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SYMPATHECTOMY DECREASES SIZE AND INVASIVENESS OF TONGUE CANCER IN RATS

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Abstract—The sympathetic nervous system plays a role in carcinogenesis wherein locally released sympathetic neurotransmitters affect proliferation, angiogenesis, vessel permeability, lymphocyte traffic and cytokine production. The present *in vivo* study was designed to investigate whether surgical sympathectomy, both unilateral and bilateral, had an effect on tumor growth, interstitial fluid pressure (IFP) and lymphatics in rat tongue cancer. We used 4-nitroquinoline-1-oxide (4-NQO) in drinking water for 19 weeks to induce tongue cancer in 20 Dark Agouti rats. After 11 weeks, one group underwent unilateral sympathectomy and another underwent bilateral sympathectomy, while the third group underwent sham surgery. By 19 weeks, tumors in the bilaterally sympathectomized (BL-SCGx) rats were significantly smaller ($P<0.05$), more diffuse in appearance and less invasive ($P<0.05$) compared with the large exophytic tumors in the sham-operated rats. The relative lymphatic area was significantly decreased ($P<0.05$) in tumors in the BL-SCGx rats compared with the sham group. Interestingly, the tumors in rats that underwent unilateral or bilateral sympathectomy had a significantly lower ($P<0.05$) IFP than those in sham rats. Lack of tyrosine hydroxylase (TH) immunoreactive nerves and few neuropeptide Y (NPY) positive fibers indicate absence of sympathetic nerve fibers in the bilateral sympathectomized group. The peritumoral lymph vessel area was correlated with the tumor size ($P<0.001$), depth of invasion ($P<0.001$), weight of rats ($P<0.005$) and IFP ($P<0.05$). In conclusion, the present study presents evidence that deprivation of sympathetic nerves decreases tumor growth in rat tongue, probably caused by decreasing IFP and lymph vessel area.

Key words: sympathetic nerves, interstitial fluid pressure, immunohistochemistry, tongue cancer, lymphatics, NPY.

Interstitial fluid pressure (IFP) is increased in solid tumors (Gutmann et al., 1992; Heldin et al., 2004) and several studies indicate that high IFP in tumors is correlated with poor prognosis (Curti et al., 1993; Milosevic et al., 2001). The mechanisms that contribute to the increased IFP are not completely known, but most likely involve increased vessel permeability, abnormal lymph vessels and interstitial fibrosis (Heldin et al., 2004). Increased vascular permeability followed by increased interstitial colloid osmotic pressure and decreased lymph drainage will both increase the tumor interstitial fluid volume according to Starling's equation, whereas increased interstitial fibrosis may lower the compliance of the tumor tissue. Even a small increase in fluid volume will raise the IFP in low compliant tissues (Aukland, 1984).

In general, the high IFP in solid tumors is associated with a poor blood supply that may act as a barrier for efficient drug delivery and lack of therapeutic effects (Jain, 1987; Heldin et al., 2004). In addition, mechanical stretch induced by the high IFP may trigger cell proliferation (Takei et al., 1997; Hofmann et al., 2006). Reduction of tumor IFP is consequently of importance in the treatment of malignant tumors.

The sympathetic nervous system (SNS) plays a significant role in inflammation and immune responses (Elenkov et al., 2000), and accumulating evidence suggests that sympathetic postganglionic nerves have an effect on vessel permeability and tumor growth. Linde et al.(1974) demonstrated increased vascular permeability after sympathetic nerve stimulation, whereas Engel (1978) reported that sympathectomy reduced plasma extravasation in the knee joint. Furthermore, sympathectomy inhibits plasma extravasation evoked by injection of bradykinin, prostaglandin and substance P (Mathison and Davison, 1994; Miao et al., 1996). Superior cervical ganglionectomy causes reduction of intraocular pressure in rabbits (Zhan et al., 1999), and mice subjected to unilateral superior ganglionectomy demonstrated slowed growth of breast cancer (Romeo et al., 1991). In humans, increased sympathetic tone due to stress increases the rate of tumor progression and cancer-related death (Kiecolt-Glaser et al., 2002).

