Epitalon and colon carcinogenesis in rats: Proliferative activity and apoptosis in colon tumors and mucosa

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Abstract. The effect of the synthetic pineal peptide Epitalon® (Ala-Glu-Asp-Gly) on proliferative activity in colon tumors, and in mucosal epithelial cells adjacent to and located far from tumors was studied in rats. To evaluate the effect of Epitalon on different stages of carcinogenesis, different treatment regimens were used: during the tumor initiation stage, during the tumor-promotion stage, or during the entire process of tumor development. Eighty 2-month-old male LIO rats were exposed weekly to five subcutaneous injections of 1,2-dimethylhydrazine (DMH) at a single dose of 21 mg/kg body weight. Rats were divided into four groups. Control rats (group 1) received saline at a dose of 0.1 ml during the entire experiment. Rats in group 2 were treated with Epitalon at a dose of 1 μg, five times a week, for 6 months, from the first injection of DMH till the end of the experiment. Rats in group 3 were treated with Epitalon after termination of the carcinogen injections. Rats in group 4 were treated with Epitalon only during the period of DMH exposure (for the first 5 weeks of the experiment). DMH induced proliferation of the secretory epithelium, and this phenomenon was accompanied by a decrease in the size of the stromal area and the area of lymph infiltration in colon tumors and in the colon mucosa adjacent to the tumors (group 1). Epitalon attenuated this effect, especially when the treatment was continued throughout the experiment (group 2). It increased the stromal areas, as well as that of lymphoid infiltration in the colon mucosa adjacent to the tumors. The intensity of lymphoid infiltration was activated in both the colon mucosa adjacent to a tumor and in the tumor. Mitotic activity of tumor cells was significantly inhibited by Epitalon when the treatment was given throughout the experiment (group 2). In parallel, a high level of apoptosis was seen in the same group. Thus, the strongest inhibitory effect of Epitalon on carcinogenesis in the colon mucosa was manifested when the treatment was continued throughout the experiment.

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Introduction

The pineal indole hormone melatonin (N-acetyl-5-methoxy-tryptamine) and the pineal peptide preparation Epithalamin have been shown to inhibit the development of spontaneous and chemically-induced neoplasms (1-3). The synthetic pineal tetrapeptide Epitalon® (Ala-Glu-Asp-Gly), which was recently structured on the basis of the amino acid sequence of Epithalamin, has also been found to reduce the incidence of spontaneous tumors in mice and chemically-induced colon tumors in rats (4,5). However, the mechanism responsible for these changes has only been partially studied.

Experimental colon cancers are manifested by higher proliferative activity and dramatically reduced apoptosis in the colonic mucosa (6). Melatonin has been shown to inhibit cellular proliferation in the rodents' colon (7). Thus, it can be suggested that the inhibitory effect of melatonin on DMH-induced colon carcinogenesis is realized, at least in part, by its influence on tissue homeostasis. Indeed, melatonin has been shown to affect apoptosis and the proliferative properties of normal and tumorous colonic tissues and several splenic lymphoid elements (8,9). The inhibitory effect of Epitalon on bowel carcinogenesis is comparable to that of melatonin (1,10).

In the present study, we compared the effects of DMH and Epitalon on the proliferative activity of tumor cells and mucosal epithelial cells located adjacent to, and far from the tumors, in an attempt to understand the mechanism undrlying the oncostatic properties of Epitalon. To evaluate the effect

