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LETTER TO THE EDITOR

Cough and transdermal long-acting β_2 agonist in Japan

Dear Editor,

Tamura and Ohta reported higher treatment compliance with the tulobuterol patch, a transdermal long-acting β_2 agonist, than with inhaled drugs in patients with asthma or COPD due to administration once daily.[1] However, we are quite concerned with the over adherence of the tulobuterol patch in Japan. The tulobuterol patch is now widely regarded as an anti-tussive agent not only for individuals with asthma, but also for those suffering from acute bronchitis or the common cold.

Parents of young children refer to these patches as "anti-tussive tape". Tulobuterol patches are frequently prescribed by pediatricians due to strong demands from parents of children suffering from cough. For young children, the transdermal drug has much better adherence and compliance than oral drugs which usually taste bitter or bad.

The anti-tussive effect of tulobuterol is primarily based on its bronchodilatory properties. Among the sensory nerves innervated in the lung, there is a general consensus that rapidly adapting receptors (PAR) and bronchopulmonary unmyelinated C-fibers are directly responsible for the initiation of cough.[2] The inflammatory mediators lowered the threshold of the sensitivities of these sensory nerves.[2] Different from receptors to chemical stimuli such as C-fibers, PAR is a mechanoreceptor which is activated by irritant stimuli and bronchospasm. Therefore, tulobuterol could restore the cough reflex threshold of PAR but not of C-fibers.

β_2 agonist, a sympathetic nerve stimulator, has a variety of side effects including burden to the heart. Due to the limited site of action and relatively larger side effects, β_2 agonist had not been used as a general anti-tussive drug prior to the development of transdermal formulations. Because the transdermal formulation of long-acting β_2 agonist is approved only in Japan and Korea, we might be the only people in the world to use long-acting β_2 agonist as a non-specific anti-tussive therapy. Evidence for effectiveness and safety is poor.

Excessive coughing is often linked to underlying pathology such as infection, asthma, gastro-esophageal reflux, and in some cases can be alleviated with disease-specific therapy.[3] However, the cause of cough may not always be definable and, in some patients, treating the underlying disease may not reduce coughing. In some cases, a non-specific anti-tussive therapy is needed for cough suppression. Currently available anti-tussive agents offer little benefit over placebo for cough relief.[4]

The rapid spread of the tulobuterol patch as a non-specific anti-tussive therapy suggests an advantage. However, due to the efficacy and safety concerns, we should reconsider the use of the transdermal long-acting β_2 agonist as a non-specific anti-tussive agent.

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Common sense, consultation, and commitment should guide collective action on global health. Common sense dictates that we focus on those most in need, usually women and very young children. Consultation with women—in their own right and because they are the engines of family and community health—is essential. Commitment to a pro-poor approach is required to achieve the Millennium Development Goals with equity. These three Cs worked well in Bangladesh in the 1990s,[2] and can provide the infrastructure necessary to address global health challenges such as HIV/AIDS.

I declare that I have no conflict of interest.

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1 Reich MR, Takemi K, Roberts MJ, Hsiao WC. Global action on health systems: a proposal for the Toyako G8 summit. *Lancet* 2008; **371**: 865–69.
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We strongly agree with Michael Reich and colleagues' proposal for global action on health systems at the Toyako G8 summit,[1] especially the need to encourage enhanced learning about health systems. Even in Japan, which Reich and colleagues raise as an example of a good health system, doctor shortages and the rapidly ageing Japanese society are contributing to a breakdown of this system.[3] Therefore efforts to achieve good health in older populations are warranted in developed countries.

Just recently, following the development of induced pluripotent stem cells by S Yamanaka, a professor at Kyoto University,[3] various Japanese ministries presented their plans to support stem-cell research. The education ministry pledged to provide ¥3 billion (US$28·7 million) to support versatile stem-cell research projects—a field that holds great potential for regenerative medicine. The Ministry of Health, Labor and Welfare plans to spend nearly ¥100 million ($958 000) to support related studies, and the Ministry of Economy, Trade and Industry is becoming involved as well.

However, since the ministries do not have residual budgets for such research, they cut the budgets for other research such as that on gerontology and health systems. Usually, basic research takes a long time to be practical in the clinical setting. The global health situation does not have that luxury. The government must balance its support for research that has an immediate effect with that for research having future prospects. We hope that the Toyako G8 summit gives the opportunity for the Japanese Government to consider this balance.

We declare that we have no conflict of interest.

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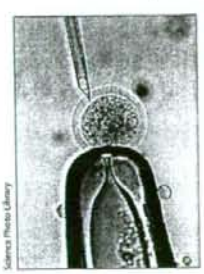

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UK Human Fertilisation and Embryology Bill

It is almost 25 years since embryo experimentation was addressed in the *Warnock Report*.[1] Major advances in human welfare could be within our grasp from this work. There has been no abuse in the UK, and the legislative framework has worked well. Against this background, we wish to state our support for the proposals in the UK Human Fertilisation and Embryology Bill that extend current methods to research with hybrid embryos. Our patients deserve the opportunity for therapeutic advances in some of the most distressing diseases. We see no new major ethical concerns: on the contrary, we believe it would be unethical not to pursue such possibilities.

Sir Leszec Borysiewicz, a catholic and chief executive of the UK Medical Research Council, has our full support in his balanced and sensible statement on the Bill[2]—brave in the face of his church's strident opposition. We are sorry that references to "Frankenstein" should be used by a major religious leader such as Cardinal Keith O'Brien, the head of the Catholic Church in Scotland. The statement does not improve rational public debate, has the potential to mislead, and represents emotional language in an area in which we acknowledge that there are sensitivities.

We declare that we have no conflict of interest.

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1 Department of Health and Social Security. Report of the Committee of Inquiry into Human Fertilisation and Embryology (*The Warnock Report*). Cm 9314. July 1984.
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Department of Error

Cohen AT, Tapson VF, Bergmann J-F, et al, for the ENDORSE Investigators. Venous thromboembolism risk and prophylaxis in the acute hospital care setting (ENDORSE study): a multinational cross-sectional study. Lancet 2008; 371: 387–94—In this Article (Feb 2), an internet link to local investigators was omitted. Details of local investigators are available at http://www.outcomes.org/ENDORSE/investigators.cfm.

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Olfactory Stimulation Using Black Pepper Oil Facilitates Oral Feeding in Pediatric Patients Receiving Long-Term Enteral Nutrition

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Patients with severe neurological disorders often require enteral nutrition (EN). Since long-term EN can cause multiple complications, reinstating the oral intake of food is beneficial. Olfactory stimulation using black pepper oil (BPO), a strong appetite stimulant, was reported to facilitate swallowing in older people. Therefore, the effects of olfactory stimulation with BPO were investigated in pediatric patients receiving long-term EN due to neurological disorders. The effects of scenting with BPO for 1 min immediately before every meal were evaluated in ten patients: 4 boys and 6 girls, aged 19-97 months (51 ± 26 months). The neurological disorders included periventricular leukomalacia (3 patients), hypoxic ischemic encephalopathy (3), Costello syndrome (1), Russell-Silver syndrome (1), Miller-Dieker syndrome (1), and cerebral palsy of unknown etiology (1). In eight of these patients, BPO intervention was continued for 3 months. Five of these eight patients showed increases in the amount of oral intake with desirable effects including facilitated swallowing movement, although complete elimination of the need for EN was not achieved. In the other three patients, BPO intervention was not effective; severe cerebral tissue loss, profound malformation or intractable seizures seemed to reduce the efficacy of BPO. In two cases, BPO intervention was discontinued due to cough or because the odor of BPO was unbearable to the family. In conclusion, olfactory stimulation with BPO facilitated oral intake in a subset of patients on long-term EN. BPO stimulation may be useful for facilitating oral intake when used in combination with conventional methods. dysphagia; food aversion; enteral nutrition; black pepper oil; cerebral palsy.
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Patients with severe neurological disorders often have problems with the oral intake of food and require the use of enteral nutrition (EN). Although EN ensures that daily nutritional requirements are met, multiple complications have been reported with long-term EN, including unexpected micronutrient deficiencies, altered gastrointestinal tract function, and reduced psychosocial stimulation (Kumode 2003; Sleigh et al. 2004; Munakata et al. 2006). Therefore, reinstating oral intake is highly beneficial even if it supplies only part of the patient's nutritional requirements. However, the introduction of oral intake is often difficult in patients receiving long-term EN as background diseases can often affect aspects of the eating process, such as appetite, perception of food, and swallowing (Ohrui 2005). In addition, children who are deprived of oral feeding experiences during developmental "sensitive periods" have poor feeding skills, resulting in intractable food aversion (Illingworth and Lister 1964; Gisel et al. 1998; Kobayashi et al. 2003).

