BRAIN RESPONSE TO INDUCED PERIPHERAL CANCER DEVELOPMENT IN RATS: DUAL FOS-TYROSINE HYDROXYLASE AND FOS-OXYTOCIN IMMUNOHISTOCHEMISTRY

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Objective. During last few decades a considerable number of data has emerged supporting the hypothesis that central nervous system might monitor and modulate tumor growth. This assumption is based on two facts: 1. immune system plays a crucial role in the development and progression of cancer; 2. immune and nervous systems communicate tightly and bidirectionally. The aim of present study was to elucidate whether tumor growth may induce detectable changes in brain structures that are involved in the response to immune challenges.

Methods. Using Fos immunohistochemistry, we investigated whether the advanced stage of cancer, induced by a single intraperitoneal injection of BP6-TU2 fibrosarcoma cells to male Wistar rats, could activate Fos expression in the nucleus of the solitary tract (NTS), amygdala and parabrachial nuclei (PBN) and also activate some of neuronal phenotypes including tyrosine hydroxylase (TH) neurons in the brainstem noradrenergic cell groups and hypothalamic oxytocinergic neurons.

Results. Twenty eight days after the initiation of tumor process we found increased Fos expression in NTS/A2, A1 noradrenergic cells, PBN as well as in the hypothalamic paraventricular, supraoptic and accessory oxytocinergic neurons. These structures are involved in the transmission of signals related to immune challenges within the brain and consequent elaboration of neuroendocrine responses.

Conclusions. The data obtained are supporting the view that the information on peripheral tumor development might be transmitted to the brain. However, further studies are necessary to be performed to reveal whether our findings can be attributed to specific effect of cancer or whether observed changes in the activity of brainstem and hypothalamic neurons reflex processes that only accompany the cancer progression.

Keywords: Brainstem noradrenergic cells – Cancer – Hypothalamic oxytocinergic cells – Immune system – Nervous system – Cancer neurobiology – Rat

Development of cancer is a complex process modulated by a number of different factors from which many remain still unknown (Hanahan and Weinberg 2000; Mareel and Leroy 2003). Cancer progression is modulated by tumor-related circumstances and also by characteristics of the host. Tumor-related conditions include...
the tumor aggressiveness that is determined by the tissue type and the degree of its dedifferentiation, functionality of apoptosis, DNA repair mechanisms, loss of contact inhibition, and ability to induce a vascular supply and metastasis. Resistance of the host depends on the immune competence (Dunn et al. 2004). It is becoming more evident that also neuro-immune mechanisms which are subordinated to the brain and behavior, play a role in the defense against cancer development and its progression (Berczi et al. 1998; Entschladein et al. 2002; Lang et al. 2006; Sephton and Spiegel, 2003). It has been hypothesized that the brain might monitor and modulate the process of tumorigenesis (Esteban et al. 2006; Gidron et al. 2005; Mravec et al. 2006; Mravec and Hulin, 2006). Based on this hypothesis as well as on experimental and clinical observations, a novel view of tumor etiopathogenesis was emerged, which has been entitled neurobiology of cancer as explained by Mravec et al. (2008). However, it is necessary to take in consideration that the interactions between the cancer and brain are considerably complicated and according to the nature of the neoplasm many variations may occur (Conti 2000).

There exist a number of studies attempting to circumscribe possible effects of psycho-neuro-immunological interactions on the genesis and progression of cancer. These publications underlined mainly the role of descending pathways represented by sympathoadrenal and hypothalamo-pituitary adrenocortical systems in the modulation of tumorigenesis (Kiecolt-Glaser and Glaser, 1999; Reiche et al. 2005; Spiegel 1999). However, the number of studies dealing with the transmission and processing of cancer-related signals by the brain, are still limited (Kerkozien et al. 1999; Konsman and Blomqvist, 2005).