Both noradrenaline (NA) and neuropeptide Y (NPY) are stored in vesicles and are released when sympathetic nerves are activated. They are shown to affect circulation, lymphocyte traffic and cytokine production (Elenkov et al., 2000). Noradrenaline synthesis depends on tyrosine hydroxylase (TH), a rate-limiting enzyme that converts tyrosine to dopamine, which is then converted to NA in vesicles. Both noradrenaline and dopamine increase migration of human breast carcinoma cells (Drell et al., 2003). Recent studies indicate that catecholamines may influence the progression of ovarian cancer and nasopharyngeal carcinoma by modulating the expression of angiogenic cytokines (Lutgendorf et al., 2003; Yang et al., 2006). Accumulating evidence that noradrenaline induces growth of vascular smooth muscle cells and adventitial fibroblast

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Abbreviations: 4-NQO, 4-nitroquinoline-1-oxide; BL, bilateral sympathectomy; IFP, interstitial fluid pressure; LYVE-1, lymphatic vessel endothelial receptor 1; NA, noradrenaline; NPY, neuropeptide Y; PA, arterial blood pressure; PBS, phosphate-buffered saline; SCC, squamous cell carcinoma; SCG, superior cervical ganglion; SNS, sympathetic nervous system; TH, tyrosine hydroxylase; UL, unilateral sympathectomy.

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(Zhang and Faber, 2001; Zhang et al., 2004) supports the idea that sympathetic nerves play a role in cell proliferation. Recently, NPY was suggested to increase vascular permeability (Kurimoto et al., 2004), promote vessel sprouting and act as a growth factor for various types of cells (Hansel et al., 2001; Pons et al., 2003; Kitlinska et al., 2005). Moreover, NPY is suggested to play a role in angiogenesis by stimulating proliferation and migration of endothelial cells (Lee et al., 2003). Taken together, there exists a vast body of experimental evidence that the sympathetic nerves affect tumor growth.

We hypothesize that surgical sympathectomy of the superior cervical ganglion (SCG) will inhibit tongue tumor growth, possibly due to reduction of IFP and tumor cell proliferation or by affecting the lymphatic/vascular supply.

EXPERIMENTAL PROCEDURES

Dark Agouti male rats, purchased from Harlan Sprague–Dawley, Inc. (The Netherlands) were housed in the animal facility at the Institute of Biomedicine, University of Bergen, Norway. The animals were kept on a 12-h light/dark cycle and maintained on standard rodent pellets with drinking water available *ad libitum*. All the experimental procedures were carried out with the approval of the Norwegian Experimental Animal Board. All efforts were made to minimize the number of animals used and their suffering.

Carcinogen treatment

In order to induce tongue cancer, 6-week-old rats were treated with 4-nitroquinoline 1-oxide (4-NQO, Sigma–Aldrich, Saint Louis, MO, USA) in drinking water at a concentration of 50 ppm for 19 weeks (Fig. 1A).

Removal of superior cervical ganglion

After 11 weeks of 4-NQO treatment the rats were randomly divided into three groups. Group 1 underwent unilateral surgical sympathectomy (UL-SCGx, $n=7$), group 2 underwent bilateral sympathectomy (BL-SCGx, $n=6$) and group 3 underwent sham surgery (sham, $n=7$). Animals were anesthetized by s.c. injection of 2.7 ml/kg Hypnorm-Dormicum (Hypnorm; Janssen Pharmaceutica, Belgium, Dormicum; F. Hoffman-La Roche AC, Switzerland). Briefly; a vertical incision was made on the ventral surface of the neck adjacent to the midline. The SCG was identified dorsal and medial to the bifurcation of the common carotid artery and dissected out with microscissors as described previously (Haug and Heyeraas, 2003). Ganglionectomy was confirmed by observing ptosis in the eyes (Bernard-Horner syndrome) on the ipsilateral side. By week 19, the rats were subjected to IFP measurements using the micropuncture technique (Fig. 1A).

