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Serotonin transporter mRNA expression in the dorsal raphe nucleus of a tumor bearing mouse

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Abbreviations: DRN, dorsal raphe nucleus; 5-HTT5, hydroxytryptamine reuptake transporter

Abstract

This study was conducted to determine if an oral squamous cell carcinoma alters mRNA expression of serotonin transporter (5-HTT) in the central nervous system. KB cell line derived from a human oral squamous cell carcinoma was inoculated into nude mice, and mRNA expression level of 5-HTT in the dorsal raphe nucleus (DRN) was examined by *in situ* hybridization when the tumor mass reached to ~10% of total body weight. Plasma leptin levels were determined by radioimmunoassay method using a commercial kit. 5-HTT mRNA level was significantly decreased in the DRN of tumor bearing mice, compared to the age-matching non-tumor control. Plasma leptin level decreased concomitantly in tumor bearing mice. These results suggest that oral carcinoma may suppress 5-HTT gene expression in the central nervous system, perhaps in relation with decreased plasma leptin level.

Keywords: depression; gene expression; *in situ* hybridization; leptin; serotonin; tumor

Introduction

Dysregulation of serotonin (5-hydroxytryptamine, 5-HT) system is known to play a role in the pathophysiology of depressive disorders (Maes and Meltzer, 1995; Mann, 1999). It has been reported that serum concentrations of tryptophan, 5-HT precursor, are significantly decreased in cancer patients (Brown *et al.,* 1989; 1991), and that this decrease correlates with depressive symptoms in cancer patients undergoing cytokine therapy (Capuron *et al.,* 2002). Depression is one of the major symptoms commonly found in cancer patients, and reported to worsen survival of the patients (Goodwin *et al.,* 2004; Klinkenberg *et al.,* 2004). However, the mechanism by which cancer induces depression in the patients is largely unknown.

5-HT reuptake transporter (5-HTT) is a member of the Na^+/Cl^--dependent membrane transporters family, controls the propagation of serotonergic signal timely by reuptake of 5-HT from the synaptic cleft immediately after its release. Pharmacologic inhibition of 5-HTT with selective 5-HT reuptake inhibitors, such as fluoxetine, enhances serotonergic transmission and decreases depression symptoms (Gorman and Kent, 1999). Gene expression of 5-HTT in the dorsal raphe nucleus (DRN), where the largest population of serotonergic neurons is located, alters by brain 5-HT level (Linnet *et al.,* 1995; Choi *et al.,* 2003). Decreased expression or lack of 5-HTT appears to correlate with the development of behavioral depression. That is, 5-HTT mRNA expression was decreased in the DRN of *ob/ob* mouse showing symptoms of behavioral depression (Collin *et al.,* 2000), and the number of 5-HTergic neurons and their neuronal activity was decreased in the DRN of 5-HTT knockout mouse exhibiting behavioral depression (Lira *et al.,* 2003).

5-HTT mRNA level in the DRN has been suggested to correlate with plasma leptin level (Jahng *et al.,* 1998; Collin *et al.,* 2000; Johansen *et al.,* 2000). It has been reported that leptin increases brain 5-HT levels in wild-type and *ob/ob* mice (Harris *et al.,* 1998; Calapai *et al.,* 1999), and that leptin receptors are localized in 5-HT or 5-HTT containing neurons in the DRN (Collin *et al.,* 2000; Finn *et al.,* 2001; Hay-Schmidt *et al.,* 2001). In this study, the DRN expression of 5-HTT mRNA and the plasma leptin level were examined in the mice bearing an oral squamous cell carcinoma, in order to define the molecular mechanism of the pathogenesis of depression in cancer subjects.

Materials and Methods

Animals

BALB/c strain male mice at 8 weeks of age were purchased (KRIBB, Taejeon, Korea) and maintained

in a consistent environment with a 12 h / 12 h light-dark cycle (light between 07:00 and 19:00). Mice received free access to standard laboratory food (Purina Rodent Chow, Purina Co., Seoul, Korea) and water (membrane filtered purified water) *ad libitum*, and were cared according to The Guide for Animal Experiments, 2000, edited by The Korean Academy of Medical Sciences, which is consistent with NIH Guideline for the Care and Use of Laboratory Animals, 1996 revised.

