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CENTRAL GLIAL ACTIVATION MEDIATES CANCER-INDUCED PAIN IN A RAT FACIAL CANCER MODEL

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Abstract—Peripheral and central glial activation plays an important role in development of pain hypersensitivity induced by inflammation and nerve injury. However, the involvement of glial cells in cancer pain is not well understood. The present study evaluated the peripheral and central glial activation and the effect of an inhibitor of glial activation, propentofylline, on pain-related behaviors in a rat facial cancer model of the growth of Walker 256B cells in the unilateral vibrissal pad until days 3–4 post-inoculation. As compared with sham animals, the facial grooming period was prolonged, the withdrawal latency to radiant heat stimulation was shortened, and the withdrawal threshold by von Frey hair stimulation was decreased at the inoculated region, indicating the development of spontaneous pain, thermal hyperalgesia and mechanical allodynia. In immunostainings for Iba1 and glial fibrillary acidic protein (GFAP), although there were no morphological changes of GFAP-immunopositive satellite glial cells in the trigeminal ganglion, Iba1-immunopositive microglia and GFAP-immunopositive astrocytes in the medullary dorsal horn showed large somata with cell proliferation. After the daily i.p. administration of propentofylline beginning preinoculation, the central glial activation was attenuated, the prolonged facial grooming was partially suppressed, and the induced allodynia and hyperalgesia from day 2 were prevented, without a change in tumor size. These results suggest that glial activation in the CNS, but not in the peripheral nervous system, mediates the enhancement of spontaneous pain and the development of allodynia and hyperalgesia at an early stage in the facial cancer model.

Key words: facial cancer, cancer pain, medullary dorsal horn, astrocyte, microglia, propentofylline.

Most patients with head and neck tumors complain of orofacial pain (Chaplin and Morton, 1999; Grond et al., 1996). Because cancer pain is sometimes intractable, even with morphine treatment (Mercadante and Arcuri, 1998; Mercadante, 1997), there is an urgent need to improve the quality of life of patients with incurable cancer and to seek means to prevent the development of cancer pain. Recently, to understand effect of tumor on the trigeminal nerve, which is concerned with nociceptive signals in the orofacial region, we developed a rat facial cancer model (FCM) by inoculating Walker carcinosarcoma 256B cells into the unilateral vibrissal pad (Harano et al., 2010; Ono et al., 2009; Nagahata et al., 2007). In the FCM, mechanical allodynia and thermal hyperalgesia are developed at the inoculated region from day 2 post-inoculation (Harano et al., 2010). As the tumor grows larger, the hypersensitive pain-receptive facial area enlarges in the facial region, and feeding difficulty, as seen clinically in orofacial cancer patients, frequently occurs on day 10 post-inoculation (Ono et al., 2009).

It is thought that the causes of pain hypersensitivity in neuropathic and inflammatory models are related to the up-regulation of neuropeptides like substance P and calcitonin gene-related peptide in inflammation (Honoré et al., 2000; Tang et al., 2007), and galanin in nerve injury (Coronel et al., 2008; Honoré et al., 2000; White et al., 1993) in the neuronal soma in the primary sensory ganglion and in the synaptic terminals in the projection sites. However, in our developed FCM, there are no changes in the neuropeptides in the medullary dorsal horn (DH), and both allodynia and hyperalgesia are less sensitive to indomethacin (Harano et al., 2010). Thus, mechanical allodynia and thermal hyperalgesia in the FCM are not mediated by the neurochemical changes due to secondary inflammation and nerve damage, which are different from other cancer pain models (Eliav et al., 2004; Nagamine et al., 2006; Peters et al., 2005; Shimoyama et al., 2002).

