

Study of the post-natal effects of chemopreventive agents on ethylnitrosourea-induced transplacental carcinogenesis in rats.

II. Influence of low-molecular-weight polypeptide factors from the thymus, pineal gland, bone marrow, anterior hypothalamus, brain cortex and brain white substance

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The influence of the polypeptide factors extracted from thymus, pineal gland, bone marrow, anterior hypothalamus, brain cortex or brain white substance on *N*-ethyl-*N*-nitrosourea (ENU)-induced transplacental carcinogenesis was studied in rats. ENU was given to pregnant rats as a single i.v. exposure at a dose of 75 mg/kg body weight on the 21st day of gestation. The polypeptide factors were given to the offspring as a series of s.c. injections, at a dose of 0.5 mg/rat/day, starting at one or 2.5 months of age and continuing throughout the whole of post-natal life. ENU induced tumors of the brain, spinal cord, peripheral nerves and kidneys in 94–98% of the offspring exposed to the carcinogen, with an average number of 2.3–2.6 tumors per rat, and an average survival time of 294 days. Post-natal thymus factor or pineal gland factor administration was followed by an increase in mean lifespan of ~2 months and a significant decrease ($P < 0.05$) in the total tumor number per tumor-bearing rat, as well as the incidence and multiplicity of spinal cord tumors. Pineal gland factor also decreased the incidence of peripheral nerve and kidney tumors and their number per tumor-bearing rat. Brain cortex factor and brain white substance factor treatment was followed by a decrease in total tumor multiplicity of 1.2- to 3.3-fold, and a decrease in incidence of brain tumors of 10 to 33% per rat in comparison to the controls. Brain cortex factor also decreased the total tumor incidence. At the same time, brain white substance factor administration increased the incidence of peripheral nerve tumors and decreased the mean lifespan. Both bone marrow factor and anterior hypothalamus factor did not have any modifying effects on any of the ENU-induced tumors and mean lifespan. Thus, our results show the possibility of attenuation of transplacental ENU-induced carcinogenesis with post-natal administration of some polypeptide substances.

Introduction

Considerable attention has been given to the study of the regulatory effects of peptides on various specialized tissues. It has been suggested that the nervous system, the organs of the immune system, the intestine and other tissues produce the regulatory peptides that mediate intercellular and intertissue co-operation, transfer of information signals through cellular

*Abbreviations: TF, thymus factor; PGF, pineal gland factor; ENU, *N*-ethyl-*N*-nitrosourea; b.w., body weight; BMF, bone marrow factor; AHF, anterior hypothalamus factor; BCF, brain cortex factor; BWSF, brain white substance factor.

membranes, and control of genetic, epigenetic and bioenergetic functions in cells (1). The polypeptide factors from various bovine tissues were extracted, partly purified and characterized as cytomedines, a new class of biological regulators of multicellular systems (1). The biological effects of these polypeptide factors are tissue-specific and are directed mostly to the tissue of origin (1). Cytomedines easily penetrate cellular membranes and may be able to regulate differentiation and proliferation of cells, and some of them may also influence the immune and hormonal systems (1). It seemed reasonable to suggest that these polypeptide factors could have a potential in cancer chemoprevention. Two out of several available polypeptide cytomedines, thymus factor (TF*) and pineal gland factor (PGF) have been studied previously (1–3). It was shown that administration of these factors prolonged the lifespan and inhibited the development of spontaneous tumors in C3H/Sn and SHR mice (1–3). TF and PGF mostly prevented mammary adenocarcinoma development, however, the effect was less pronounced with leukemias and other tumors. The inhibitory effect of TF and PGF on the development of mammary tumors induced by 7,12-dimethylbenz[*a*]anthracene was also shown in rats (4,5). Exposure to TF and PGF also decreased the incidence of mammary gland, uterus, ovary and thyroid gland tumors, and leukemias induced by total-body X-ray irradiation (6). A number of available reports on the inhibitory effect of various preparations of thymus and pineal gland on virus-, chemical- or radiation-induced carcinogenesis and tumor growth have been reviewed (7–10). In this paper, the results of a study on the effect of polypeptide factors extracted from bovine thymus, pineal gland, anterior hypothalamus, bone marrow, brain cortex and brain white substance on post-natal development of tumors initiated transplacentally by *N*-ethyl-*N*-nitrosourea (ENU) in rats, are presented.