Recently, Ebihara et al. (2006) reported that olfactory stimulation using black pepper oil (BPO), a strong appetite stimulant, safely improves swallowing function in older people with swallowing dysfunction regardless of their level of consciousness. Olfaction is a primitive sense, and human infants have been shown to be responsive to olfactory stimuli (Schaal et al. 2004). This led us to speculate that intervention with BPO may facilitate oral intake in pediatric patients, which has not been addressed in previous studies.

The objective of this preliminary study was to assess whether BPO stimulation facilitates oral intake in pediatric patients receiving prolonged EN despite a continuous rehabilitation program for oral intake.

Patients and Methods

This study was performed from September 2006 to September 2007 at Takuto Rehabilitation Center for Children, Sendai, Japan. Before the intervention, physical condition and risks for aspiration were assessed. The assessment included observing the cough reflex and swallowing of saliva and a swallowing test with water and thickened 5%-glucose solution. A videofluoroscopic examination was performed in patients suspected of aspiration. Inclusion criteria for the trial comprised: (i) patients with chronic EN for more than 10 months despite a 6-month trial of a standard oral rehabilitation program; (ii) low risk of aspiration—specifically, only patients with a cough reflex and no dysphagia, or slight dysphagia for fluid only were included in the trial. Patients who had undergone tracheolaryngeal separation surgery were included because they had no risk of aspiration.

Ten patients {4 boys and 6 girls; aged 19-97 (51 ± 26 [mean ± S.D.]) months} with prolonged EN were included in this trial (Table 1). The duration of EN was 50 ± 27 months. In the swallowing test, taking water induced an occasional cough in Patients 4 and 7; in the videofluoroscopic examination, no intratracheal aspiration was observed in Patient 4, while Patient 7 aspirated a slight amount of water, but none of the thickened solution. Since Patients 1 and 8 had undergone tracheolaryngeal separation, no risk of aspiration existed despite disorganized swallowing. The parents or guardians of all patients gave their informed consent to participate in the study. The study protocol was approved by the ethics committee of Takuto Rehabilitation Center for Children, Sendai, Japan.

Olfactory stimulation using BPO was performed as reported previously with some modifications (Ebihara et al. 2006). Oral feeding was attempted after starving once or twice per day. Nasal stimulation with 100 μl of BPO (Yamamoto Perfumery, Osaka) was accomplished by administering BPO to the nostrils with a filter paper stick for 1 min just before the meal. In cases with tracheolaryngeal separation, BPO was directed to the nasal cavity by gentle fanning, in which case the BPO was also inevitably inhaled through the tracheostomy. Oral feeding was then attempted with pureed foods, and the amount of each meal was recorded. In bedridden patients, oral feeding was performed in the head-up tilt position. After the meal, liquid enteral nutrition was injected. In cases in which the amount of oral intake exceeded 100 g, the amount of oral intake was subtracted from the subsequent enteral nutrition. During oral feeding, a thin NG tube (6-8 French units) was left in place, considering the burden on pediatric patients and the risks of mal-location of the tube tip accompanied by frequent reinsertion of the tube. The effects of BPO were evaluated after 3 months of daily BPO trials. The statistical significance of changes in the amount of oral intake was determined using the Mann-Whitney's U-test.

Table 1. Clinical features of patients included in the BPO intervention trial.

Patient	Age/Sex	Diagnosis	Developmental delay (DQ)	Motor disabilities	Epilepsy (AED)	Neuro-imaging	Problems in swallowing	Dysphagia	Onset of EN	Type of EN	Operation
1	2y6m/M	Sequelae of HIE	Severe (DQ 22)	Bedridden	+ (PB)	Atrophy of basal ganglia	Poor swallowing movement	+	Birth	G	Gastrostomy, tracheolaryngeal separation
2	7y/M	PVL	Severe (DQ 34)	-	-	White matter atrophy	Food aversion	-	Birth	NG	Tracheostomy
3	2y5m/F	Costello syndrome	Severe (DQ 33)	Bedridden	-	Normal	Food aversion	-	Birth	NG	-
4	3y/M	Sequelae of HIE	Severe (DQ 26)	Bedridden	+ (CZP)	Cortical atrophy	Occasional choking, delayed oral stage	-	2y1m	NG	-
5	5y3m/F	Sequelae of HIE	Severe (DQ 6)	Bedridden	+ (VPA, DZP)	White matter atrophy	No concern about food, delayed oral stage	-	Birth	G	Gastrostomy
6	3y10m/M	Russell-Silver syndrome	Normal (DQ 91)	-	-	Normal	No concern about food, delayed oral stage	-	Birth	G	Gastrostomy, Nissen fundoplication
7	8y1m/F	Miller-Dieker syndrome	Severe	Bedridden	+ (VPA)	Lissencephaly	Slight aspiration of fluid	+	Birth	G	Gastrostomy
8	1y7m/F	PVL	Severe	Bedridden	+ (VPA, CZP, ZNS)	White matter atrophy	Poor swallowing movement	+	Birth	G	Gastrostomy, tracheolaryngeal separation
9	5y6m/F	PVL	Borderline (DQ 79)	-	-	White matter atrophy	No concern about food, delayed oral stage	-	Birth	IOE	-
10	3y4m/F	CP, MR (unknown etiology)	Severe	Bedridden	+ (ZNS, VPA)	Cortical atrophy	No concern about food, delayed oral stage	-	Birth	NG	-

BPO, black pepper oil; y, year(s); m, month(s); F, female; M, male; HIE, hypoxic ischemic encephalopathy; PVL, periventricular leukomalacia; CP, cerebral palsy; MR, mental retardation; DQ, developmental quotient; AED, antiepileptic drug; PB, phenobarbital; CZP, clonazepam; DZP, diazepam; VPA, valproate; ZNS, zonisamide; G, gastrostomy; NG, nasogastric tube; IOE, intermittent oroesophageal tube.

Results

Table 2 shows the results of the BPO intervention. Eight of the ten patients successfully completed 3 months of the intervention. The intervention was discontinued before 3 months in two patients because the patients' family found the odor of BPO unbearable (Patient 9) or frequent coughing occurred on smelling the BPO (Patient 10), although BPO did not elicit any other serious complications in any patient tested. The coughing during BPO stimulation in Patient 10 was caused not by increased salivation, but by stimulation of the airway by BPO. The body weights of the eight patients showed normal growth, with values before and after the BPO intervention of 12.3 ± 3.7 and 12.6 ± 4.0 (mean ± S.D.) kg, respectively.

Of the eight patients who completed 3 months of the BPO intervention, five responded to the intervention and showed a distinct increase in oral intake (Patients 1 through 5), although complete cessation of EN was not achieved. As shown in Table 2, the increase was accompanied by desirable effects, such as facilitated appetite, reduced drooling, and distinct swallowing movements. In Patient 5, the amount of oral intake was temporarily reduced when the number of seizures increased during the intervention, although the amount of intake was eventually increased. In Patient 6, the average amount of oral intake was increased slightly but was not stable among meals. Patients 7 and 8 did not respond to BPO. Patient 7 had Miller-Dieker syndrome with profound cerebral malformation and Patient 8 had severe white matter volume loss. As summarized in Fig. 1, BPO intervention significantly increased the amount of oral intake ($p = 0.016$). As described above, the intervention was discontinued before 3 months in Patients 9 and 10, and their data are not plotted in Fig. 1.

Discussion

This study investigated the effects of BPO stimulation in pediatric patients receiving long-term EN who did not respond to a conventional oral feeding rehabilitation program. Although complete discontinuation of EN was not achieved, the 3-month BPO intervention facilitated oral intake in these patients.

Swallowing is an elaborate mechanism that enables the ingestion of fluids and solid foodstuffs without aspiration. This mechanism is regulated by a widespread neuronal network spread over the cerebrum, cerebellum, and brain stem (Humbert and Robbins 2007). Therefore, various diseases

Table 2. Summary of the results of BPO intervention.

Patient	Result	Observation after 3 months of the BPO trial	Adverse effects
1	Effective	Increased oral intake, reduced drooling, obvious swallowing movements	None
2	Effective	Increased oral intake, shows an interest in food (sniffing, eating by himself)	None
3	Effective	Increased oral intake, facilitated oral stage	None
4	Effective	Increased oral intake, choking during meals disappeared, facilitated oral stage	None
5	Effective	Increased oral intake, facilitated oral stage	None
6	Equivocal	Increased oral intake, but not stable	None
7	Not obvious	No obvious changes	None
8	Not obvious	No obvious changes	None
9	Discontinued	No obvious changes	Family could not tolerate the odor of BPO
10	Discontinued	No obvious changes	Frequent coughing during BPO stimulation

affecting the central nervous system are frequently accompanied by swallowing dysfunction. Among the brain areas, the insular cortex has been shown to play a major role in the mechanism of the effects of BPO. The smell of BPO potently stimulates the insular cortex and orbitofrontal cortex (Ebihara et al. 2006). Functional imaging studies have revealed that the insular cortex plays significant roles in both the sensation of appetite and in the generation of voluntary swallowing (Tataranni et al. 1999; Humbert and Robbins 2007). Therefore, BPO may exert beneficial effects on both appetite and swallowing *via* activation of the insular cortex. In addition, piperine, a major source of BPO flavor, is an agonist of the transient receptor potential vanilloid 1 receptor (TRPV1) causing systemic release of substance *P* (SP) (Szallasi 2005; Ebihara et al. 2006). SP has been shown to facilitate the swallowing process. Indeed, serum SP levels are increased by the smell of BPO (Yamaya et al. 2001). Stimulation with BPO may also improve swallowing function through the action of SP.

