhibit gastric carcinogenesis (28).


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