Materials and methods

Animals

Outbred albino rats from the Rappolovo Animal Farm of the Russian Academy of Medical Sciences and outbred LIO rats from the Petrov Research Institute of Oncology (St Petersburg) were used in the experiments. The characteristics of these rats have been described elsewhere (12,13). Rats were kept in steel cages, six in each, under a 14/10 h light/dark regimen at $22 \pm 2^\circ\text{C}$. They received standard laboratory chow and tap water *ad libitum*.

Polypeptide factor

The polypeptide factors were obtained from Cytomed (St Petersburg) and the Medical Preparation Factory (St Petersburg). They were prepared from bovine thymus, pineal gland, bone marrow, anterior hypothalamus, brain cortex and brain white substance by standard methods described in detail elsewhere (2,14). The main stages of the preparation procedure were as follows. The native tissues were kept in acetone at -4°C for 48 h. After the acetone was poured off, the tissues were homogenized and extraction was performed in 3% acetic acid at a ratio of 1:6 (v/v) in the presence of ZnCl_2 for 72 h. After the final extraction and centrifugation for 20 min at 3000 r.p.m., acetone was added to the supernatant (-4°C ; 1:8). After precipitate formation the acetone was poured off. The precipitate was treated with acetone and ether in the filter until a white powder was formed. The powder was dissolved, sterilized and lyophilized. Study of the prepared substances by ion-exchange chromatography on carboxyl cationite (Biocarb, Moscow, Russia), gel filtration on

Table I. Effects of TF, PGF, BMF and AHF on ENU-induced transplacental carcinogenesis in rats

Treatment	Effective no. of offspring	Tumor incidence, no. of tumors and average no. of tumors/rat						Mean survival, days ($M \pm SEM$)
		Total	Brain	Spinal cord	Peripheral nerves	Kidney	Others	
ENU	89	87 (97.8%)	54 (60.7%)	55 (61.8%)	37 (41.6%)	33 (37.1%)	7 (7.9%)	294 ± 7
		235	83	65	43	36	8	
		2.64 ± 0.15	0.93 ± 0.11	0.73 ± 0.07	0.48 ± 0.04	0.40 ± 0.04	0.09 ± 0.04	
ENU + TF	52	47 (90.4%)	34 (65.4%)	15 (28.8%)*	22 (42.3%)	13 (25.0%)	5 (9.6%)	355 ± 13*
		105	48	16	23	13	5	
		2.02 ± 0.12*	0.92 ± 0.09	0.31 ± 0.06*	0.44 ± 0.06	0.25 ± 0.03*	0.10 ± 0.03	
ENU + PGF	56	49 (87.5%)*	33 (58.9%)	19 (33.9%)*	15 (26.8%)*	7 (12.5%)*	3 (5.4%)	359 ± 12*
		93	44	21	18	7	3	
		1.66 ± 0.09*	0.79 ± 0.06	0.37 ± 0.06*	0.32 ± 0.06*	0.12 ± 0.03*	0.05 ± 0.03	
ENU + BMF	49	46 (93.9%)	32 (65.3%)	28 (57.1%)	15 (30.6%)	15 (30.6%)	8 (16.3%)	305 ± 12
		125	49	30	17	17	12	
		2.55 ± 0.19	1.00 ± 0.10	0.61 ± 0.07	0.35 ± 0.07	0.35 ± 0.07	0.24 ± 0.10	
ENU + AHF	52	51 (98.1%)	36 (69.2%)	36 (69.2%)	17 (32.7%)	18 (34.6%)	7 (13.5%)	280 ± 9
		143	54	41	19	21	8	
		2.75 ± 0.19	1.04 ± 0.12	0.79 ± 0.09	0.37 ± 0.06	0.40 ± 0.06	0.15 ± 0.06	

Experiments were performed in rats from the Rappolovo Farm (St Petersburg).

*Significantly different from control group given only ENU; $P < 0.05$.

Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) and electrophoresis on cellulose layers (Filtrak, Germany) showed that all the substances were complexes of polypeptides with molecular weights of 1000–10 000 Da (1,2,15) with a profile that was specific to the tissue of origin. Contamination by other tissue components contributed 1% of the total. The powder preparations were dissolved in normal saline and used in all the experiments.

Experiments

Two sets of experiments were carried out. Carcinogen ENU, 100% pure, was purchased from Sigma Chemical Co. (St Louis, MO, USA) and stored at 4°C. In the first experiment pregnant rats from the Rappolovo Animal Farm, 3–4 months old, were given a single i.v. injection of ENU dissolved in saline, 75 mg/kg body weight (b.w.), on the 21st day after conception. Their descendants of both sexes were randomized and divided into five groups. In the control group the rats exposed to ENU were not given any additional treatment during their post-natal life. In the other four groups the rats were treated with one of the polypeptide factors during all of their post-natal life. Aliquots of TF, PGF, bone marrow factor (BMF) or anterior hypothalamus factor (AHF), dissolved in 0.5 ml saline, were injected s.c. at 0.5 mg/rat/day for five consecutive days once a month from the age of 2.5 months up to the time of natural death.

In the second experiment pregnant LIO rats, 3–4 months old, were given a single i.v. injection of ENU in saline (75 mg/kg b.w.) on the 21st day after conception. The offspring of both sexes were randomized and divided into three groups. In the control group the rats exposed to ENU were not given any additional treatment. In the other two groups the rats were treated with brain cortex factor (BCF) or brain white substance factor (BWSF) throughout their post-natal life. Both preparations, dissolved in 0.5 ml saline, were injected s.c. at a dose of 0.5 mg/rat/day for five consecutive days once every 3 weeks starting at the age of one month until natural death occurred.

Pathological examination

Animals were observed until their natural death or were killed when moribund. All animals were autopsied and all organs with macroscopic lesions, in all cases the brain, spinal cord, peripheral nerves and kidneys, were fixed in 10% buffered formalin and examined histologically. Paraffin slices 5–7 µm thick were stained with hematoxylin and eosin. Tumors discovered were classified according to IARC recommendation (16).

Statistics

Experimental results were statistically processed according to IARC recommendation (17). The Student's *t*-test and chi-square test were also performed. The statistical significance of any apparent increases or decreases of effect with regard to polypeptide substances was assessed by combining the tests for fatal and incidental tumors (17).

Results

The results of the two sets of experiments are presented in Tables I and II. Since there were no significant differences between male and female nervous system and kidney tumor

incidence, or tumors at other sites, the data were compiled irrespective of rat sex.

As shown in Tables I and II, ENU mostly induced tumors of the brain, spinal cord, peripheral nervous system and kidneys. The majority of brain and spinal cord tumors were classified as oligodendrogliomas, mixed oligoastrocytomas and astrocytomas. Ependymomas, meningiomas and glioblastomas were rarely present. Tumors of the peripheral nervous system were mainly localized in the nervi trigemini and rarely in the plexus lumbosacralis, plexus brachialis, radices of the spinal cord or other tissues. The majority of the peripheral nervous system tumors were malignant schwannomas and, in some cases were mixed malignant tumors with both sarcomatous and neurogenic components. Almost all kidney neoplasias were classified as mesenchymal tumors, and in some cases as epithelial adenocarcinomas.

The incidence of spontaneous tumors (pituitary and thyroid adenomas, mammary fibroadenomas and adenocarcinomas, leukemias and others) characteristic for the rat strains used has been reported before (12,13). The rate of these tumors in the present experiment was not significantly changed by transplacental ENU, either with or without modifying factors. Their total incidence is shown in Tables I and II. We have never found nervous system and kidney tumors developing spontaneously in the rat strains used in this experiment (12,13).

None of the modifying agents used in these experiments had any toxic effects in rats. All animals developed normally and there were no significant differences in body weight when animals exposed to ENU were given peptide preparations compared with those treated with peptides alone.