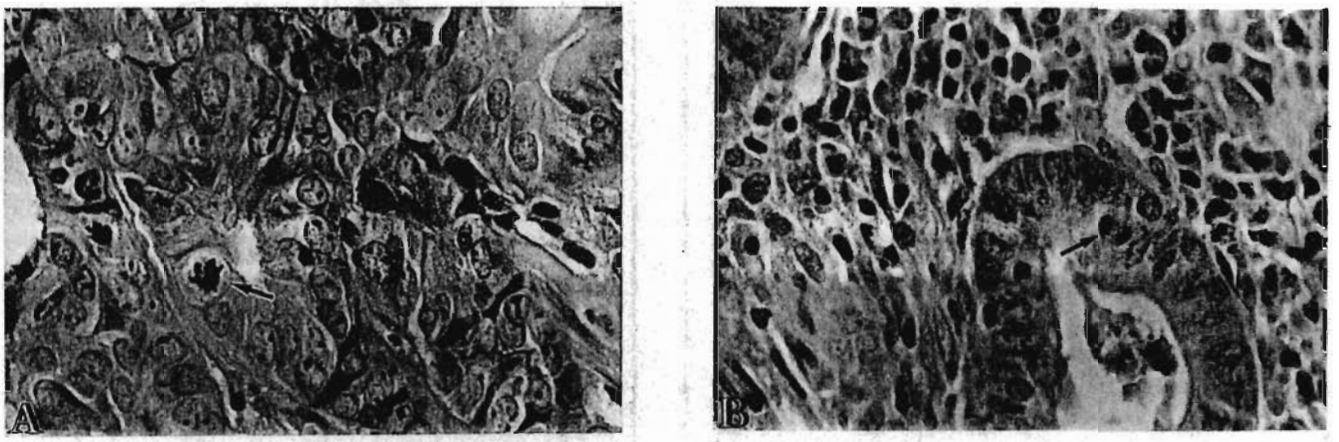

Figure 1. Well-differentiated adenocarcinoma of the colon obtained from a control rat exposed to DMH. Note the different phases of mitosis: metaphase (A) and anaphase (B). H&E. x800.

of Epitalon on different stages of carcinogenesis, different regimens of Epitalon treatment were used: during the tumor-initiation stage, during the tumor-promotion stage, or during the entire process of tumor development (5).

Materials and methods

Chemicals. 1,2-Dimethylhydrazine dihydrochloride (DMH) was purchased from Sigma Chemical Co. (St. Louis, MO). The synthetic pineal peptide Epitalon was obtained from Dr G.I. Grigoriev, Institute of Bioregulation and Gerontology, St. Petersburg, Russia.

Animals. Two-month-old outbred male LIO rats from the Animal Department of Central Research Roentgeno-Radiological Institute, St. Petersburg, were used in the study. Animals were kept under a standard light/dark regimen (12:12 h) at 22±2°C. They received standard laboratory chow and tap water *ad libitum*.

Tumorigenic experiments. Eighty rats were exposed weekly to five subcutaneous injections of DMH at a dose of 21 mg/kg body weight (calculated as a base). Under this regimen, the carcinogen induced colon tumors in most of the rats (1). DMH was *ex tempore* dissolved in normal saline and neutralized with sodium bicarbonate (pH 7.0). Rats were divided into four groups. Control rats (group 1) received saline at a dose of 0.1 ml during the entire experiment, lasting 6 months. Rats from groups 2, 3 and 4 were treated subcutaneously with Epitalon at a single dose of 1 µg five times a week. Rats in group 2 were treated with Epitalon for 6 months, from the first injection of DMH till the end of experiment. Rats in group 3 were treated with Epitalon for about 5 months, after termination of carcinogen injections till the end of the experiment. Rats in group 4 were treated with Epitalon only during the period of DMH exposure (for the first 5 weeks of the experiment). All animals were weighed weekly. The experiment was terminated 6 months after the first injection of carcinogen, and all rats were sacrificed by ether vapor.