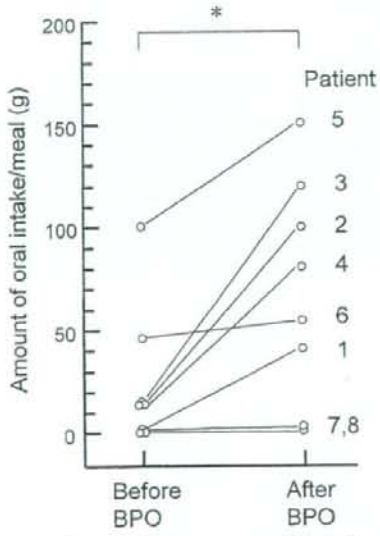

Fig. 1. Changes in the amount of food ingested orally.
Plots represent the average amount of oral intake during 1 week of observation before and after 3 months of BPO therapy. Lines connect the plots from the same patient. Numbers beside the plots indicate the patient numbers, corresponding to those in Table 1. Asterisks indicate statistical significance (Mann-Whitney's U-test, $p < 0.05$). Data from Patients 9 and 10 are not included in the figure and statistics because their therapies were discontinued prior to 3 months (see text).

In this study, BPO intervention had beneficial effects on oral intake in patients affected in various regions of the brain, including the cortex, white matter, and basal ganglia. Magnetic resonance imaging (MRI) showed that the patients in whom BPO was effective had preserved cerebral tissue volume irrespective of the severity of their developmental disabilities. However, Patient 8 in whom BPO was not effective showed severe and diffuse white matter loss. Patient 7, who had Miller-Dieker syndrome, also did not respond to BPO. In Miller-Dieker syndrome, cerebral tissue volume is maintained but the tissue is severely malformed (lissencephaly) due to an abnormal Lis1 gene (Dobyns et al. 1993). Impairment of the Lis1 gene causes disorganization of the olfactory bulb (Hirotsune et al. 1998), resulting in inefficient olfaction, which would render BPO stimulation ineffective. Overall, BPO intervention seemed to be generally effective, although patients with severe cerebral tissue loss or profound malformation may not show a response to this appetite stimulant. Control of seizures also seemed to be important for the efficacy of BPO.

The effect of BPO seemed to be correlated with appetite in this study, and caused an increase in the oral intake. In addition, an improvement in swallowing movements was observed during the BPO intervention, as shown in Table 2. In elderly patients with dysphagia, BPO stimulation shortened the latency of the swallowing reflex and reduced pooling in the recessus piriformis, both of which imply ameliorated swallowing dysfunction (Ebihara et al. 2006). Although complete functional measurements of swallowing were not performed to reduce the burden on the patients, BPO may remediate swallowing dysfunction in pediatric cases. Although olfactory stimulation with BPO should be applied carefully in patients

with dysphagia, further investigation of the effects of BPO on dysphagia are warranted based on our results.

In conclusion, olfactory stimulation with BPO is a potentially effective means to facilitate oral intake in pediatric patients with prolonged EN. Although the effect of BPO was limited, it was still beneficial, especially in patients who did not respond to conventional rehabilitation intervention. BPO stimulation may be useful for facilitating oral intake when used in combination with conventional methods. Further investigations are required to determine the efficacy, application, and limitations of the BPO intervention.

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Reduced tumor growth in a mouse model of schizophrenia, lacking the dopamine transporter

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The incidence of cancer in patients with schizophrenia has been reported to be lower that in the general population. On the other hand, it is well established that patients with schizophrenia have a hyper-dopaminergic system and dopamine has the ability to inhibit tumor angiogenesis. Therefore, in order to investigate the molecular mechanisms responsible for the lower cancer risk in schizophrenic patients, we used a mouse model of schizophrenia, which shows hyper-dopaminergic transmission in the nerve terminals of dopaminergic neurons. Here, we hypothesized that tumor growth was reduced in a mouse model of schizophrenia, lacking the dopamine transporter (DAT), and investigated tumor growth and angiogenesis in DAT knockout mice. The subcutaneous tumor in mice inoculated with cancer cells was smaller in $DAT^{-/-}$ mice than in the wild type ($p < 0.05$); however, the level of plasma dopamine in $DAT^{-/-}$ mice was lower than that of control littermates. Using human umbilical vascular endothelial cells (HUVEC), we examined dopamine signaling through dopamine D_1 receptor (D_1R) and D_2R. Dopamine stimulation slightly decreased the surface expression of vascular endothelial growth factor receptor-2 (VEGF-R2) but induced the phosphorylation of VEGF-R2 through Src in HUVEC. In addition, $DAT^{-/-}$ mice had less D_1R. Both pharmacological and genetic interruption of D_1R showed inhibited tumor growth. These results suggest that modulation of the dopaminergic system may contribute to cancer therapy.

Key words: schizophrenia; dopamine; dopamine receptors; dopamine transporter; angiogenesis; VEGF-R2

The risk of cancer among patients with schizophrenia has been discussed.[1,2] The majority of studies in the last decade suggested that patients with schizophrenia are protected against cancer in general, despite increased smoking[3,4] and drinking habits in this population.[5]

The dopamine transporter (DAT) is believed to control the temporal and spatial activity of released dopamine by the rapid uptake of neurotransmitters into presynaptic terminals. $DAT^{-/-}$ mice, which showed behavioral abnormalities, neuroendocrine dysfunction, and altered sensitivity to certain drugs,[6,7] was proposed as an animal model of schizophrenia[8] and attention-deficit hyperactivity disorder.[9]

Blood supply is essential for solid tumors and tumor growth highly depends on angiogenesis, the formation of new capillaries from pre-existing blood vessels.[10] Therefore, the angiogenic process is an essential early step in the progression of malignant tumors. In the conventional view, angiogenesis is mediated by the local proliferation and migration of vessel wall-associated endothelial cells that emerge from their resting state in response to angiogenic growth factor, such as vascular endothelial growth factor and basic fibroblast growth factor.[10] Recently, several experimental works suggests that traditional neurotransimitters, such as dopamine, acetylcholine and noraderenaline, may also contribute to solid tumor progression by modulating tumor angiogenesis.[11–14] However, it is still not clear whether the abnormally transmitted neurotransmitter in psychiatric disorders affects tumor angiogenesis or not.

Dopamine D_1 and D_5 receptors are classified as D_1-like, and D_2, D_3 and D_4 receptors as D_2-like receptors.[15] In endothelial cells, dopaminergic stimulation *via* dopamine D_2 receptor (D_2R) was reported to prevent angiogenesis.[11,16,17] On the other hand, the stimulation of dopamine D_1 receptor (D_1R), which also exists in endothelial cells, release GTP-binding protein coupled βγ subunits, resulting in activation of Src kinase proteins. Src kinase proteins are known to transactivate protein kinase receptors, such as the epidermal growth factor receptor.[18,19] Therefore, the overall effect of dopaminergic stimulation on angiogenesis and endothelial cell functions is still being debated. To elucidate the relationship between the dopaminergic system and cancer progression, we investigated tumor growth in DAT knockout mice as an animal model of schizophrenia.

Material and methods

Animals

Six- to 9-week-old male mutant mice lacking DAT and littermate wild-type mice were obtained from heterozygous crosses with an Sv129/C57BL/6 mixed genetic background. The details of the generation of DAT knockout mice have been described previously.[6] Four- to six-week-old male $D_1R^{-/-}$ mice with a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). In every mutant mice group, we generated homozygous, heterozygous, and wild types by crossing adult heterozygotes. DNA extracted from tail biopsies was genotyped using PCR. Mice were group housed (2–4 per cage) with food and water *ad libitum* in a room maintained at 22 ± 2°C and 65 ± 5% humidity under a 12 hr light-dark cycle. The animals were killed with an overdose of urethane (20 g/kg). All animal experiments were performed according to the Animals Act (scientific procedures) 1986 and approved by the local ethics panel at Tohoku University School of Medicine.

