

Chemical sympathectomy suppresses fibrosarcoma development and improves survival of tumor-bearing rats

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Both experimental and clinical data indicate that the sympathetic nervous system may affect the development of certain tumors. To test this, in the present study we combined *in vivo* and *in vitro* approaches to study the effect of the sympathetic nervous system on proliferation of BP6-TU2 fibrosarcoma cells. First, we investigated the effect of 6-hydroxydopamine-induced sympathectomy on tumor development and survival of tumor-bearing rats. One week after chemical sympathectomy, we injected the BP6-TU2 fibrosarcoma cells intraperitoneally into male Wistar rats. The sympathectomy significantly reduced the incidence of intraperitoneal tumors and resulted in significantly improved survival of tumor-bearing rats compared to those with intact sympathetic innervation. Using immunohistochemical methods, we found neuron-specific enolase immunopositive structures within fibrosarcoma tissue, indicating innervation of tumors. Finally, an *in vitro* study showed elevated proliferation of BP6-TU2 fibrosarcoma cells in response to adding norepinephrine to the culture medium. Our findings indicate that sympathetic nerves directly potentiate the proliferation of BP6-TU2 fibrosarcoma cells in rats.

Key words: BP6-TU2 fibrosarcoma cells; chemical sympathectomy; innervation; norepinephrine; neuron-specific enolase; sympathetic nervous system.

The broad and dense innervation of tissues and organs by the sympathetic nervous system participates in the precise regulation of bodily functions during both physiological and pathological conditions [1]. Furthermore, several findings indicate that the sympathetic nervous system represents a critical factor that may also affect processes related to tumor development, progression, and formation of metastasis [2]. Recent evidences propose various putative pathways and mechanisms by which the sympathetic nervous system might realize its effects on tumor growth. Sympathetic nerves represent one of the most important pathways by which the brain can modulate the activity of immune cells via neurotransmitters (norepinephrine, neuropeptide Y) released into innervated immune organs [3, 4]. Modulation of immune cells proliferation, angiogenesis, vessel permeability, lymphocyte traffic, and cytokine production by immune cells create the basis for proposing that there is an indirect effect of the sympathetic nervous system on processes related to carcinogenesis as well as the development of metastases [5]. For example, sympathetic innervation

of the primary and secondary lymphoid organs, as well as the presence of β - and α -adrenergic receptors on different types of immunocompetent cells may allow the sympathetic nervous system to markedly influence the initial phase of the immune response to tumor cells by modulation of the production and mobilization of immune cells [3, 4]. Moreover, norepinephrine may also down-regulate the cytotoxicity of natural killer cells, which are particularly important in the elimination of metastatic tumor cells [6, 7]. There is also a growing body of evidence that psychological and behavioral factors that alter the activity of the sympathetic nervous system may in turn affect the motility of tumor cells and invasion [8]. Distress and depression are associated with important processes of carcinogenesis, including poorer repair of damaged DNA, an increase in the frequency of sister chromatid exchange, and alterations in the processes related to apoptosis [6].

Furthermore, in the last few years it has been suggested that the sympathetic nervous system might also influence tumor growth directly. This assumption is based on evidence

of the innervation of certain tumors [9]. Likewise, tumors have been shown to initiate their own innervation by releasing neurotrophic factors including nerve growth factor, brain-derived growth factor, and vascular endothelial growth factor [10]. These and other factors play a role in forming a direct “neural-tumor” connection called neuro-neoplastic synapses [11].

The aim of the present study was to investigate the effect of chemical sympathectomy on intra-abdominal tumor development and survival of tumor-bearing rats. The sympathectomy was induced by intraperitoneal pretreatment with the neurotoxin 6-hydroxydopamine (6-OHDA), which selectively destroys sympathetic nerve endings. Tumor growth was then induced by an intraperitoneal injection of BP6-TU2 fibrosarcoma cells into Wistar rats. Moreover, we also investigated the presence of neural structures in tumor tissue, as well as the effect of the norepinephrine on the *in vitro* proliferation of BP6-TU2 fibrosarcoma cells.