Effects of TF, PGF, BMF and AHF

Results showed that TF increased the mean lifespan by ~2 months and decreased by 1.3-fold the total number of tumors per tumor-bearing rat in comparison to those in the ENU-only control group (Table I). Treatment with TF was followed by a 33% decrease in the incidence of spinal cord tumors and by a 2.4-fold decrease in their multiplicity. The number of kidney tumors per rat in rats exposed to ENU and TF was decreased by 1.6-fold compared to the group exposed to ENU only.

The inhibitory effect of PGF on ENU-induced transplacental

Table II. Effects of BCF and BWSF on ENU-induced transplacental carcinogenesis in rats

Treatment	Effective no. of offspring	Tumor incidence, no. of tumors and average no. of tumors/rat						Mean survival, days ($M \pm SEM$)
		Total	Brain	Spinal cord	Peripheral nerves	Kidney	Others	
ENU	101	95 (94.0%)	73 (72.3%)	47 (46.5%)	30 (29.7%)	18 (17.8%)	8 (7.9%)	304 ± 6
		236	122	53	34	19	8	
		2.34 ± 0.12	1.21 ± 0.08	0.52 ± 0.04	0.34 ± 0.04	0.19 ± 0.04	0.08 ± 0.02	
ENU + BCF	46	37 (80.4%)*	26 (56.5%)*	17 (37.0%)	11 (23.9%)	6 (13.0%)	—	288 ± 11
		77	37	21	13	6	—	
		1.67 ± 0.10*	0.80 ± 0.07*	0.46 ± 0.07	0.28 ± 0.07	0.13 ± 0.03	—	
ENU + BWSF	47	46 (97.9%)*	27 (57.4%)*	18 (38.3%)	21 (44.7%)*	9 (19.1%)	4 (8.5%)	270 ± 10*
		90	34	19	23	10	4	
		1.91 ± 0.10*	0.72 ± 0.07*	0.40 ± 0.07	0.49 ± 0.07	0.21 ± 0.07	0.08 ± 0.03	

Experiments were performed in rats from the Petrov Research Institute of Oncology (St Petersburg).

*Significantly different from control group given only ENU; $P < 0.05$.

carcinogenesis was more pronounced than that of TF. PGF increased the average lifespan by 2 months; decreased the total tumor incidence by 10.3% and the number of tumors per tumor-bearing rat by 1.6-fold in comparison to the control (ENU only) group. Treatment with PGF decreased the incidences of spinal cord tumor by 27.9%, peripheral nerve tumors by 14.8% and kidney tumors by 24.6%. PGF also diminished the multiplicities of tumors at these locations by 2.0-, 1.5- and 3.3-fold, respectively, as compared to the control group.

However, neither TF nor PGF affected the incidence or multiplicity of brain tumors, which were the most common type of tumor in rats exposed to ENU transplacentally.

Thus, post-natal exposure to TF and PGF attenuated the transplacental carcinogenic effect of ENU in rats.

Table I shows that neither BMF nor AHF had any effect on the incidence of any kind of tumor or on average lifespan.

Effects of BCF and BWSF

Table II shows that LIO rats from the Petrov Research Institute of Oncology, exposed to the same dose of ENU as the other rats developed more tumors of the brain and less tumors of the spinal cord, peripheral nerve and kidney, as compared to rats from the Rappolovo Animal Farm. BCF decreased the total tumor incidence by 13.6% and the number of tumors per tumor-bearing rat by 1.4-fold, in comparison to the ENU-only control group. Exposure to BCF also decreased the incidence of brain tumors by 15.8% and their multiplicity by 1.5-fold as compared to the control group.

Treatment with BWSF was followed by a decrease in the total tumor multiplicity of 1.2-fold, in brain tumor incidence of 14.9% and multiplicity of 1.7-fold, in comparison to the control group. However, at the same time this treatment led to an increase in the incidence of peripheral nerve tumors by 15.0% and shortened the average lifespan of rats by 1 month as compared to the control group.

Discussion

It has been shown that after transplacental exposure to even low doses of ENU in rats, preneoplastic lesions in the target tissues (brain) could be detected in offspring within the first few weeks of their post-natal life (18), when we started treatment with the various polypeptide factors. Therefore, their effect could presumably be realized at stages of promotion and progression of tumorigenesis.