Cell culture

Lewis lung carcinoma (LLC) cells were purchased from the American Type Culture Collection (Manassas, VA). LLCs were cultured in high glucose DMEM containing 10% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Human umbilical vascular endothelial cells (HUVEC) were purchased from Kurabo (Osaka, Japan) and were cultured in EC growth medium (Kurabo).

In vivo *tumor models*

LLCs were injected (1×10^6 cells/animal) subcutaneously (s.c.) into the flank of male 6- to 9-week-old wild-type mice, $DAT^{+/-}$, $DAT^{-/-}$, $D_1R^{+/-}$ and $D_1R^{-/-}$ mice on day 0. In tumor growth rate models, saline, GBR12909 (10 mg/kg), SCH23390 (0.3 mg/kg) or domperidon (1 mg/kg) was injected intraperitoneally (i.p.) every 2 days. Tumor size was quantified daily as

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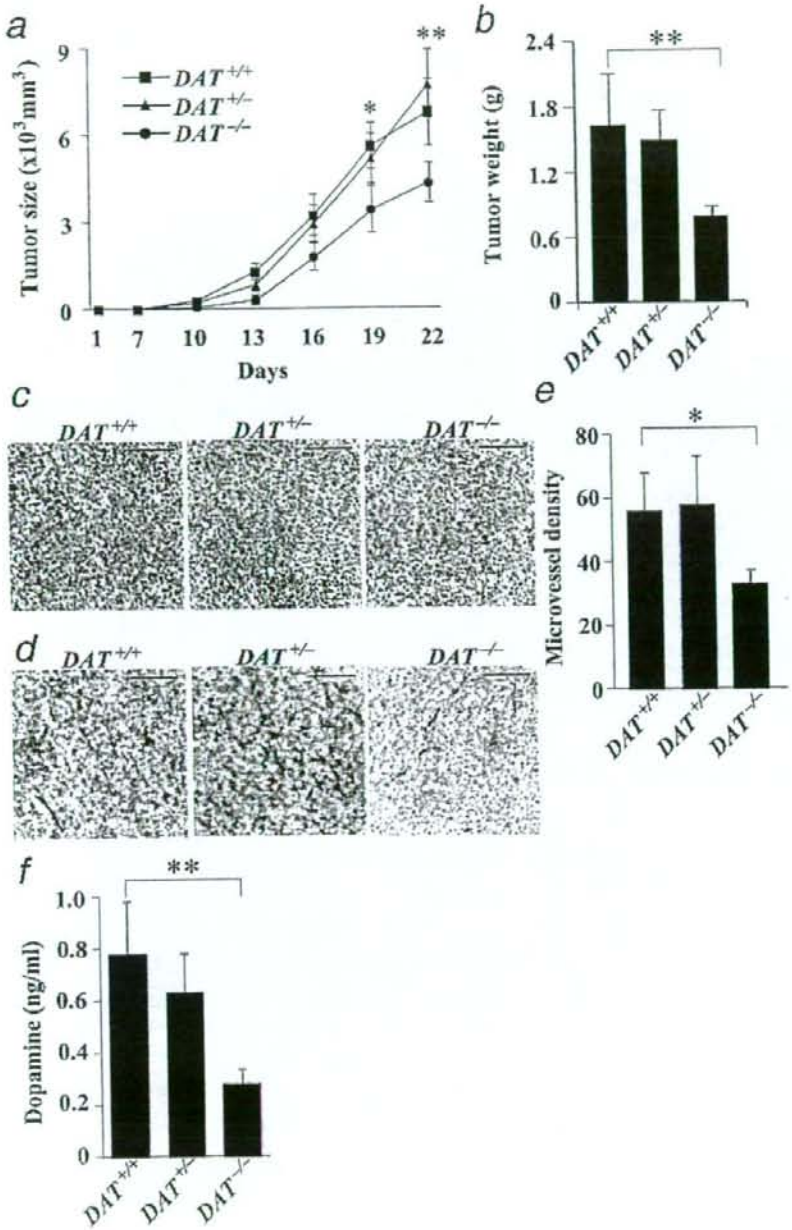

FIGURE 1 – Effects of DAT on tumor growth of LLC in mice. (*a*) A total 1×10^6 LLC cells were implanted into $DAT^{-/-}$ (circle), $DAT^{+/-}$ (pyramidal shape), and $DAT^{+/+}$ (square). Tumor volumes were calculated from tumor measurements scored on the indicated day. Results are presented as the mean tumor volume ± s.e.m. (*b*) On day 22 after implantation, the mice were killed, tumors were collected, and wet weight was determined. (*c*) Bars represent 100 μm. Hematoxylin and eosin-stained sections of tumors. (*d*) Representative sections of tumors stained for factor VIII as a vascular endothelial marker (×200 magnification). (*e*) $DAT^{-/-}$ receiving LLC tumors exhibited significantly decreased angiogenesis and MVD. (*f*) Plasma levels of dopamine. The levels of plasma dopamine were significantly different between $DAT^{+/+}$ and $DAT^{-/-}$ animals. $DAT^{+/+}$, $n = 10$; $DAT^{+/-}$, $n = 10$; $DAT^{-/-}$, $n = 9$; *, $p < 0.05$ compared to the value for $DAT^{+/+}$ and $DAT^{+/-}$ mice; **, $p < 0.01$ compared to the value for $DAT^{+/+}$ mice and $DAT^{+/-}$ mice.

width^2 × length × 0.52. Mice inoculated with LLCs were killed on day 22 and tumors were collected, weighed and sized.

Expression analysis

RT-PCR/RNA was prepared from dissected tissues of adult mice or LLC or HUVEC and treated extensively with DNase. Human whole brain RNA was purchased from Ambion (Austin, TX). Reverse transcription and amplification were carried out as described previously.[20,21] The oligonucleotide primers (Invitrogen, Carlsbad, CA) used for amplification of the dopamine receptor subtypes D1 and D2 were reported previously.[20] PCR was performed with cDNA prepared from 5 ng of RNA in 25-μl reactions for 37 cycles.

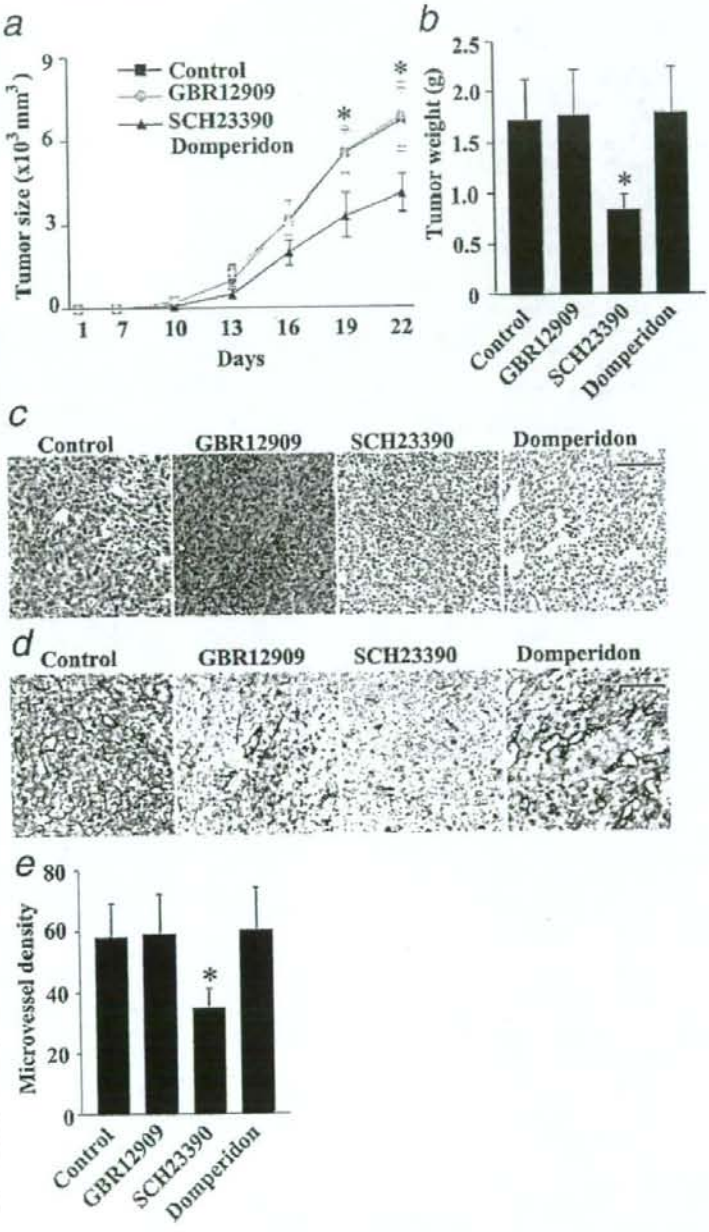

FIGURE 2 – Effect of DAT inhibitor and DA receptor inhibitors on tumor growth of LLC in mice. (*a*) Mice were injected s.c. with LLC on day 0 and were treated with saline (red square), GBR12909 (green circle), SCH23390 (blue pyramidal shape) or domperidon (yellow rhombus) from day 6, every 2 days. Tumor volumes were calculated from tumor measurement scored on the indicated day. Results are presented as the mean tumor volume ± s.e.m. (*b*) On day 22 after implantation, the mice were killed, tumors were collected and wet weight was determined. (*c*) Bars represent 100 μm. Hematoxylin and eosin-stained sections of tumors. (*d*) Representative sections of tumors stained for factor VIII as a vascular endothelial marker (×200 magnification). (*e*) SCH23390-treated mice exhibited significantly decreased angiogenesis and MVD. *, $p < 0.05$ compared to the value for control mice.