Materials and methods

Animals. Eighty-eight male Wistar rats, obtained from AnLab (Prague, Czech Republic), were housed 4 per cage and maintained under controlled conditions (12 hr light-dark cycle – lights on at 6:00 am, ambient temperature 22 ± 2 °C and $55 \pm 10\%$ humidity). Animals had free access to tap water and standard pellet rat chow. The animals were handled daily and protected from external noises to minimize possible stress. All experimental procedures were approved by the Animal Care Committee of the Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovakia.

Experimental protocol. Before the experiment, the rats were acclimatized to the animal room for 7 days. They were then randomly divided into 4 experimental groups based on the type of treatment: I) animals injected with tumor cells only (BP6-TU2, $n = 36$); II) sympathectomized rats injected by tumor cells (BP6-TU2 + 6-OHDA, $n = 36$); III) sympathectomized rats without injection of tumor cells (6-OHDA, $n = 8$); and IV) untreated absolute controls (AC, $n = 8$). All experimental procedures were performed between 8:00 – 13:00 am. During the experiment, body weight (3 times per week), water and food intake (2 times per week) were recorded and the health status and survival of rats was monitored daily.

Chemical sympathectomy. A chemical sympathectomy was performed in conscious animals (BP6-TU2 + 6-OHDA and 6-OHDA group) by intraperitoneal injection of 6-OHDA (100 mg/kg body weight, Sigma-Aldrich, Germany) over two consecutive days. The 6-OHDA was dissolved in sterile saline containing 0.1% of the antioxidant ascorbic acid (Sigma-Aldrich, Germany). This treatment has been shown to induce the destruction of peripheral sympathetic nerve endings after 3-5 days and this effect lasted for at least 21 days [12]. Efficiency of the sympathectomy was confirmed by presence of ptosis in the sympathectomized rats (Claude

Bernard-Horner’s syndrome) as well as by the presence of blood in urine immediately after 6-OHDA application, indicating the destruction of sympathetic nerve endings in the urinary tract.

Injection of tumor cells. One week after chemical sympathectomy, BP6-TU2 fibrosarcoma cells were intraperitoneally injected into the animals in the BP6-TU2 and BP6-TU2 + 6-OHDA groups. Tumor cells were cultured in RPMI 1640 medium containing 10% fetal calf serum plus antibiotics (kanamycin and streptomycin) at 37°C, in 5% CO₂. Seventy-two animals weighting 185 ± 12 g were injected with a single dose of 0.5×10^6 BP6-TU2 fibrosarcoma cells in 2.0 ml of RPMI 1640 medium per rat without anesthesia. Control animals did not receive an injection of cells.

Tumor development. Animals in all groups were observed until the last animals with tumors had died. Any animals that were injected with BP6-TU2 cells that did not develop tumors as determined by examining the abdomen for tumor protrusion were sacrificed after the 79th day following injection to confirm the lack of tumor formation and growth.

Immunohistochemistry. Tumor tissue samples were taken from one randomly chosen rat found to have tumor developed. The animal was killed by decapitation 28 days after injection of BP6-TU2 tumor cells. The tumors were then removed and fixed in phosphate-buffered 4% formaldehyde (pH 7.0), and routinely processed for immunohistochemistry via paraffinization. The tumors were then cut into 3 µm thick sections. Prior to immunohistochemistry, the sections were deparaffinized and rehydrated in phosphate-buffered solution (PBS). Before immunohistochemical analysis, microwave epitope retrieval was performed (10 mM citrate buffer, pH 6.0; 20 minutes at 96°C). The slides were then subsequently incubated for 1 hour with mouse anti-neuron-specific enolase (Dako, Glostrup, Denmark) diluted 1:50 in DAKO Real antibody diluent (Dako, Glostrup, Denmark). After 3 rinsing steps of 5 minutes each in PBS, sections were incubated for 30 minutes with Histofine antibody polymer conjugated with horseradish peroxidase (Nichirei Biosciences, Tokyo, Japan). After 3 rinsing steps of 5 minutes each in PBS, the peroxidase activity was visualized with diaminobenzidine (Dako, Glostrup, Denmark) using the protocol supplied by the manufacturer and the sections were then counterstained with hematoxylin.