Micropuncture measurements of IFP

Anesthesia was induced by i.p. administration of 0.1 ml/100 g mebumal (50 mg/ml sodium phenobarbital, Svaneapoteket, Bergen). The rats were placed on a servocontrolled heating pad to maintain constant body temperature and tracheotomized to facilitate free breathing. A femoral vein was catheterized for supplemental anesthesia and an artery for continuous recording of systemic arterial blood pressure (PA) with a Spectramed pressure transducer and Gould recorder. The head was immobilized, the lower jaw opened, and the tongue gently pulled out in position to measure IFP in the tumor as previously described (Tonder and Kvinnsland, 1983).

In short, micropuncture was performed with sharpened glass pipettes under the guidance of a stereomicroscope; the pipette was slowly advanced 500–1000 μm into the tissue. Simultaneous repeated measurements of IFP in the tongue tumor and PA were performed. The rats were killed by rapid i.v. injection of 50–100 mg sodium phenobarbital and the tongues were thereafter excised. The most posterior part of tongue comprising the main tumor was fixed overnight in 4% paraformaldehyde containing 0.2% picric acid for histological studies. After cryoprotection with 30% sucrose, the specimens were stored at −80 °C until sectioning. Hematoxylin—eosin-stained sections were prepared and reviewed for classification as well as measurements of the depth of invasion of the tumors.

Immunohistochemistry

Serial cryostat sections (40 μm) of the tumor tongues were cut and mounted on gelatin-coated slides. Sections were rinsed in phosphate-buffered saline (PBS) several times and incubated with methanol containing 0.3% hydrogen peroxide to inactivate endogenous peroxidase activity. Preincubation was done in 2.5% normal goat serum (Vector Laboratories, Inc., Burlingame, CA, USA) for 1 h. In order to visualize sympathetic nerves, the sections were incubated in polyclonal antibody raised in rabbit, against NPY (dilution 1: 4000, Peninsula Laboratories, San Carlos, CA, USA) for 72 h at 4 °C or monoclonal antibody raised in mouse, against TH (dilution 1:2000; Neuromics, Minneapolis, MN, USA). Lymph vessels were visualized by incubating the sections in polyclonal antibody raised in rabbits against lymphatic vessel endothelial receptor-1 (LYVE-1) (dilution 1:1000; Abcam, Cambridge, UK) for 72 h at 4 °C. Following several rinses in PBS, secondary antibody incubation was in goat anti-rabbit or horse anti-mouse biotinylated immunoglobulin G (1:100, Vector Laboratories) for 1 h or overnight as previously described (Haug et al., 2001). Antigen–antibody complexes were detected using the avidin–biotin peroxidase (ABC) kit (Vectastain ABC kit; Vector Laboratories) and visualized by nickel enhanced 3, 3′-diaminobenzidine (Sigma–Aldrich). Finally, the sections were counterstained with Richardson's stain, coverslipped and evaluated using a photomicroscope. Controls for the specificity of the immunoreactions were routinely included by substitution of the primary or secondary antiserum with PBS.

Quantification

Depth of tumor invasion. The invasive depth of the tumors was measured from the surface of the adjacent normal epithelium to the deepest reaching front of infiltration in central serial hematoxylin–eosin-stained sections. The measurements were performed at magnifications of 4× or 10×, using a Nikon E600 microscope and a Nikon DXM 1200F digital camera (Nikon, Tokyo, Japan) connected to an image analysis program (NIS-Elements, AR 2.30; Nikon) containing the required features. The mean values of the invasive depth in millimeters were calculated for each rat.

Relative area of lymph vessel. LYVE-1 immunoreacted sections were scanned at low magnification and areas with the highest lymph vessel density, "hot spots," were chosen in the intratumoral and peritumoral region as well as in dysplastic epithelium and adjacent submucosa. Four hot spots per section from two to three sections at least 40 μm apart were selected. LYVE-1-positive vessels that were located in tumor parenchyma were identified as intratumoral lymph vessels. Peritumoral lymph vessels were defined as LYVE-1-positive vessels located at the tumor periphery within a distance of 250 μm from the tumor edge, because the highest density of lymph vessels was observed within this area. The sections were examined using a Nikon E600 microscope with a Nikon DXM 1200F digital camera (Nikon). Relative area occupied by the lymph vessels was determined by morphometric analyses with an image analysis program (NIS-Elements, AR 2.30). The area examined per field was 0.07 mm^2 in the peritumor and dysplastic tongue submucosa, at a magnifica-