Immunoprecipitation and Western blot analysis

2×10^5 HUVECs were seeded in 10 cm dishes, cultured for 2 days, serum-starved (0.1 % serum) for 24 hr, and then treated with either dopamine (1 μM) and/or SCH23390 (10 nM) and/or domperidon (10 nM), followed 5 min later by the addition of 10 ng/ml vascular endothelial growth factor (VEGF) (R&D Systems, Minneapolis, MN). Cells treated with or without dopamine or SCH23390 or domperidon or VEGF were suspended in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA) containing protease inhibitors (20 mg/ml leupeptin, 1 mg/ml pepstatin A and 1 mM PMSF) and then sonicated on ice. Cell extracts, obtained by centrifugation

at 16,000*g* for 15 min, were incubated with anti-phosphotyrosine mAb (Upstate Biotechnology, Lake Placid, NY) at 4°C for 3 hr. Protein G-Sepharose 4 Fast beads (20 µl of wet volume) incubation was performed at 4°C for 1 hr. After the beads were washed with lysis buffer, the bound proteins were eluted by boiling the beads in SDS sample buffer for 10 min. The sample was subjected to SDS-PAGE, followed by Western blotting using anti-vascular endothelial growth factor receptor-2 (VEGF-R2) Ab (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemistry

When the diameter of the tumor was 1 cm, tumor tissues were fixed in 10% formalin, embedded in paraffin and sectioned. They were blocked with 10 % normal goat serum and incubated with polyclonal anti-human factor VIII-related Ag Ab (Dako Japan, Kyoto, Japan). Subsequently, the sections were incubated first with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and then with the ABC kit (Vector Laboratories), then detected by 3-amino-9-ethylcarbazole (Vector Laboratories), and counterstained with hematoxylin.

Determination of microvessel density (MVD)

Intratumoral microvessel density was determined as previously described.[21] In brief, intratumoral vessels were stained immunohistochemically with anti-human factor VIII-related Ag Ab. The image that contained the highest number of microvessels was chosen for each section by initial scan at 100× magnification, and then the vessels were counted in the selected image at 200× magnification. At least 4 fields were counted for each section, and the highest count was taken. Two independent investigators evaluated the number of vessels.

Flow cytometry

FITC-labeled control mouse IgG_1 and PE-labeled anti-human VEGF-R2/KDR mAb were purchased from BD PharMingen (San Diego, CA). To determine cell-associated VEGF-R2, 1×10^5 HUVECs were treated with 1 µM dopamine or 10 nM SCH23390 or 10 nM domperidon for 5 min at 37°C in a humidified 5% CO_2 atmosphere. HUVECs were treated with trypsin-EDTA and suspended in PBS. The cells were first incubated with unlabeled anti-CD16/32 mAb (eBiosience, San Diego, CA) to block nonspecific binding to FcgR. After washing, the cells were incubated on ice with a mixture of FITC-, PE- and nonlabeled Abs. After washing again, the cells were subjected to flow cytometry on a FACScan (BD Biosciences), and the data were analyzed with CellQuest software (BD Biosciences). For all samples, dead cells were excluded from the analysis by proridium iodide staining.

Measurement of dopamine

Dopamine was measured in the plasma of $DAT^{-/-}$, $DAT^{+/-}$ and $DAT^{+/+}$ mice. Prepared samples from blood were used for the assay of dopamine by high-performance liquid chromatography with electrochemical detection.

Other products

Dopamine, GBR12909, SCH23390 and domperidon were purchased from Sigma (St. Louis, MO).

Data analysis

Statistical analysis of the results was performed using ANOVA with Fisher's least significant difference test for multiple comparisons. A value of $p < 0.05$ was considered significant.

Results

To investigate whether the natural differences in dopaminergic reactivity among $DAT^{-/-}$, $DAT^{+/-}$ and $DAT^{+/+}$ mice, are associated with differences in tumor development, we evaluated tumor growth using a cancer animal model, a mouse inoculated with LLCs s.c. As shown in Figure 1, tumors in $DAT^{-/-}$ mice were significantly smaller than tumors in $DAT^{+/-}$ or wild-type mice (Fig. *1a* and *1b*). H&E staining of the tumor tissues revealed a decrease in tumor tissue vessels from $DAT^{-/-}$ mice (Fig. 1*c*). To confirm the endothelial cells, we stained paraffin sections immunohistochemically using an Ab against factor VIII-related Ag (Fig. 1*d*). Factor VIII-related Ag is a well-established cell surface marker of vascular endothelial cells.[22] Compared with control mice, we found a decreased number of tumor vessels in $DAT^{-/-}$ mice. The difference in MVD between control and $DAT^{-/-}$ mice was statistically significant (Fig. 1*e*). To get more insight into the possible contribution of changes in peripheral catecholamines to the observed effect of deletion of *DAT* on LLC tumors, we determined the concentration of norepinephrine, epinephrine, and dopamine in plasma from $DAT^{-/-}$, $DAT^{+/-}$ and $DAT^{+/+}$ animals. There were no differences in plasma epinephrine and norepinephrine (data not shown). In contrast, the level of plasma dopamine was dramatically reduced ($p < 0.01$) in $DAT^{-/-}$ compared with wild-type mice (Fig. 1*f*).

We investigated whether the DAT inhibitor or dopamine agonist influenced tumor growth. LLCs were inoculated into the flank of C57BL/6 mice s.c. on day 0. From day 6 after tumor identification, we injected GBR12909, a DAT inhibitor; SCH23390, a D_1R inhibitor; domperidon, a D_2R inhibitor; or saline i.p. every 2 days. Compared with saline treatment, GBR12909 and domperidon treatment did not inhibit tumor growth (Figs. 2*a* and 2*b*); however SCH23390 decreased tumor growth (Figs. 2*a* and *b*). H&E staining of the tumor tissues revealed a decrease in tumor tissue vessels from mice with SCH23390 treatment (Figs. 2*c*). To confirm the endothelial cells, we stained paraffin sections immunohistochemically using an Ab against factor VIII-related Ag (Fig. 2*d*).

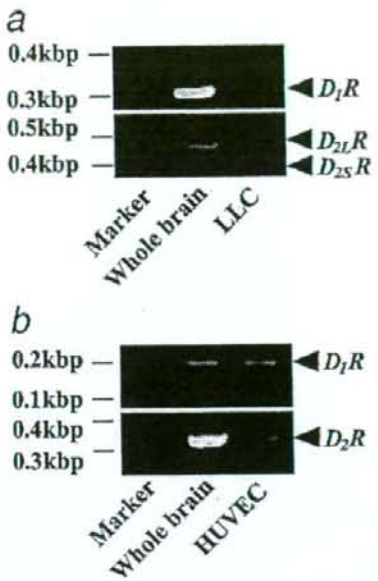

FIGURE 3 – Dopamine receptor expression in LLC and HUVEC. (*a*) D_1R and D_2R expressions were determined using RT-PCR, but D_2R was not expressed in LLC. D_2R exists as 2 alternatively spliced isoforms differing in the insertion of a stretch of 29 amino acids in the third intracellular loop ($D_{2S}R$ and $D_{2L}R$). Brain-derived RNA was used as a positive control. (*b*) D_1R and D_2R were expressed in HUVEC.