Tumor inoculation

KB cell line, which was derived from a poorly differentiated squamous cell carcinoma in the mouth floor of 54-year-old man, was prepared at a 5×10^7 cells/ml concentration. One ml of the KB cell culture was subcutaneously inoculated into the dorsal area of mice. The non-tumor control group received 1 ml of saline instead. Tumor weight in grams was estimated by an empirically-derived formula: length× width×1.33/100 (McCarthy and Daun, 1993). Tumor masses were excised at sacrifice, weighed and then processed for H/E staining to validate the tumor development in each animal (data not shown).

In situ hybridization

Tumor bearing mice were sacrificed for 5-HTT *in situ* hybridization when the tumor mass reached to -10% of total body weight (n = 6). Age-matching non-tumor mice were processed parallel as the control groups (n = 6). Mice were overdosed with sodium pentobarbital (Hallym Pharmaceutical Co., Seoul, Korea) and transcardially perfused first with 100 ml of heparinized isotonic saline containing 0.5% $NaNO_2$ (Sigma Co., Saint Louis, MO), followed by 100 ml of ice-cold 4% paraformaldehyde (Sigma Co.) in 0.1 M sodium phosphate buffer. Brains were immediately removed, post-fixed for 2 h, and transferred into 30% sucrose (Sigma Co.) for cryoprotection. Forty micron coronal sections were cut on a freezing, sliding microtome (HM440E, Microm Co., Germany). Alternate sections through the rostral-caudal extent of the raphe nucleus (between bregma -7.64 and -8.80 mm; Paxinos and Watson, 1986) were collected into 20 ml glass scintillation vials containing ice-cold 2×SSC (0.3 M NaCl, 0.03 M Na Citrate). *in situ* hybridization was performed with 5-HTT cDNA probe (a 0.8 kb EcoR1 restriction fragment; Jahng *et al.,* 1998) as previously described (Choi *et al.,* 2003).

Plasma leptin level

Cardiac blood (n = 12) was collected into the microtubes containing 20 ml of heparin, rapidly after exposing the heart with an overdose of sodium pentobarbital, and centrifuged at 2,000 rpm for 20 min to separate the plasma. The plasma leptin level was determined by radioimmunoassay method using a commercial kit (Mediagnost mouse/leptin kit, Aspenhaustr, Reutlingen, Germany).

Image analysis and statistics

Images on the autoradiographic films were digitized with a Zeiss Stemi-2000 stereoscope attached to a Dage-MTI CCD 72 camera and MCID image analysis system (MCID, Imaging Research Inc., Ontario, Canada). Messenger RNA expression level was determined by quantifying the mean relative optical density of pixels with densities of at least 2 S.D. above the mean density of the image background (mRNA pixels). For each section, the mean background value was subtracted from the mean mRNA pixel value. The mRNA pixel values were averaged across 5 sections from each individual mouse and the average mRNA value of each mouse then averaged across all mice within each group. The average mRNA value of the tumor group was then converted to relative value to the control group. All data were analyzed by unpaired t-test using StatView software (Abacus, Berkeley, CA).

Results and Discussion

5-HTT expression

Tumor mice were sacrificed when the tumor mass reached to -10% of total body weight, the raphe nucleus sections of brain tissues were processed for *in situ* hybridization with 5-HTT cDNA probes. 5-HTT *in situ* signals on the autoradiographic films of tumor mice appeared to be decreased compared with the age-matching non-tumor mice (Figure 1A). Quantificational analysis of the *in situ* signals showed a significant ($P < 0.05$) reduction in 5-HTT mRNA expression level in tumor mice, *i.e.* -65% of non-tumor mice (Figure 1B). It has been reported that gene expression level of 5-HTT in the DRN may positively correlate with the brain 5-HT level (Linnet *et al.,* 1995; Choi *et al.,* 2003), and that the transgenic mouse lacking 5-HTT expression exhibits decreases in the activity and number of serotonergic neurons in the DRN (Lira *et al.,* 2003). These reports together with our result suggest that the brain 5-HT system may be down-regulated in the tumor bearing mouse. Dysregulation in the brain 5-HT system is known to play a role in the pathophysiology of depressive disorders (Maes and Meltzer, 1995; Mann, 1999). Indeed, both obese (*ob/ob*) mouse, which exhibits decreased expression of 5-HTT in the DRN, and 5-HTT knockout mouse present behavioral depression (Collin *et al.,* 2000; Lira *et al.,* 2003). These reports support the idea that this tumor animal model may present behavioral depression. Depression is one of the major symptoms commonly found in cancer patients, and reported to worsen survival of the patients (Goodwin *et al.,* 2004; Klinkenberg *et al.,* 2004). However, the brain mechanism by which cancer induces depression in the patients is not known. Further studies on the analyses of brain 5-HT level and behavioral depression in this animal model will provide valuable information about the pathogenesis of depression in cancer patients.