Glial activation has recently been raised as another mechanism of pain hypersensitivity (Inoue and Tsuda, 2009; Sessle, 2007; Takeda et al., 2007, 2009). Activated glial cells have manifested large somata with many thick processes and cause abnormal neuronal activities due to the release of various chemical mediators (e.g. pro-inflammatory factors, brain-derived neurotrophic factor, and glutamine) in inflammatory and neuropathic models (Coull et al., 2005; Inoue and Tsuda, 2009; Okada-Ogawa et al., 2009; Takeda et al., 2007, 2009; Tsuda et al., 2003). In the trigeminal and dorsal root ganglions (TRG and DRG), activated satellite glial cells release several inflammatory and immune mediators in response to inflammation and nerve damage and influence other sensory neurons within the ganglion (Gunjigake et al., 2009; Takeda et al., 2007, 2009; Woodham et al., 1989). In the medullary and spinal DH, inflammation and nerve damage have been reported to induce the activation of microglia and/or astrocytes (Coull et al., 2005; Dubner and Ruda, 1992;

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Abbreviations: ATF3, activating transcript factor 3; DH, dorsal horn; DRG, dorsal root ganglion; EXT, tooth-extraction; FCM, facial cancer model; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; PPF, propentofylline; TRG, trigeminal ganglion.

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Inoue and Tsuda, 2009; Okada-Ogawa et al., 2009; Tsuda et al., 2003). Although similar glial activation has also been reported in bone cancer models (Honoré et al., 2000; Peters et al., 2005; Schwei et al., 1999; Zhang et al., 2005), it is possible this induction is caused by secondary inflammation and nerve injury due to bone destruction (Peters et al., 2005; Zhang et al., 2005). Interestingly, microglia activation in the DH has been recently reported to be induced by the intense electrical stimulation of C-fibers without nerve damage (Hathway et al., 2009), suggesting inflammation/nerve injury-independent central glial activation. Our previous study shows that the FCM showed the prolongation of facial grooming, which is a sign of spontaneous pain (Ono et al., 2009). Hence, the central glial activation may be operative in the mechanism of cancer pain in the FCM, independent of inflammation and nerve damage.

In the present study, to examine this possibility, we investigated appearances of glial cells in the TRG and the medullary DH and measured the effect of an inhibitor of glial activation, propentofylline (PPF), on pain-related behaviors in the FCM. Because our previous studies showed that day 3–4 post-inoculation in the model was suitable experimental days as the early stage of cancer pain induction (Ono et al., 2009; Harano et al., 2010), the present study was performed until the days to investigate mechanisms at development of facial cancer pain.

EXPERIMENTAL PROCEDURES

Experimental animals

Young male Wistar rats (50–60 g, $n=37$) were used in the present study. Rats were exposed to a light-dark cycle (L:D, 12:12-h) and kept in a temperature- and humidity-controlled room (22–25 °C and 40–60%) with food and water *ad libitum*. All experiments were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and were approved by the Animal Experiment Committee of the Kyushu Dental College. Efforts were made to minimize the number of animals used and animal suffering.

GFP-gene transfection into cancer cells

To confirm tumor size and metastasis in the FCM, green fluorescent protein (GFP) gene-transfected cancer cells were used, similar to the cells used in other cancer models (Hoffman and Yang, 2006; Yang et al., 2000). Walker carcinosarcoma 256B cells (kindly provided by Shionogi Pharm. Co., Japan) were cultured in 1.5 ml of culture medium (MEM alpha; Invitrogen, CA, USA) without serum and antibiotics in four 35-mm culture dishes (Falcon, NJ, USA) at the time of transfection. The transfection of pAcGFP-C1 DNA (Takara Bio, Shiga, Japan) was performed in Opti-MEM medium (Invitrogen, CA, USA) using GeneJuice (Merck, Germany), according to the manufacturer's protocol. After the transfection, cancer cells were incubated in culture medium with fetal bovine serum (10%), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a CO_2 incubator at 37 °C. Subsequently, cells were passaged in 98-well plates, and they were cultured in the presence of 100–1000 μg/ml of Geneticin (Wako, Japan) for the selection of cells that highly expressed GFP.