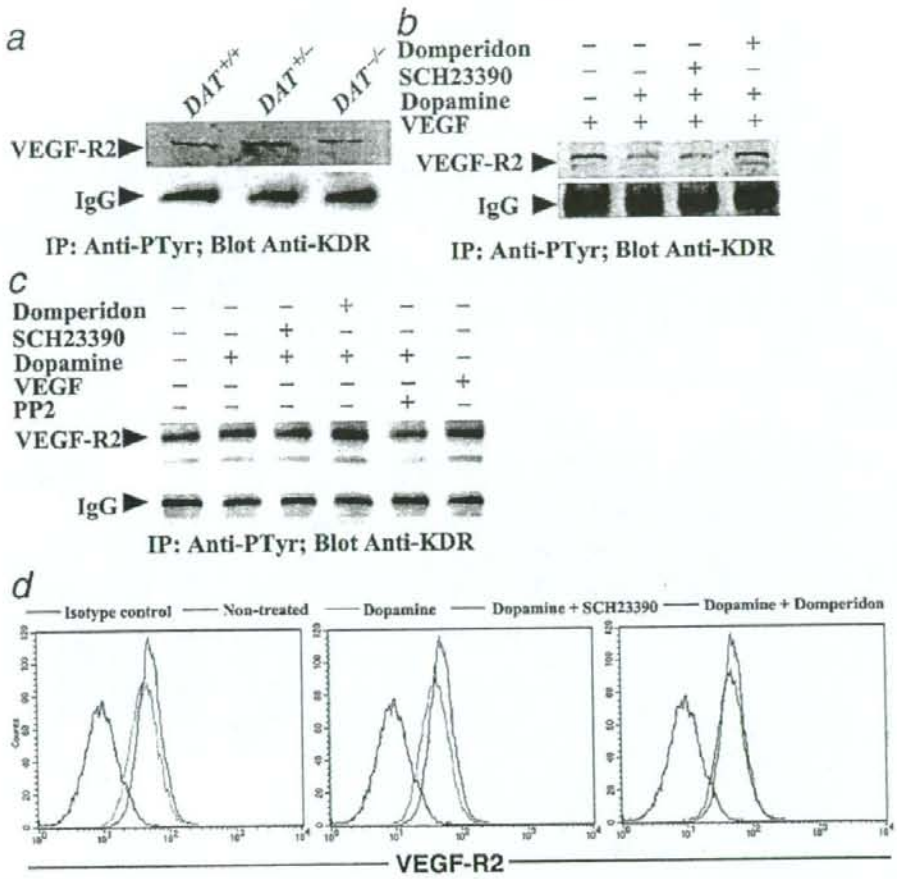

FIGURE 4 – D_1R stimulated phosphorylation of VEGF-R2 *via* Src, but D_2R stimulation induced internalization of VEGF-R2. (*a*) Tumors from $DAT^{-/-}$ decreased phosphorylation of VEGF-R2. Tumors from $DAT^{+/-}$ had no effect. Each tumor was collected for extraction, immunoprecipitation with antibodies to phosphotyrosine and immunoblotting with antibodies to VEGF-R2. (*b*) Effects of dopamine on VEGF-induced phosphorylation of VEGF-R2 in cultured HUVEC. (*c*) Effects of dopamine on phosphorylation of VEGF-R2 *via* Src. Pretreatment with SCH23390, domperidon or PP2 for 1 hr. Dopamine or VEGF was added to cultured HUVEC. Cells were collected for extraction, immunoprecipitation with antibodies to phosphotyrosine and immunoblotting with antibodies to VEGF-R2. (*d*) Effects of dopamine on cell-surface VEGF-R2 expressed by FACS.

Compared with control mice, a decreased number of tumor vessels in mice with SCH23390 treatment were found. The difference in MVD between control and SCH23390-treated mice was statistically significant (Fig. 2*e*).

We confirmed dopamine receptor expression in LLC and HUVEC. We used RT-PCR to analyze the mRNA expression in LLC and HUVEC. LLC expressed only D_1R. HUVEC expressed D_1R and D_2R (Figs. 3*a* and 3*b*). Dopamine stimulation could not induce or reduce cAMP and had no effect on cell proliferation in LLC (data not shown). We could not detect the expression of *DAT* mRNA in LLC and HUVEC (data not shown).

Tumors from $DAT^{-/-}$ mice showed significantly lower levels of VEGF-R2 phosphorylation (Fig. 4*a*), suggesting that reduced vascularization of the tumor in $DAT^{-/-}$ mice was the result of the inhibited phosphorylation of VEGF-R2. We also confirmed that dopamine inhibited VEGF-induced phosphorylation of VEGF-R2 in HUVEC (Fig. 4*b*). However, we found that, in the absence of VEGF, 1 μM of dopamine alone induced the phosphorylation of VEGF-R2 in HUVEC (Fig. 4*c*). To elucidate the receptors involved in dopamine-induced phosphorylation of VEGF-R2 tyrosine kinase, we conducted a blocker study using SCH23390 and domperidon. SCH23390 inhibited the dopamine-induced phosphorylation of VEGF-R2, suggesting that dopamine-induced phosphorylation of VEGF-R2 was through D_1R. Since VEGF-R2 tyrosine kinase was known to be phosphorylated from the outside of the VEGF-R2 tyrosine kinase axis by Src kinase,[23] we investigated the involvement of Src kinase using PP2, a specific Src kinase antagonist. PP2 completely inhibited the phosphorylation of VEGF-R2 by dopamine stimulation (Fig. 4*c*). These results suggested that dopamine stimulation in peripheral vessels induced the phosphorylation of VEGF-R2 through Src *via* D_1R. On the other hand, it has been reported the involvement of VEGF-R2 internalization by stimulation of D_2R in endothelial cells.[11] Therefore, we estimated endothelial cell surface VEGF-R2 expression using

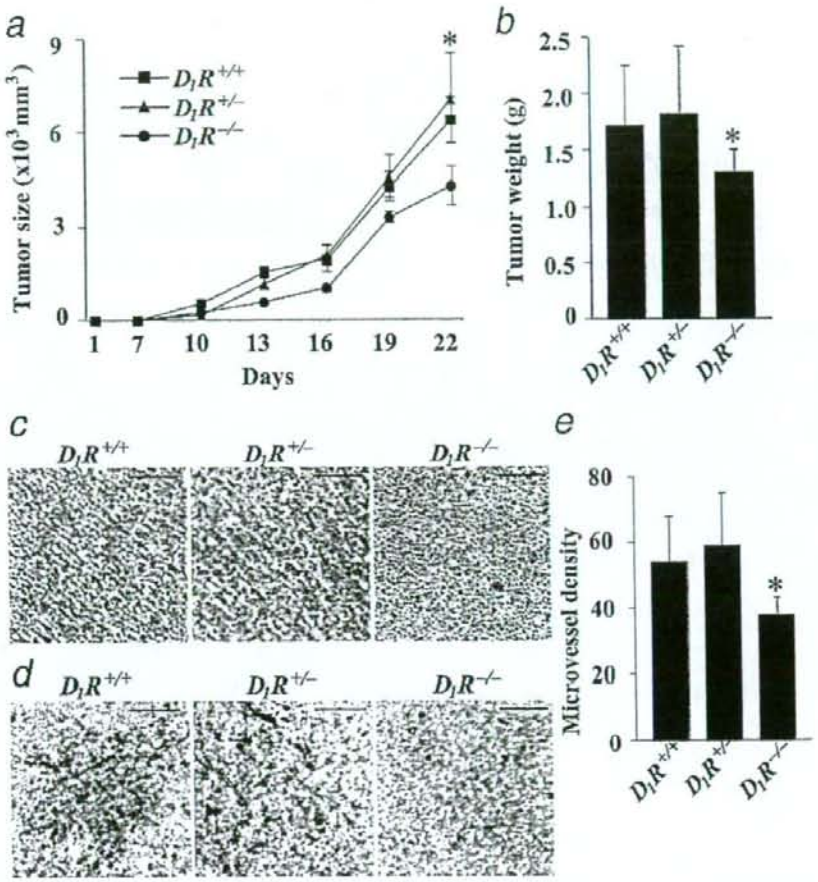

FIGURE 5 – Effects of D_1R on tumor growth of LLC in mice. (*a*) A total 1×10^6 LLCs were implanted into $D_1R^{-/-}$ (circle), $D_1R^{+/-}$ (pyramidal shape), and $D_1R+/+$ (square). Tumor volumes were calculated from tumor measurements scored on the indicated day. Results are presented as the mean tumor volume ± s.e.m. (*b*) On day 22 after implantation, the mice were killed. Tumors were collected, and wet weight was determined. (*c*) Bars represent 100 μm. Hematoxylin and eosin-stained sections of tumors. (*d*) Representative sections of tumors stained for factor VIII as a vascular endothelial marker (×200 magnification). (e) $D_1R^{-/-}$-receiving LLC tumors exhibited significantly decreased angiogenesis and MVD. $D_1R^{+/+}$, $n = 8$; $D_1R^{+/-}$, $n = 10$; $D_1R^{-/-}$, $n = 9$; *, $p < 0.05$ compared to the value for $D_1R^{+/+}$ and $D_1R^{+/-}$ mice.