In vitro experiment. Tumor cells were seeded at the concentration of 500 cells/well in 500 µl of RPMI 1640 medium on 48-well plates (Sigma-Aldrich, Germany) and incubated at 37 °C and 10% CO₂. Stock solutions of norepinephrine (Sigma-Aldrich, Germany) were prepared in distilled water and were added once to 24 wells to obtain concentrations of 100 µM/well, while the other 24 wells were used as a control. Cell growth was monitored daily by adding a trypsin solution (5 mM EDTA in PBS, 0.025% trypsin solution, Sigma-Aldrich, Germany) to a randomly chosen well to dislocate the adherent monolayer of fibrosarcoma cells for counting in a Bürker chamber. The arithmetical average was calculated from the

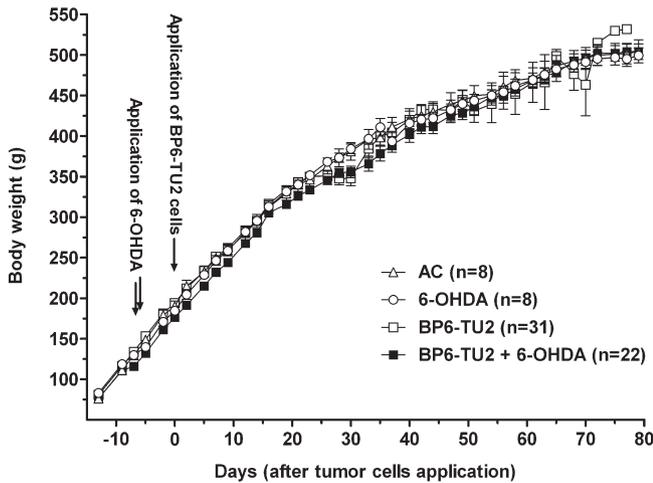


Figure 1. Time course of body weight gain of rats: (Δ) controls (AC), (\circ) animals pretreated with 6-OHDA without injection of tumor cells (6-OHDA), (\square) animals injected with tumor cells with intact sympathetic nerves (BP6-TU2), (\blacksquare) sympathectomized animals injected with tumor cells (BP6-TU2 + 6-OHDA). The arrows mark the days when 6-OHDA and BP6-TU2 cells were injected. Each value is the mean \pm SEM.

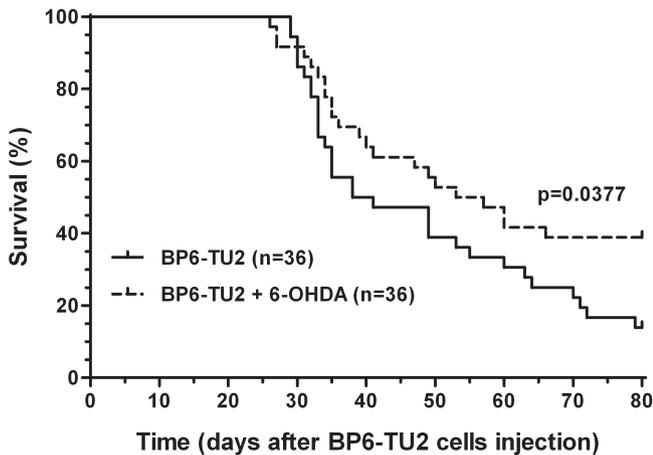


Figure 3. Survival curves of rats injected with tumor cells with an intact sympathetic nervous system (—) and sympathectomized rats injected by tumor cells (---).

cells counts of 3 wells for each group. This monitoring was performed for 7 days.

Statistical analyses. The data were analyzed using GraphPad Prism, Version 5. Significant differences between and within the groups were determined by using a two-way ANOVA using treatment and time as factors as well as a Student's *t*-test when appropriate. Comparison of the survival curves was analyzed by Log-rank test. Data are expressed as the mean value \pm SEM and $P < 0.05$ was considered statistically significant.