The aim of present study was to elucidate whether the advanced stage of tumor growth, induced by a single intraperitoneal injection of BP6-TU2 fibrosarcoma cells, may evoke detectable changes in the brain structures that are involved in the response to immune challenge, including brainstem viscerosensory relay nuclei (noradrenergic cell groups A1, A2, A5, A6, parabrachial nucleus and brainstem circumventricular organ area postrema), hypothalamic neuroendocrine centers (paraventricular, supraoptic, accessory nuclei) and such center for emotional events as amygdala, by employing Fos immunohistochemistry. In selected areas also TH and OXY phenotypic identifications of activated neurons were performed by using Fos/TH and Fos/OXY dual immunohistochemistry. Materials and Methods

Animals. All experiments were carried out in male Wistar rats (obtained from AnLab, Prague, Czech Republic). During the experiment, the animals were kept in an animal facility under controlled conditions (12 h light/12 h dark cycle, lights at 06:00 h; temperature, 22±1 °C) and with free access to tap water and standard pelleted rat chow. The experiments were performed between 08:00-14:00 h and the animals were protected from all external noises or other possible stressful stimuli. All experimental procedures were approved by the Animal Care Committee of the IEE SAS Bratislava, Slovak Republic. The investigation conforms also to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Induction of tumors. Rat fibrosarcoma cell line (BP6-TU2) routinely cultured in RPMI 1640 medium containing 10 % FCS plus antibiotics was used. Animals (n=25) weighing 150±20 g were injected intraperitoneally by a single injection of 0.5 x 10^6 amount of the fibrosarcoma cells dispersed in 2.0 ml of serum-free RPMI 1640 medium. Control rats (n=12) were exposed to the same volume of the serum-free medium. The tumors developed in 68 % of animals that received intraperitoneal injection of BP6 cells, while in 32 % of injected rats no tumor mass was macroscopically detectable in the abdominal cavity. During the experiments, 11 % of animals failed to survive the process of tumor growth. At the end of experiment, i.e. 28 days after cancer cells implantation, the rats were sacrificed by intracardial perfusion with fixative. The process of tumor growth was considered to be effective when 28 days after the cancer cells implantation a tumor mass was developed in the abdominal cavity. No metastases were detected in tumor-bearing animals which showed a 6.5 % loss of body weight when compared to control animals at the day of perfusion.

Imunohistochemistry. The rats were anesthetized by pentobarbital (50 mg/kg, Spofa, Prague, Czech Republic) and perfused transcardially with 250 ml fixative solution consisting of 0.1 M phosphate buffer (PB, pH 7.4) containing 4 % paraformaldehyde. Then the brains were removed, postfixed in the same fixative overnight at 4 °C and infiltrated with 15 % sucrose in 0.025 M PB for 48 h at 4 °C. Quickly frozen brains in cold isopentane (-30/-40 °C) were sectioned into 30 µm thick coronal sections over the hypothalamus, caudal midbrain, and brainstem areas in a Reichert cryocut device adjusted to −16 °C. The sections were collected as free floating in a cold (+4 °C) PB.
The sections were washed in PB and preincubated with 3 % H₂O₂ for 30 min. Then they were incubated with a polyclonal Fos protein antiserum (No 94012, 1:2000), in 0.1 M PB containing 4 % normal goat serum (Gibco, Grand Island, NY, USA), 0.5 % Triton X-100 (Koch-Light Lab. Ltd., Colnbrook, Berks, England), and 0.1 % sodium azide (Sigma Chemical Ltd., St. Louis, MO, USA) for 48 h at 4 °C. After several washes in PB, the sections were incubated with biotinylated goat-antirabbit IgG (1:500, VectorStain Elite ABC, Vector Lab., Burlingame, CA, USA) for 90 min. Next PB rinses were followed by incubation with avidin-biotin peroxidase complex (1:250) for 90 min. PB washes were followed by rinsing in 0.05 M sodium acetate buffer (SAB, pH 6.0). The Fos reaction was visualized with 0.03 % 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) in SAB containing 0.003 % H₂O₂ and 2.5 % nickel ammonium sulfate (Sigma). After several washes in PB, the Fos-positive sections were incubated with TH (1:2000) or OXY (1:2000) antibodies using the same procedure as described above. The TH and OXY immunoreactivities were developed by 0.0125 % DAB chromogen dissolved in 0.05 M Tris (pH 7.4) containing and 0.003 % H₂O₂ until an appropriate tawny color was reached. Finally, the sections were rinsed in 0.05 M Tris buffer followed by 0.05 M SAB buffer, mounted into 0.1 % of gelatine dissolved in 0.0125 M SAB, air-dried, coverslipped with Permount (Sigma), and examined under Leica DMLS light microscope. Immunostaining of negative control, which did not show any antisera immunolabeling, included substitution of the primary antisera with normal rabbit serum, and sequential elimination of the primary or secondary antibody from the staining series.