FACS analysis. FACS analysis revealed that in the absence of VEGF, dopamine slightly reduced the surface expression of VEGF-R2 on HUVEC. The cells treated with both dopamine and domperidon recovered the surface expression of VEGF-R2 (Fig. 4*d*). These results suggested that D_2R stimulation induced VEGF-R2 internalization in the absence of VEGF, whereas, in the presence of VEGF, downstream of VEGF signaling was activated by D_1R stimulation.

Both D_1R and D_2R are reported to be down-regulated in $DAT^{-/-}$ mice.[24] Since extracellular dopamine concentration was significantly lowered in $DAT^{-/-}$ mice, it was speculated that reduced tumor growth in $DAT^{-/-}$ mice was due to reduced D_1R stimulation in $DAT^{-/-}$ mice, resulting in the inhibition of dopamine-induced VEGF-R2 phosphorylation. Although this hypothesis is supported by SCH23390-induced tumor growth inhibition (Fig. 2*a*), further investigation of the hypothesis using $D_1R^{-/-}$ mice was conducted. To investigate whether differences among $D_1R^{-/-}$, $D_1R^{+/-}$ and wild-type mice are associated with differences in tumor progression, we analyzed tumor growth in these mice. Tumors from $D_1R^{-/-}$ mice were significantly smaller than tumors from $D_1R^{+/-}$ or wild-type mice (Figs. 5*a* and 5*b*). H&E staining of the tumor tissues revealed a decrease in tumor tissue vessels from $D_1R^{-/-}$ mice (Fig. 5*c*). To confirm the vessels, we stained paraffin sections immunohistochemically using an Ab against factor VIII-related Ag (Fig. 5*d*). Compared with control mice, we found a decreased number of tumor vessels in $D_1R^{-/-}$ mice. The difference in MVD between control and $D_1R^{-/-}$ mice was statistically significant (Fig. 5*e*). These observations suggest that reduced tumor growth in $D_1R^{-/-}$ mice is the result of reduced vascularization of the tumor *in vivo*.

Discussion

According to the hypothetical role of dopamine in schizophrenia,[25] we investigated tumor growth in $DAT^{-/-}$ mice, which are a

genetic model of persistent hyperdopaminergia.[26] Tumor growth was inhibited in $DAT^{-/-}$ mice that also had less peripheral dopamine; moreover, the DAT inhibitor could not inhibit tumor growth. We revealed that dopamine stimulation reduced the surface expression of VEGF-R2 on HUVEC *via* D_2R but induced the phosphorylation of VEGF-R2 through D_1R *via* Src *in vitro*. Finally, we investigated whether D_1R expression in tumor angiogenesis influenced tumor growth *in vivo*; in $D_1R^{-/-}$ mice, tumor growth was reduced. These results showed that it was important for reduced tumor growth not only to induce D_2R stimulation but also to prevent D_1R stimulation.

In our study, we focused on $DAT^{-/-}$ mice as a schizophrenic model, which receive the most attention and, in our opinion, hold the most promise for yielding insights into the complex nature between cancer and schizophrenia. Contrary to our expectations, $DAT^{-/-}$ mice, which have hyperdopaminergia in the central nervous system (CNS), had less peripheral serum dopamine. Moreover, it has been reported that $DAT^{-/-}$ mice have less D_1R in the CNS.[24] Although patients with schizophrenia may have a hyperdopaminergic brain, systemic D_1R density was reduced in schizophrenia.[27] These observations suggest that not only the hyperdopaminergic state but also less D_1R might reduce tumor growth, resulting in the possible protection of patients with schizophrenia against cancer.

$D_1R^{-/-}$ mice exhibit normal coordination and locomotion, although they displayed significantly decreased behavior.[28] $D_1R^{-/-}$ mice are growth retarded and die shortly after weaning age.[28] The distribution of peripheral D_1R was exhibited in blood vessels, kidney and adrenal gland.[29] However, the precise roles of D_1R are not really elucidated in peripheral organs. In neural cells, receptors coupled to the Gs family of G proteins, such as D_1R, are characterized by their abilities to trigger adenyl cyclase-mediated cAMP formation.[30] Activation of Gs-coupled D_1R in SK-N-MC human neuroblastoma cells increased JNK activity in a cAMP and PKA-dependent manner.[31] There was a report that Gs-linked receptors are also capable of stimulating this kinase *via* an alternative pathway, in which Gβγ subunits serve as the primary players in the signal transduction. In COS-7 transfected with D_1R, the Gβγ subunits released from Gs and Gi cooperated, using a Gβγ/Src-dependent pathway to mediate the JNK activation. On the other hand, D_1R signaling suppressed the gastrin-releasing peptide-preferring bombesin receptor (GRPR) and mediated JNK activation by down-regulating D_1R signaling the cAMP-dependent protein kinase in the phospholipase C pathway.[32] In HUVEC, the release of Gβγ subunits activated Src kinase proteins, which, in turn, transactivate protein kinase receptors.[33,34]

Src, a proto-oncogene, has been strongly implicated in the growth, progression and metastasis of a number of human cancers.[34] Activation of Src stimulates VEGF protein production from various types of cell lines, and Src cooperates with VEGF receptors (KDR/Flk-1) in endothelial cells, resulting in stimulation of endothelial proliferation.[35] Thus, efforts to reduce the growth and spread of cancers have recently focused on inhibiting Src activity.[36] Activated G protein *via* a neurotransmitter such as dopamine activates an Src family kinase.[33] We also showed this pathway could contribute to a dopamine-induced signaling pathway to phosphorylate VEGF-R2 in endothelial cells. Then the tumor growth might be accelerated by angiogenesis which was induced by activation of Src through D_1R signaling. Although dopamine stimulation through D_2R reduced the surface expression of VEGFR-2,[11] our data showed that D_2R antagonist treatment did not influence tumor growth. There was a report that internalization of VEGF-R2 was mediated by a distinct mechanism involving PKC.[37] Our results suggest that D_2R stimulation with the concerned PKC led to the down regulation of VEGF-R2, but the down regulation of VEGF-R2 might discontinue later.

Our study showed that the stimulation of D_1R might accelerate tumor angiogenesis in patients with solid tumors; therefore, peripheral D_1R could be a molecular target for cancer therapy.

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Serum C-Reactive Protein Even at Very Low (<1.0 mg/l) Concentration Is Associated with Physical Performance in a Community-Based Elderly Population Aged 70 Years and Over

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Key Words
Physical performance · Inflammation · High-sensitivity C-reactive protein

Abstract
Background: Although several studies have reported that C-reactive protein (CRP) is associated with physical performance, few studies have evaluated the relationships between CRP and physical performance among subjects who had a very low range of CRP. Therefore, it is still unclear whether a lower CRP is favorably associated with physical performance even within a very low range. ***Objective:*** The aim of this study was to investigate the relationships between CRP and physical performance among a Japanese population with a low serum CRP concentration (CRP <1.0 mg/l). ***Methods:*** We designed a cross-sectional survey for 775 persons aged 70 years and older living in Japan. High-sensitivity CRP was measured using a nephelometric method. The subjects whose serum CRP concentrations were higher than 10.0 mg/l were excluded. Physical performance was assessed using a 10-meter maximum walk test, leg extension power, and a timed 'up and go' test. ***Results:*** The median value (interquartile range) of CRP was 0.55 (0.29–1.20) mg/l. After adjustment for potential confounding factors, an inverse relation of CRP with the 10-meter maximum walk test and leg power was observed in all subjects (p for trend = 0.10 and 0.04, respectively). For subjects who had a CRP <1.0 mg/l, these inverse relations were unchanged (p for trend = 0.03 and 0.02, respectively). ***Conclusions:*** Serum CRP concentration is favorably related to physical performance, even within a very low range in a community-based elderly population aged 70 years and over. The findings suggest that maintaining as low CRP levels as possible may potentially maintain better physical performance.

Introduction

Aging is associated with decreased skeletal muscle mass, quality and function [1–4] that negatively impact quality of life and may eventually compromise independence [5, 6]. An accelerated decline in muscle mass and

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strength with aging is probably one of the major causes of disability in late life [7, 8].

A chronic inflammatory state has been proposed that may be detrimental by accelerating the progression of medical conditions that result in functional decline and disability [9, 10]. Furthermore, a direct role of inflammation in the development of disability can be hypothesized based on the catabolic effects that proinflammatory cytokines may have on muscles [11]. A biological mechanism recently proposed to underlie the decline in physical function is chronic inflammation [9, 12]. Therefore, relatively high-inflammatory levels have been hypothesized to play a role in the reduction of skeletal muscle mass and physical function among the elderly.