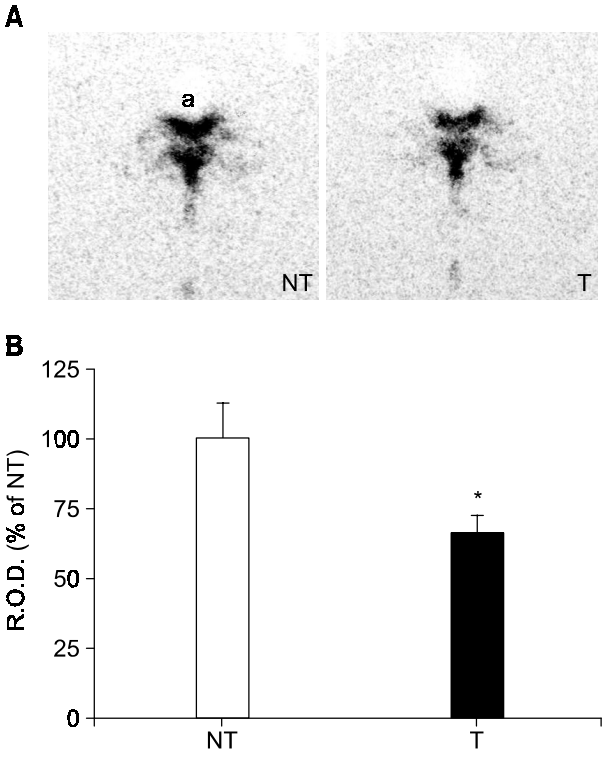

Figure 1. 5-HTT *in situ* hybridization in the dorsal raphe nucleus (DRN) of mouse bearing a human oral squamous cell carcinoma (T) and the age matching non-tumor control (NT). Mice were sacrificed for *in situ* hybridization when the tumor mass reached to -10% of total body weight. (A) Autoradiography of *in situ* signals on X-ray films. (B) Relative optical density (R.O.D.) of the *in situ* signals. 5-HTT mRNA expression was significantly decreased in the DRN of tumor mice, compared to the non-tumor controls. a; acquiduct, $*P < 0.05$ vs. non-tumor control.