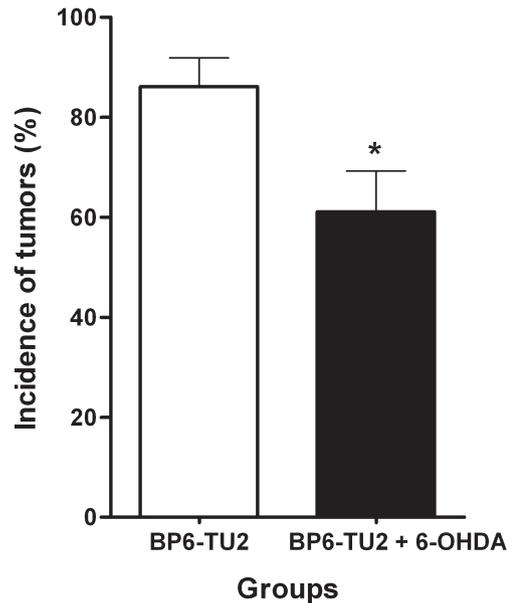


Figure 2. The percentage of tumor incidence in rats with an intact sympathetic nervous system injected by tumor cells (BP6-TU2, \square ; $n = 36$) and sympathectomized rats injected with tumor cells (BP6-TU2 + 6-OHDA, \blacksquare ; $n = 36$). Each value is the mean \pm SEM. Statistical significance compared to matched group * - $p < 0.05$.

Results

Body weight of animals. As shown in figure 1, regardless the presence of tumors in animals of the BP6-TU2 and BP6-TU2 + 6-OHDA groups, we did not find any significant differences in body weight between rats in groups injected by tumor cells (BP6-TU2 and BP6-TU2 + 6-OHDA) and controls (AC and 6-OHDA group).

Incidence of tumors. Sympathectomy significantly reduced incidence of tumors ($p = 0.0152$). We found tumor masses in the abdominal cavity of 31 of 36 animals (87%) with an intact sympathetic nervous system injected with tumor cells (BP6-TU2 group) compared to 22 of 36 rats (61%) in the group that received 6-OHDA before injection of BP6-TU2 cells (Fig. 2). We did not find any metastasis in tumor-bearing rats.

Survival of tumor-bearing animals. A significant difference between the survival of the tumor-bearing rats with intact sympathetic nerves (BP6-TU2 group) and the sympathectomized tumor-bearing rats (BP6-TU2 + 6-OHDA group) was observed ($p = 0.0377$). We found that the survival during the experiment for animals that underwent chemical sympathectomy was significantly improved in comparison with animals that did not receive 6-OHDA pretreatment (Fig. 3).

Immunohistochemistry. Tumors induced by application of BP6-TU2 fibrosarcoma cells have the morphological characteristics of fibrosarcomas and are characterized histologically by the presence of monomorphic spindle-shaped tumor cells with mild nuclear atypia. Immunohistochemical staining

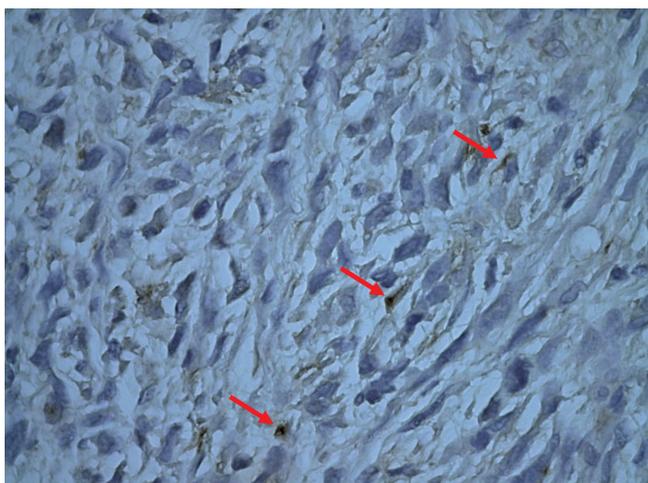


Figure 4. Micrograph showing the presence of neuron-specific enolase immunopositivity in fibrosarcoma tumor tissue. Arrows indicate localization of putative nerves (horse radish peroxidase, diaminobenzidine, 400x)

of neuron-specific enolase confirmed the presence of nerve structures in the investigated tumor tissue indicating its innervation (Fig. 4).