Evaluation of immunostainings. The cell counting was performed on 30 µm thick serial coronal sections employing a computerized system that included Leica DMLS light microscope equipped with a Canon digital camera (PowerShot S40). The quantitative assessment was performed from the captured images on a computer screen obtained from 4-8 brain sections/ rat from each brain area selected, i.e. Fos only in the parabrachial nucleus, amygdala, and NTS; Fos/TH colocalizations in the A1, A2, A5, A6, A7 and the area postrema brainstem cell groups, and Fos/OXY colocalizations in the paraventricular, supraoptic, and accessory nuclei of the hypothalamus. The percentage of activated TH or OXY neurons was calculated from the ratio of the amount of TH or OXY perikarya displaying Fos signal deducting from the total TH or OXY immunolabeled perikarya x 100.

Antibodies. Fos antiserum (No 94012) was raised against the N-terminal peptide similar to 2-17 of the rat Fos protein according to the protocol described elsewhere (MIKKELSEN et al. 1998) and was provided by Dr. J.D. Mikkelsen (NeuroSearch A/S Ballerup, Denmark). The polyclonal antisera to oxytocin and tyrosine hydroxylase were purchased from Chemicon International, Inc. (Temicula, CA, USA), cat # AB 911 and cat # AB 151, respectively.

Statistical evaluation. All data represent the mean ± SEM. For statistical comparisons t-test was used, p<0.05 being considered as statistically significant.

Results

Effect of tumors on single Fos expression. In the amygdala, hypothalamus (Fig. 1A, C), PBN (Fig. 2A), and brainstem of controls, i.e. animals injected intraperitoneally with serum-free medium, single Fos labeled perikarya occurred only sporadically. In contrast to controls, a clear accumulation of Fos immunoreactivity was found in PVN (Fig. 1B), SON (Fig. 1D), several accessory nuclei (Fig. 3A), PBN (Fig. 2B), NTS/A2 (Fig. 2C) and A1 (Fig. 2D) noradrenergic cell groups in the tumor-bearing rats. In the amygdalar subdivisions and A7 noradrenergic cell group no visible response to tumor growth was observed (not shown), i.e. the amount of the Fos labeled neuronal perikarya in these areas did not exceed the amount of Fos labelings observed in the control animals.

Effect of tumors on the hypothalamic oxytocinergic neurons. Hypothalamic sections of control rats injected intraperitoneally with serum-free medium, showed only a sparse Fos expression over the whole hypothalamus, however none of OXY perikarya exhibited Fos immunoreactivity.

Oxytocin immunoreactive neurons expressing Fos (Fos/OXY) were analyzed in hypothalamic paraventricular nucleus, SON, and magnocellular accessory (Acc) cell groups. The greatest incidence of Fos/OXY colocalizations was found in PVN (Fig. 4A) and adjacent periventricular nucleus (Fig. 3B) of tumor-bearing rats, while in SON (Fig. 4B) and Acc (Fig. 3A,C) the Fos/OXY colocalizations were observed less frequently. Among the Acc magnocellular nuclei mainly the circular (Fig. 3A) and perifornical ones (Fig. 3C) revealed Fos/OXY double stainings.

Effect of tumors on the brainstem noradrenergic neurons. Incidence of TH-immunoreactive neurons expressing Fos immunoreactivity (Fos/TH) was count-
Fig. 1 Effect of 28 days lasting tumor growth on the Fos expression in the PVN and SON neurons. In the PVN (A) and SON (C) of controls only scattered Fos is observable while in the PVN (B) and SON (D) of tumor-bearing rats many cells display Fos immunoreactivity. Abbreviations: V – 3rd ventricle; OCH – optic chiasm.

Fig. 2 Effect of 28 days lasting tumor growth on the Fos expression in the parabrachial nucleus and Fos/TH colocalizations in the NTS/A2 and A1 cell groups. Parabrachial nucleus of tumor-bearing rats (B) contains markedly more Fos profiles than controls (A). In the NTS/A2 (C) and A1 (D) areas of tumor-bearing rats many free Fos and a few colocalized Fos/TH (arrows) are visible. Abbreviations: pcs – superior cerebellar peduncle.
ed in the noradrenergic cell groups localized in pons (A5, A6, A7) and medulla oblongata (A1, NTS/A2). Brainstem sections from control rats injected i.p. with serum-free medium, showed only a sparse Fos expression in many brain areas including the medulla oblongata. However, none of the investigated noradrenergic cell groups exhibited Fos/TH colocalizations in controls (not shown).