Pathological investigation. All animals were autopsied. The intestine was opened longitudinally. The position and size of each tumor were recorded. All tumors and colon mucosa were fixed in 10% neutral formalin and, after routine histological processing, were embedded in paraffin. Sections (3 µ-thick) through the middle part of each tumor were stained with hematoxylin and eosin (H&E). Proliferative activity of the epithelial cells and condition of the stroma were studied in the mucosa adjacent to a tumor (<1 cm away) and located far from the tumor (≥3 cm) as well as in the tumors themselves. The distant mucosa served as an internal control. Proliferative activity was evaluated from the whole slide at a magnification of x150. Mitotic index (MI) was calculated as the percentage of mitotic cells per 10,000 µm² of mucosa or tumor. The cells were analyzed with an ocular grid, at a magnification of x600. In each sample 1,000-3,000 cells were evaluated. The stromal areas were evaluated using a 10,000 µm² micrometric net in 10 randomly chosen fields of tumors and colon mucosa. The rate of lymphoid infiltration was calculated by determine the number of lymphocytes present as a percentage of the total number of cells in the same areas. The relationship between tumorigenesis and apoptosis was studied by comparing the rate of apoptosis in tumors obtained from different rat groups. The apoptotic index (AI) was determined using the ApopTag marker (Oncor, Inc., Gaithersburg, MD) and was calculated as the percentage of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells per 1,000 cells (11). All data were analyzed statistically by Student's t-test.

Results

DMH stimulated mitotic activity of the epithelial cells in the mucosa adjacent to a tumor (Fig. 1). In tumors, this parameter was sevenfold that in the mucosal epithelial cells located far from the tumor (Table I). Epitalon inhibited mitotic activity of the epithelial cells adjacent to a tumor and especially that of tumor cells when the treatment was given throughout the experiment (group 2, Table I).

Significant differences were found in the AI of tumors obtained from rats treated under the different regimes: long-term treatment with Epitalon (throughout the experiment, group 2) resulted in a significant increase in the number of apoptotic cells in tumors (Fig. 2; Table II).

Table I. Effects of Epitalon on the rate of mitotic activity in the colon (the number of mitotic cells per 10,000 μm^2 of the stroma, mean ± SE).

Rat groups	Areas of the mucosa located far from a tumor (A)	Areas of the mucosa adjacent to a tumor (B)	Areas of colon adenocarcinomas (C)
1	0.23±0.08	0.43±0.08	1.62±0.13[a]
2	0.23±0.09	0.23±0.07	1.03±0.13[a,b]
3	0.25±0.09	0.22±0.07	1.36±0.13[a]
4	0.22±0.07	0.40±0.08	1.66±0.14[a,c]

All rats were exposed to DMH. Rat groups: 1, Control, non-treated. 2, Treated with Epitalon from the first injection of DMH till the end of experiment, for 6 months. 3, Treated with Epitalon after the last injection of DMH till the end of experiment. 4, Treated with Epitalon only during the exposure to DMH, for the first 5 weeks of experiment. [a]Significantly different from areas A and B, $p<0.01$. [b]Significantly different from group 1, $p<0.01$. [c]Significantly different from group 2, $p<0.01$.