Preparation of FCM and administration of propentofylline

The GFP-expressing Walker 256B cells were harvested via trypsinization and washed with FBS-containing culture medium to inactivate trypsin activity, followed by washing three times with phosphate-buffered saline. After cell counting using a hemocytometer, cell density was finally set to be 4×10^7 cells/ml in phosphate-buffered saline. The rats were anesthetized with thiopental (50 mg/kg) and then injected with cancer cells (4×10^6 cells in 0.1 ml, as FCM; $n=24$) or phosphate-buffered saline alone (0.1 ml, as sham; $n=9$) into the right vibrissal pad. PPF (Sigma-Aldrich, MO, USA; 10 mg/kg, in saline) or vehicle (saline only, 0.5 ml) was administrated i.p. 30 min before the inoculation of the cancer cells (day 0) and daily after the observations of pain-related behaviors until day 3 post-inoculation (each $n=9$). The concentration of PPF was selected based on prior studies demonstrating the clear suppression of glial activation in nerve injury models of rat and mice (Sweitzer et al., 2001; Tawfik et al., 2008).

The GFP-expressing tumor tissues in all FCM rats were excited by 475-nm, blue LED (Twin Light, RelyOn Ltd., Japan), and captured using a digital camera (PowerShot A650 IS; Canon, Japan) through a 515-nm long pass filter. In some FCM rats, while observing GFP fluorescence with the excitation of blue LED, the tumor tissue was taken out and weighed after removing as much of the surrounding, non-fluorescent tissues as possible (non-administered FCM, $n=6$ and PPF- and vehicle-administered FCM, $n=4$ each).

Behavioral observations for pain tests

The behaviors of the sham and FCM groups were observed on day 0 (pre-inoculation of cancer cells) and day 3 post-inoculation (sham, $n=5$ and FCM, $n=6$). The measurements included facial grooming periods, head withdrawal latencies after radiant heat, and head withdrawal thresholds to von Frey hair stimulation (Semmes-Weinstein monofilament set, Muromachi, Japan; pressures 0.16, 0.4, 0.6, 1.0, 1.4, and 2.0 g) against the inoculated and contralateral skin surfaces of the vibrissal pads, as described in our previous studies (Harano et al., 2010; Ono et al., 2009). The von Frey hair test was performed near the eyelid and at the lower lip of the ipsilateral side. Facial grooming was measured as the amount of rubbing and/or scratching on the inoculated facial region using the forepaws for 10 min. The grooming of regions other than the inoculated facial region was not evaluated. The radiant heat stimulus was a focused 7×7 mm^2 beam of light from a modified thermo-stimulator (DPS-705, Dia Medical System Co., Japan), and the aperture was 10 cm from the stimulation site. When measuring the head withdrawal latencies after radiant heat and the head withdrawal thresholds to von Frey hair stimulation, each measurement was repeated five times at 2 min intervals and averaged after the exclusion of maximal and minimal values. The latter mechanical withdrawal threshold during each test period was described as a percentage of that prior to the inoculation of cancer cells (day 0). In PPF- and vehicle-administrations (each $n=9$), these pain-related behaviors were observed daily from day 0 (pre-inoculation) to day 3.

Immunofluorescence for the TRG

To examine nerve injury and satellite glial cell activation in the primary sensory ganglion, immunofluorescences for activating transcript factor 3 (ATF3, as nerve injury marker) and glial fibrillary acidic protein (GFAP, as satellite glial cell) were performed for the ipsilateral TRG in sham and FCM on day 4 post-inoculation (each $n=4$). The ipsilateral TRG was quickly removed under i.p. deep anesthesia with thiopental (50 mg/kg) and immersed in 4% paraformaldehyde in phosphate buffer for 1 h. After treatment with 30% sucrose overnight, 8-μm-thick sections of the TRG were made horizontally using a cryostat (CM1900, Leica Microsystems,