In tumor-bearing animals, the highest degree of colocalizations of TH/Fos neurons was observed in NTS/A2 noradrenergic cell group (Fig. 2C) (8.0 %, p < 0.05). Substantially lower incidence of TH/Fos double labeled perikarya was seen in the cells of A1 noradrenergic cell group (Fig. 2D) (1.4 %) of the lateral reticular nucleus. Other noradrenergic cell groups, including A5 and A7, exhibited less than 1 % of Fos/TH colocalizations in the tumor-bearing rats (Fig. 5).

The number of Fos activated cells in locus coeruleus (LC, A6), with respect to the high density of TH immunoreactive cells, was not counted as a ratio of Fos/TH colocalizations but only as a total number of superimposed Fos profiles over the LC territory delineated by TH cell perikarya. In the tumor-bearing animals, the Fos profiles occurred only sporadically in LC, i.e. 5-6 Fos profiles/1 LC area. Likewise LC, area postrema also revealed only a limited number of Fos activated cells, i.e. 2-3 Fos profiles/1 AP area/section (not shown).

**Discussion**

The aim of this study was to reveal more information regarding possible interactions between the tumor tissues and brain. Our attempt was to assess whether the peripheral tumor growth lasting 28 days and induced by direct intraperitoneal implantation of fibrosarcoma cells may affect some brain areas that are sensitive to immune challenges. As indicator of cell activation, Fos protein immunohistochemistry was employed (Hoffman et al. 1993). Although this protoontogen is usually
used for the identification of acute cell responses, many studies, including ours (Pirnik et al. 2003) clearly show that Fos protein presence can also be used as a marker of chronic as well as repeated stimulations of nerve cells (Hebert et al. 2005).

In the present study we demonstrate that 28 day lasting peripheral experimental tumor process shows increased Fos expression of neurons in several brain areas including TH immunoreactive cells in the NTS/A2, A1 noradrenergic cells and PBN as well as in OXY synthesizing cells in the hypothalamic paraventricular, supraoptic, and accessory nuclei. Since these structures are involved in the transmission of signals related to immune challenges within the brain and in the elaboration of appropriate neuro-endocrine responses, we imply that the brain may receive information about cancer development at the periphery and it can probably also modulate its progression.

Involvement of the nervous system in the monitoring and modulation of development and progression of cancer has been indicated by several clinical and experimental data (Erin et al. 2004; Erin et al. 2006; Hodgson et al. 1998; Hodgson et al. 1999; Leo and Bonneau, 2000). General approach is focused on the role of sympathoadrenal system and hypothalamo-pituitary adrenocortical axis in the modulation of tumor progression by the brain (Antoni et al. 2006; Bendlivajeh et al. 2007). Although some published data deals also with the hypothesis that the brain may monitor the process of tumor growth (Gidron et al. 2005; Mravec et al. 2006; Mravec and Hulin, 2006) studies concerning the transmission of tumor-related signals to the brain are rather sporadic.

Immunohistochemical mapping of Fos expression in tumor-bearing rats (Kergozién et al. 1999) demonstrate that peripheral tumor growth may stimulate neu-

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Fig. 4 Effect of 28 days lasting tumor growth on the Fos/OXY colocalizations in the PVN and SON neurons. In both the PVN (A) and SON (B) of tumor-bearing rats several OXY cell display Fos immunoreactivity (arrows). Inserted pictures show a detailed view on the Fos/OXY double labeled perikarya. Abbreviations: V – 3rd ventricle; OCH – optic chiasm.

Fig. 5 Effect of 28 days lasting tumor growth on the Fos expression in tyrosine hydroxylase immunoreactive cells in brainstem of controls (C; n=3), and tumor-bearing (T; n=4) rats. Each value is the mean ± SEM. Statistical significance compared to matched control group: * - p<0.05. Abbreviations: NTS – nucleus of the solitary tract; NTSc – nucleus of the solitary tract; commissural part; A1, A5, A7 – brainstem noradrenergic cell groups.
rons in central nervous system. Thus, Konsman and Blomquist (2005) have revealed changes in the activity of forebrain structures indicating that these changes are related to the tumor-associated anorexia-cachexia. Besides animal studies, changes in the brain activities have been also described in patients suffering from cancer (Tashiro et al. 2000). However, the interpretation of the findings obtained in patients remains still difficult.