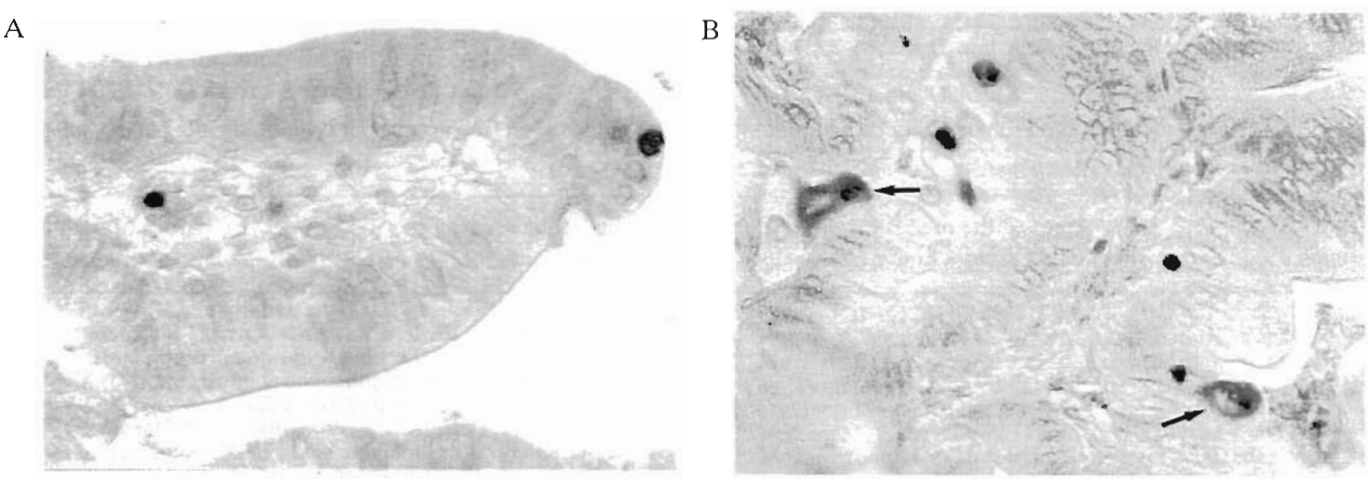

Figure 2. Colon tumors obtained from a control rat (A) and a rat treated with Epitalon (B). Note the abundance of apoptotic cells (arrows) in tumors from the experimental rat. TUNEL reaction. x400.

Table II. Apoptotic index (AI) in colon tumors obtained from different rat groups (mean ± SE).

Rat groups	AI
1[a]	1.64±0.27
2	12.74±1.19[b]
3	2.76±0. 32
4	2.17±0.23

[a]See footnotes at Table I. [b]Significantly different from the other groups, $p<0.01$.

The morphometric study showed that DMH-induced colon carcinogenesis is accompanied by a decrease in the size of the stromal area and in that of the lymphoid infiltration into tumors and in the mucosa adjacent to the tumors, relative to mucosal areas located far from the tumors (Table III). Epitalon attenuated this effect, especially when the treatment was continued for the entire experimental period. A comparison between the different experimental groups showed that Epitalon increases the stromal areas and the intensity of lymphoid infiltration both in the colon mucosa adjacent to a tumor and in the tumors themselves (Tables III and IV).

Discussion

As previously shown, Epitalon inhibits chemically-induced colon carcinogenesis in rats (5). We found that Epitalon inhibited mitotic activity of the epithelial cells adjacent to a tumor and especially that of the tumor cells themselves when the treatment was given throughout the experiment. Mucosal samples near tumors showed a small, non-significant increase in mitotic activity relative to the more distant samples (12). The mean number of mitoses/crypt has been found to be similar for controls and adenomas but greater for cancers, and particularly for familial adenomatous polyposis (12). Although hyperproliferation of the normal colorectal mucosa in patients with sporadic adenomas has been described (13), no differences were found in the crypt-cell proliferation state between patients with sporadic adenomas and controls (12). Epithelial cell proliferation reflects the progression of carcino-

Table III. Effects of Epitalon on the stromal areas and lymphoid infiltration in the colon (% of the entire area of the mucosal layer at the slide, mean ± SE).

Rat groups	Areas of the mucosa located far from a tumor (A)		Areas of the mucosa adjacent to a tumor (B)		Areas of colon adenocarcinomas (C)	
	Stroma	Lymph infiltration	Stroma	Lymph infiltration	Stroma	Lymph infiltration
1	26.1±1.3	14.9±1.3	21.3±1.2[a]	11.5±0.7	17.0±1.7[a]	6.5±0.9[b]
2	32.2±1.0[c]	16.2±0.9[d]	28.0±2.1[c]	14.9±1.6[c,e]	18.3±1.4[b]	6.4±0.8[b]
3	28.8±1.3	12.5±0.9	29.2±1.4[c]	11.7±0.9	17.8±1.2[b]	5.4±0.7[b]
4	32.2±1.7[c]	13.8±0.4	24.2±1.2[a]	9.75±0.6[f]	18.7±1.3[b]	6.8±0.7[b]

Rat groups: see footnotes at Table I. [a]Significantly different from A, $p<0.05$-0.01. [b]Significantly different from A and B, $p<0.01$. [c]Significantly different from group 1, $p<0.05$-0.01. [d]Significantly different from groups 3 and 4, $p<0.05$. [e]Significantly different from groups 1, 3 and 4, $p<0.05$. [f]Significantly different from areas A and C, $p<0.01$.