In vitro study. After adding norepinephrine to growth medium containing BP6-TU2 fibrosarcoma cells we observed a significant increase in the proliferation of tumor cells following the sixth day after treatment. At the end of *in vitro* study, number of BP6-TU2 cells treated by norepinephrine exceeded number of untreated BP6-TU2 cells by 75.5 % (Fig. 5).

Discussion

In the present study, the role of the sympathetic nervous system in BP6-TU2 fibrosarcoma cell proliferation was investigated by three approaches. We studied 1) the effect of chemical sympathectomy on the proliferation of BP6-TU2 fibrosarcoma cells in Wistar rats, 2) the presence of neural structures in tumor tissue, and 3) the influence of norepinephrine on BP6-TU2 fibrosarcoma cells growth *in vitro*. In the sympathectomized rats injected by BP6-TU2 fibrosarcoma cells we found a markedly reduced incidence of tumors and significantly prolonged survival in comparison with rats with an intact sympathetic nervous system injected by BP6-TU2 fibrosarcoma cells. We also found positive immunohistochemical signals for the general neuronal marker, neuron-specific enolase, in tumor tissue. Moreover, using this *in vitro* approach we found that norepinephrine significantly increased the growth of BP6-TU2 cells.

Several studies highlight the role of the sympathetic and parasympathetic nervous systems in the development of tumors and metastases. For example, our results are in accordance with the study of Tatsuta et al. [13] who showed that prolonged intraperitoneal treatment with 6-OHDA attenuated the incidence of N-methyl-N'-nitro-N-nitrosoquinidine

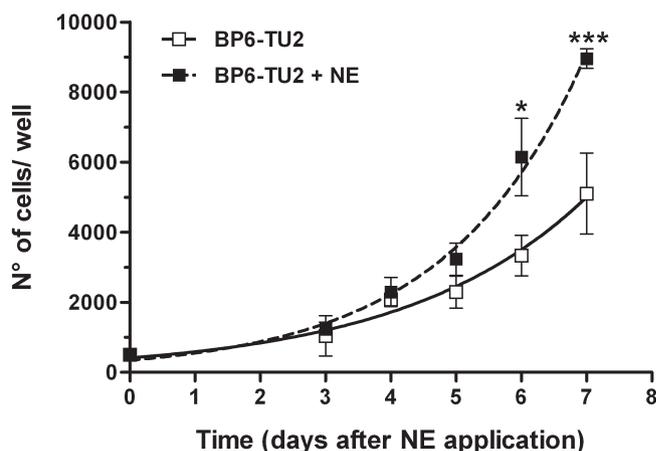


Figure 5. *In vitro* growth of BP6-TU2 fibrosarcoma cells with (■) and without (□) 100 μ M norepinephrine (NE) added to the medium. Each value is the mean \pm SEM. Statistical significance compared to matched group * - $p < 0.05$; *** - $p < 0.001$.

(MNNG) induced gastric cancer in rats. Likewise, Raju et al. [5] demonstrated that bilaterally sympathectomized rats develop significantly smaller, more diffuse in appearance, and less invasive tumors compared with sham operated rats. The above-mentioned authors have suggested that their findings may reflex decreased interstitial fluid pressure and lymph vessel area. Grzanna et al. [14] studied the effects of 6-OHDA on tumor development and growth in mice with murine LPC-1 plasmacytoma cells. They found that sympathectomy significantly altered the growth pattern of tumors [14]. In another study of Raju et al. [2] they found that sympathetic denervation significantly alters the gene-expression profile of tumor cells. They found 280 genes differentially expressed in tumors with intact sympathetic innervation compared with the bilaterally sympathectomized tumors. Alterations were found in genes associated with cell adhesion and signaling structure, proliferation, metabolism, lymphangiogenesis, growth and development, and immunity. However, it is necessary to take in consideration also the fact that in sympathectomized animals the activity of the parasympathetic nervous system may prevail. It is known that parasympathetic nervous system, particularly the vagus nerve, exerts potent anti-inflammatory effects [15]. Therefore, improved survival of sympathectomized tumor-bearing rats may reflect not only reduced sympathetic influence but also prevalent effect of intact parasympathetic nerves.