Single Fos expression was markedly elevated in NTS and PBN, less distinctly in the area postrema, but it was completely missed in the amygdala of tumor-bearing rats. Our attempt was to reveal Fos response in TH and OXY neuronal phenotypes involved in the response of tumor growth in the brainstem noradrenergic cell groups and in the hypothalamic paraventricular, supraoptic and accessory nuclei. Actually, these structures are known to participate significantly in the transmission and processing of signals related to immune challenges (Gaykema et al. 2007; Hollis et al. 2004; Masek et al. 2003; Yang et al. 2000). Moreover, oxytocin has been shown to have immunomodulatory properties (Yang et al., 1997) and his relevance to tumorigenesis come out from his role in suppressing the proliferation of tumor cells (Cassoni et al. 2004).

From our experiments several assumptions may be drawn as related to the monitoring and modulation of tumor progression by the brain. Since the tumor occurrence was restricted to the abdominal cavity, we assume that the increased Fos incidence in NTS/A2 and A1 TH labeled neurons in tumor-bearing animals may indicate the transfer of peripheral signals to NTS by the vagus nerve. Actually, the vagus nerve quite densely innervates the peritoneal cavity and NTS neurons represent the relay station for immune signals carried by ascendent vagal pathway (Goehler et al. 2000). We suppose that increased Fos immunoreactivity in NTS/A2 and the activation of TH immunolabeled neurons in NTS/A2 may indicate that the vagus nerve could be involved in the transmission of information related to peripherally localized tumor progression and that catecholaminergic cells are also a part of this process. It is well documented that visceral fibers of the vagus nerve contain a variety of sensory receptors (Paintal 1973) and that the vagal sensory neurons themselves express mRNA for IL-1 receptors (Ek et al., 1998). Therefore, we assume that cytokines produced by immune cells, as a response to tumor proliferation, might activate the sensory afferents of the vagus nerve, which might subsequently transmit signals from the abdominal cavity to the nucleus of the solitary tract.

However, our data do not allow to determine, whether the increased Fos immunoreactivity in NTS neurons may reflect solely the immune system reaction to tumor growth, or whether the activation of these neurons represents a reaction to activated abdominal mechanoreceptors as a consequence of the tumor mass growth in abdominal cavity in which even the changes in food passage or other concurrent processes accompanying tumor proliferation may interfere. Moreover, it is necessary to take into consideration that besides of vagal afferent pathway activation, the role of primary afferent fibers of neurons localized in dorsal root ganglia of abdominal spinal cord cannot be excluded regarding the activation of NTS/A2 noradrenergic neurons (Nance and Sanders, 2007). In addition, the tumor-related signals might reach the NTS cells also by a humoral way (Quan and Banks, 2007). However, this case seems to be less likely, since we found only very limited Fos immunoreactivity in the area postrema cells of tumor-bearing rats when compared to matched controls. Eventually, we can speculate that the faint increase of Fos expression in area postrema neurons might reflect an adaptation of neurons to long lasting activation to cancer-related humoral changes. A1 noradrenergic neurons also participate in the transmission of immune signals to higher brain structures, including parabrachial nucleus, hypothalamus, and amygdala (Gaykema et al. 2007). There remains a question whether low levels of Fos expression in A1 cells might indicate the involvement of these neurons in the transmission of cancer-related signals from the lower brainstem to the forebrain structures. Parabrachial nucleus is an important structure involved in the transmission of immune signals within the brain (Buller et al. 2004). Increased Fos expression in these neurons in tumor-bearing rats might indicate involvement of PBN in the transmission of tumor-related signals to forebrain structures.

We also found increased Fos expression in PVN of tumor-bearing rats. It is well known that particularly the PVN neurons contribute significantly to central response elicited by systemic immune challenge (Buller et al. 2003; Yang et al. 1997). Moreover, PVN represents an important nodal point for the coordination of autonomic, endocrine, and immune systems activities (Wrona 2006). Therefore we suggest that the activation of the PVN neurons may be one of the acceptable facts indicating that the brain might elaborate a neuroendocrine-immune response to tumor growth.
In conclusion, our findings support the notion that specific brain areas may be informed about the tumor progression at the periphery, i.e. far from the brain, which could provide a basis for a neurobiological view on cancer (Mravec et al. 2008). However, further studies including selective blockade of different types of peripheral receptors, using a wider scale of activation markers and different time intervals, are necessary to be performed to reveal whether our findings can be attributed to specific effect of cancer or whether observed changes in the activity of brainstem and hypothalamic neurons reflex processes that only accompany the cancer progression (increased intraperitoneal pressure, anorexia-cachexia, ect.).

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