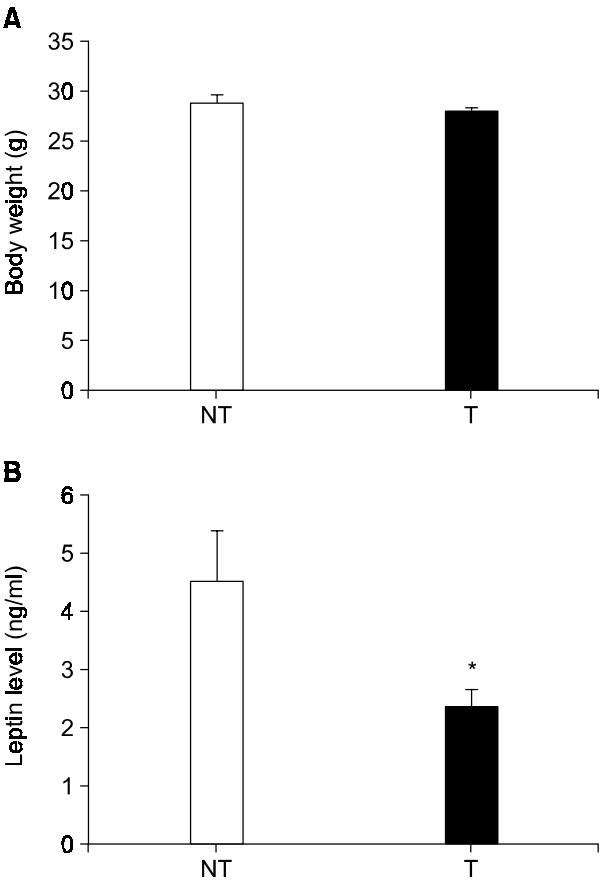

Figure 2. Body weight (A) and plasma leptin level (B). Tumor mice were sacrificed for the plasma leptin assay when the tumor ratio reached to -10% of total body weight. Real body weight of the tumor mice (T), which the tumor weight was subtracted from total body weight, was not significantly reduced compared with the non-tumor controls (NT), however, the plasma leptin levels of tumor mice significantly decreased. $*P < 0.05$ vs. non-tumor control.

Plasma leptin and 5-HTT expression

Plasma leptin levels were analyzed when the tumor ratio reached to -10% of total body weight. The plasma leptin levels of tumor mice were markedly decreased ($P < 0.05$) compared with the age-matching non-tumor mice (Figure 2B), concomitantly with the decrease of 5-HTT mRNA level in the DRN. This result concurs with previous reports that 5-HTT mRNA expression is decreased in *ob/ob* mouse lacking functional leptin (Collin *et al.,* 2000) and in anorexia (*anx/anx*) mouse exhibiting decreased level of the plasma leptin (Jahng *et al.,* 1998; Johansen *et al.,* 2000). Leptin increases brain 5-HT levels in wild-type and *ob/ob* mice (Harris *et al.,* 1998; Calapai *et al.,* 1999) and 5-HT turnover in the brain of wild-type mouse (Calapai *et al.,* 1999). Leptin receptors are localized in the 5-HT (Finn *et al.,* 2001; Hay-Schmidt *et al.,* 2001) or 5-HTT (Collin *et al.,* 2000) containing neurons of the DRN where the most of 5-HT neurons are located in the brain. Leptin which was administrated into the lateral ventricle is accumulated in serotonergic neurons of the raphe nucleus (Fernandez-Galaz *et al.,* 2002). Leptin-induced hypophagia, a major behavioral effect of leptin, is inhibited by serotonergic agents, which suppress the brain 5-HT system (Yamada *et al.,* 2003). These reports all suggest that plasma leptin may activate the brain 5-HT system, perhaps through its stimulatory effect on the 5-HT and/or 5-HTT neurons in the DRN. Together with our result, it is suggested that decreased expression of 5-HTT in the DRN of this tumor mouse may be, at least partly, due to a decrease in the plasma leptin level.

Body weight and plasma leptin

Initial body weight of tumor mice at tumor inoculation did not differ from non-tumor mice (24.817±0.359 g vs. 25.440±0.432 g). Real body weight was calculated by subtracting the tumor weight from total body weight at sacrifice. Real body weight of tumor mice did not significantly differ from the age-matching

non-tumor mice (Figure 2A). Circulating levels of leptin, a peptide hormone synthesized in adipose tissue, are positively correlated to the body mass index (Maffei *et al.,* 1995). For an example, decreased plasma level of leptin in anorexia mutant (*anx/anx*) correlates with a significant weight loss compared with its wild type littermates (Jahng *et al.,* 1998). However, in this study the plasma leptin level was significantly decreased in the tumor bearing mouse despite no significant decrease in body weight, compared to the non-tumor control. Leptin release is reported to be regulated by several factors, such as glucocorticoids or insulin stimulates, but catecholamine inhibits its release (Fruhbeck *et al.,* 1998). Further study is required to determine what caused a reduction in plasma leptin level in this tumor mouse model.

In summary, 5-HTT expression was decreased in the DRN of mouse bearing a human oral squamous cell carcinoma. This decrease may correlate with a reduction in the plasma leptin level in this mouse model. This is the first report demonstrating that oral carcinoma may decrease 5-HTT gene expression in the central nervous system. This animal model can be used for future studies on the pathogenesis of depression in cancer subjects.

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