The polypeptide factors TF, PGF, BCF and BWSF inhibited

the development of tumors induced by transplacental exposure to ENU in rats. The mechanisms of anticarcinogenic effect of these factors are not clear. The fact that two among all tested polypeptide preparations, BMF and AHF, had no significant influence on tumor development allows us to suggest that the preventive effect of some of them was not observed due to non-specific causes only. Failure of some of the agents to inhibit the development of brain tumors is in accordance with this conclusion.

AHF administration depresses T cell immunity and so serves as a form of negative control to the immunostimulating activity of TF and PGF (2). AHF had no anticarcinogenic effect in our experiment. BMF, which stimulates B cell immunity (15), also failed to modify the transplacental carcinogenic effect of ENU in rats. It is suggested that immunomodulating agents do not influence the development of brain tumors in experimental animals (19). Our data show that TF, PGF, AHF and BMF administration had no influence on the development of transplacentally ENU-induced brain tumors.

It is likely that all of these polypeptide preparations exerted multiple effects on the organism. A major effect of TF is the stimulation of T cell immune function (14) and this mechanism might contribute to the anticarcinogenic action of TF. A number of immunostimulatory peptides with hormone-like activity were isolated from thymus (20), and TF, being a complex mixture of thymic peptides, has some of these activities. It was shown that various immunostimulatory agents such as BCG vaccine (21), chronic toxoplasma infection (22), and bovine and human albumins (23,24) did not inhibit the development of tumors in rats exposed transplacentally to ENU. The anticarcinogenic effect of TF using the same model might indicate a more powerful or specific immunostimulatory activity of this factor.

PGF can also stimulate T cell immunity (2,8,9). At the same time the normalizing influence of PGF on some hormonal—metabolic shifts, which develop during carcinogenesis and aging (such as elevation of threshold of hypothalamic sensitivity to homeostatic influence of peripheral hormones; alterations in lipid and carbohydrate metabolism; decrease in glucose tolerance; hyperinsulinemia; hypercholesterolemia; and metabolic immunodepression), may play an important role in the mechanism of the anticarcinogenic effects of this preparation (2,8,9). It has been shown that the above-mentioned hormonal—metabolic shift progressively developed in offspring after transplacental exposure to methylnitrosourea (25)

or diethylstilbestrol (26). It is worth noting that in this study as well as in our previous work (2–6,8), the anticarcinogenic effects of PGF were more pronounced, similar to those of TF.

The ability of polypeptides extracted from central nervous tissue to normalize differentiation and regulate proliferation of brain glial cells (1) was a reason for BCF and BWSF testing of their anticarcinogenic activity. Nerve growth factor, to which BCF and BWSF may be related, inhibited the development of preneoplastic and neoplastic lesions in the nervi trigemini in rats after transplacental exposure to ENU (27). In our experiment, treatment with BCF and BWSF limited their effects to the nervous system only. They were suppressive for brain tumors, but had no effect (a once only BWSF) on peripheral nerve tumors. BCF also has the ability to stimulate T cell immunity similar to that of TF (28), and this may contribute to its chemopreventive effect. Since AHF was extracted from brain tissue, it served as a control for the specificity of BCF and BWSF in our study.

Knowledge of the chemical structure and mechanism of action of these polypeptide factors is necessary, and there is no doubt that several polypeptides could be prospective anticarcinogenic agents. Hypotheses of the role of immunostimulation and/or correction of hormonal–metabolic shifts for inhibition of transplacental carcinogenesis, suggested by some of our data, may now be further investigated. This ongoing effort will be important in establishing criteria for high cancer risk in humans exposed to chemicals (29) and in understanding the role of regulatory peptides in the realization of transplacental carcinogenesis. Finally, it is worth noting that the commercial drug forms of TF (thymalin) and PGF (epithalamin) are permitted by the Pharmaceutical Committee of the Russian Ministry of Health for medical use, and some results from treatment of cancer patients with these substances have already been reported (30,31).

Acknowledgements

We would like to thank Dr Lucy Anderson (NCI, Frederick, MD, USA) for helpful discussions.

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Received on August 22, 1995; accepted on May 8, 1996