It is well established that norepinephrine, the principal neurotransmitter of sympathetic nerves, is an etiological factor in some types of cancer [16]. Three aspects of the body's noradrenergic system are relevant to this assumption. 1) Norepinephrine circulates in the blood and is released by both peripheral and central sympathetic nerve terminals allowing it to access tissues and organs throughout the body. 2) Many different cells possess adrenergic receptors on their

outer surface and are therefore responsive to norepinephrine. 3) By binding to its extracellular receptors, norepinephrine affects intracellular second messenger systems that could influence processes related to carcinogenesis, proliferation of tumor cells, angiogenesis and development of metastases [16]. Yang et al. [17] have shown that norepinephrine treatment upregulates the production of vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), and interleukin 6 (IL-6) in C8161 cells by inducing gene expression of the mentioned molecules. This suggests that norepinephrine may stimulate the aggressive potential of melanoma tumor cells, in part by inducing the production VEGF, IL-8, and IL-6 [17]. However, the role of norepinephrine in tumor neo-angiogenesis is still not completely understood [18]. Our *in vitro* study demonstrated a direct effect of norepinephrine on the growth of BP6-TU2 cells, which was markedly stimulated on the sixth day after the treatment. Some of the cancer cells are known to express receptors for several neurotransmitters, including adrenergic receptors [11]. Likewise, it has been shown that β_2 -adrenergic receptors are highly expressed in oral squamous cell carcinoma and this expression is significantly correlated with tumor size and cervical lymph node metastasis [19]. Moreover, β_2 -adrenergic receptors are also expressed in human breast cancer and their stimulation is associated with increased cell proliferation [20]. Bastian et al. [21] have also clearly shown that norepinephrine potentiates migratory activity of human PC-3 prostate, SW 480 colon, and MDA-MB-468 breast carcinoma cells. In contrast, the same authors have shown that human ES-2 ovarian carcinoma cells have reduced migratory activity after norepinephrine treatment [21]. Data from our *in vitro* study support the assumption of a direct stimulatory effect of norepinephrine on BP6-TU2 tumor cells; most likely via occupation of adrenergic receptors expressed by the tumor cell itself.

It has been demonstrated that surgical or chemical sympathectomy might alter immune responses in rodents [3]. However, the sympathetic nervous system may exert both pro- and anti-inflammatory influence by regulating immunity at a regional level through innervation of immune organs including the spleen, thymus and lymph nodes [4, 22]. In addition to the innervation of immune organs, the sympathetic fibers may also participate in the innervation of tumor tissue itself. Although, it has been generally assumed that tumors are not innervated, Seifert and Spitznas [9] found nerve fibers within an adenoma of the ciliary body epithelium of the eye and later in tumors of the urinary bladder [23]. Because norepinephrine stimulates proliferation of BP6-TU2 cells *in vitro*, we suggest that the presence of neural tissue in BP6-TU2 tumors might create a basis for our observed direct sympathetic effect on proliferation of BP6-TU2 cells *in vitro* that may participate in the higher tumor incidence and shorter survival in animals with intact sympathetic nerves injected by tumor cells. Since we used a general neuronal marker, neuron-specific enolase, for detection of innervation of tumor tissue, it is not possible to distinguish whether detected nervous structures are of

sympathetic or parasympathetic origin. However, because sympathetic nerves innervate a majority of tissues and blood vessels, we suggest that positive immunohistochemical staining for this neuronal marker indicates the sympathetic phenotype of the nerves innervating fibrosarcoma tissue.

In summary, our data indicate that the sympathetic nervous system plays an important role in modulation of proliferation of BP6-TU2 fibrosarcoma cells in rats. Based on our findings of decreased incidence of tumors and prolonged survival in sympathectomized rats, along with our observations of the presence of nervous structures in fibrosarcoma tissue, and findings of norepinephrine-induced stimulation of BP6-TU2 fibrosarcoma cell proliferation *in vitro*, we suggest that the sympathetic nervous system exerts a stimulatory effect on the proliferation of BP6-TU2 fibrosarcoma cells in rats most likely directly by norepinephrine released locally within tumor tissue.

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