Table IV. Effects of Epitalon on the intensity of lymphoid infiltration in the colon (the number of lymphoid cells per 10,000 μm^2 of the stroma, mean ± SE).

Rat groups	Areas of the mucosa located far from a tumor (A)	Areas of the mucosa adjacent to a tumor (B)	Areas of colon adenocarcinomas (C)
1	132.3±10.7	100.5±3.9[a]	41.1±4.9[b]
2	135.2±9.8	121.7±10.9	53.3±6.8[b]
3	95.9±9.2[c]	97.3±4.6	37.3±4.8[b]
4	101.3±4.0[c]	101.0±5.1	42.1±6.0[b]

Rat groups: see footnotes at Table I. [a]Significantly different from A, $p<0.05$. [b]Significantly different from A and B, $p<0.01$. [c]Significantly different from groups 1 and 2, $p<0.05$.

genesis. The histogenesis of hyperplastic polyps with atypia involves the hyperplastic polyp-carcinoma sequence, whereas the development of tubulovillous or serrated adenomas may involve the tubulovillous adenoma-carcinoma or serrated adenoma-carcinoma sequence (14).

Long-term treatment with Epitalon resulted in a significant increase in the number of apoptotic cells in tumors. Similar data were obtained in our previous study (9). We observed an increase in the AI of colon tumors in rats exposed to DMH alone as compared with the AI in the colon mucosa of the same rats, as well as controls (11). It has been suggested that apoptosis is inhibited during the progression of colon tumorigenesis (15). In azoxymethane-induced rat colon carcinogenesis, a decrease in the expression of the apoptotic repressor bcl-2 and an increase in the expression of the apoptosis accelerator, bax protein, have been shown (16). Our data appear to be relevant to this observation.

Cell kinetic status affects the macroscopic morphology of colorectal neoplasms (17). AI is closely associated with macroscopic morphology in adenomas but not in carcinomas. The MI is relatively constant among the three macroscopic types in adenomas and carcinomas, although overall MI is significantly higher in carcinomas than in adenomas, whereas AI does not differ. The macroscopic morphology of colorectal adenomas is determined by the apoptosis and not by the proliferation, and high apoptosis found in depressed adenomas implies low net growth.

We have observed a significant decrease in the mean square of stromal areas and of lymphoid infiltrates in the colon mucosa adjacent to a tumor and in tumors of rats exposed to DMH alone, as compared to intact controls. The treatment with Epitalon was followed by a reduction in these parameters. The strongest inhibitory effect of Epitalon occurred when the treatment was continued throughout the experimental period (group 2).

Similar data have been obtained in our previous study on the effect of melatonin on proliferative activity in the colon mucosa and colon tumors (9). The multiplicity of DMH-induced colon tumors in rats was significantly reduced after treatment with melatonin and this effect was correlated with an inhibition of mitosis and stimulation of apoptosis. These data are in agreement with observations of the inhibitory effect of melatonin on proliferative activity in rodent colon cells (7), grafted hepatoma cells (18) and human peripheral

blood lymphocytes (19). Melatonin decreased cell proliferation, and increased the AI in colon cancer cells (2). Moreover, melatonin significantly decreased the proliferation-to-apoptosis ratio, an additional parameter confirming the inhibition of tumor growth (2).

The finding in the present study is in accordance with our previous observations that the inhibitory effect of Epitalon on colon cancer is manifested at various stages of carcinogenesis (5,6) and especially when it is applied during the entire process of tumor development. It is suggested that Epitalon can act both directly and indirectly through the stimulation of pineal function (20). Our data confirm the hypothesis of the preventive role of the pineal gland in cancer development, realized through the action of melatonin as well as of the pineal peptides.

Acknowledgments

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