C-reactive protein (CRP) is a classical acute-phase marker and a member of the pentraxin family of innate immune response proteins [13]. The concentration of CRP in serum is generally <2 mg/l but increases by as much as 1,000-fold in response to stimuli such as tissue injury or inflammation [14]. Following removal of the inflammatory stimulus, CRP levels decline rapidly. These features have made CRP useful as a clinical marker of an inflammatory process. Recent studies have particularly focused on CRP as measured by a high sensitivity assay (hsCRP) [15]. High-sensitivity CRP detects the same CRP molecule as older CRP tests, but its lower limit of detection is substantially lower and it can therefore detect lower levels of inflammation [15].

Several epidemiological studies assessed the relationship between CRP and physical performance in an elderly population [9, 16–19]. Although these observational studies have demonstrated that there is an inverse association of serum CRP concentrations with physical performance, the serum CRP concentration in these studies was higher than it is in the Japanese [20–25]. Our previous study showed that a higher consumption of fish may be contributing to the lower serum CRP concentrations among the older Japanese population [26]. Moreover, although the CRP concentrations of <1.0, 1.0–3.0 and >3.0 mg/l have been associated with low, intermediate and high risk, respectively, for coronary heart disease (CHD) [27], serum CRP concentration may be positively associated with a preclinical inflammation status even within a very low range. However, few studies have reported the relationship between a very low range of serum CRP concentration and physical performance. Therefore, it is still unclear whether the serum CRP concentration is associated with physical performance even within a very low range.

Thus, to investigate whether lower serum CRP relates to a favorable physical performance even at concentrations <1.0 mg/l, we designed a cross-sectional study in a Japanese elderly population.

Subjects and Methods

Study Participants

Our study population was composed of subjects aged 70 years and older who were living in the Tsurugaya area of Sendai, one of the major cities in the Tohoku area of Northern Japan. At the time of the study in 2002, there were 2,730 individuals aged 70 years and older living in Tsurugaya. All of these individuals were invited to participate in a comprehensive geriatric assessment, which included medical status, physical function, cognitive function and dental status and 1,178 of them accepted, giving their informed consent for data analysis. The protocol of this study was approved by the Institutional Review Board of the Tohoku University Graduate School of Medicine.

We excluded subjects whose hsCRP had not been measured (n = 29). Those subjects whose serum CRP concentrations were higher than 10.0 mg/l (n = 35) were also excluded, because people with acute inflammatory conditions frequently have serum CRP concentrations ≥10.0 mg/l [28]. In addition, subjects who did not complete the measurement on the physical performance test were excluded (n = 89), as were all potential subjects with notable comorbidity factors that might influence the frequency and degree of physical activity by self-reported arthritis (n = 163) or a history of stroke (n = 39), as well as 48 subjects with peripheral arterial disease (PAD; lowest leg ankle brachial index, ABI, <0.90). As a result of these exclusions, the final study population was composed of 775 subjects [age 75.9 ± 4.7 years (mean ± standard deviation, SD); men: 43.0%].

Measurement of Serum CRP

The CRP concentrations were determined using an immunotechnique on a Behring BN II analyzer (Dade Behring, Tokyo, Japan). The BN II high sensitivity assay utilizes a monoclonal antibody coated on polystyrene particles and fixed-time kinetic nephelometric measurements [29]. The detection limit of this assay is 0.02 mg/l.

Physical Performance Tests

Physical performance was measured with three tests: 10-meter maximum walk test, leg extension power and a timed up and go test. The physical performance tests were measured by a well-trained physiotherapist as follows:

- Ten-meter maximum walk test [30]: Each participant was asked to walk 10 m at maximum walking speed. A stopwatch was used for timing, and a counter was used to obtain the number of steps. To eliminate periods of acceleration and deceleration, the subjects started their laps 3 m before the beginning of the walkway and concluded them 3 m beyond its end. The test was repeated three times, and the data of the fastest walk were recorded. These data were used to determine each subject's maximum walking speed in meters per second.

- Leg extension power: The participants were placed well back on a seat, and the waist was fixed with a belt. The knee joint was angled at 90°. The isometric contractions lasted for 5 s each and were separated by 15-second rest intervals. Peak power was detected, calculated, and recorded in watts by a microcomputer. The average of the two highest measurements among 5 trials was recorded as 'isometric strength performance' (Aneropress 3500, Combi Wellness, Tokyo). To minimize differences in body mass, leg extension power was expressed as the average peak of the leg relative to body weight (W/kg).
- Timed 'up and go' test [31]: The participants were seated in a free-standing padded armchair (46 cm high) and asked to rise (with or without using the arm rests), walk to a mark 3 m away, turn around, and walk back to the chair and sit down. The time between rising from the seat and making contact with the back of the seat was measured in seconds. This test was repeated three times and the time of the fastest trial was recorded.

Assessment of Other Variables

Anthropometrics (height, body weight) were recorded using a standardized protocol. Body mass index (BMI) was calculated as weight (kg)/height (m)^2. Blood pressure (BP) was measured at home with an HEM747IC device (Omron Life Science Co. Ltd, Tokyo, Japan), which uses the cuff-oscillometric method to generate a digital display of systolic and diastolic pressures. The mean of 15.6 ± 10.5 (SD) BP measurements were used as the BP values. Participants who did not measure their home BP on at least 3 days were treated as having missing information on hypertension. The ABI was measured using established methods [32]. The lowest leg ABI was used in this study.

Blood samples were drawn from the antecubital vein of the seated subject with minimal tourniquet use. Specimens were collected in siliconized vacuum glass tubes containing sodium fluoride for blood glucose, and no additives for albumin, lipids and CRP analyses.

Total cholesterol (T-C), high-density lipoprotein cholesterol (HDL-C) concentrations and blood glucose concentrations were measured by enzymatic methods (T-C, Denka Seiken, Tokyo, Japan; HDL-C, Daiichi Pure Chemicals, Tokyo, Japan; blood glucose, Shino-Test, Tokyo, Japan). Information on smoking status, drinking status, use of medication and histories of prior CHD, cancer and stroke were obtained from the questionnaire survey. The drug information was confirmed by a well-trained pharmacist. All individuals were told to bring their own drug to the scene of the conduct, and were checked and recorded by pharmacist. The 30-item Geriatric Depression Scale (GDS) [33] was used to assess depressive symptoms. Cognitive functioning was measured with the Mini-Mental State Examination (MMSE) [34]. The mean daily intake of nutrients including energy and n-3 polyunsaturated fatty acids (n-3 PUFA), was obtained from a brief self-administered diet-history questionnaire [35]. Detailed information is provided in our previous reports [26].

Definitions of Variables

We categorized the study participants on the basis of the recently proposed cutoff points for CRP as having low concentrations (<1.0 mg/l) or high concentrations (at least 1.0 mg/l) [35, 36].

Hypertension was defined as a home systolic BP (SBP) of 135 mm Hg or over and/or a home diastolic BP (DBP) of 85 mm Hg or over or use of antihypertensive agents [37]. Diabetes was defined as a casual blood glucose concentration of 200 mg/dl or over or current use of an antidiabetic medication. Hypercholesterolemia was defined as a concentration of T-C of 220 mg/dl or over, or current use of nonstatin lipid-lowering agents. We treated statin agents as independent confounding factors because they have been reported to lower CRP concentrations [38].

Physical activity (PA) was assessed first by a self-reported single-item question on whether the participant obtained any PA in the past year. If yes, questions were asked about the frequency and duration of walking, brisk walking, and sports. PA was then classified into 3 categories based on the frequency and duration in the participant: (1) High, at least 3–4 times per week for at least 30 min each time; (2) Low, reporting some activity in the past year, but not enough to meet high levels, and (3) None, no PA. PA was then further classified into six levels based on the above three categories and each physical activity such as walking, brisk walking, and sports: (1) Level 1, no walking, no brisk walking, no sports; (2) Level 2, low walking, no brisk walking, no sports; (3) Level 3, high walking, no brisk walking, no sports; (4) Level 4, any walking, low brisk walking, no sports; (5) Level 5, any walking, high brisk walking, no sports; (6) Level 6, any walking, any brisk walking, low or high sports. Detailed information is provided in previous reports [39]. Finally, the subjects were divided into two categories: level 3 or lower or higher than level 3. A GDS score of ≥11 was used to indicate depressive symptoms [40]. An MMSE score of <26 was used to indicate cognitive impairment [41].

Statistical Analysis

Descriptive data are presented as means (95% confidence interval, 95% CI) or percentages. The values of the physical performance measurement were used as the dependent variable and the serum CRP concentration level as the independent variable. The CRP levels were categorized as follows: CRP ≥1.0 mg/l and the tertiles of CRP <1.0 mg/l. The differences in variables among the CRP groups were examined by analysis of covariance (ANCOVA) for continuous variables or by multiple logistic regression analysis for variables of proportion after adjustment for age and sex. ANCOVA was used to examine the relation of CRP with physical performance after adjustment for age, sex, BMI, serum albumin concentration, hypercholesterolemia (nonstatin drugs), low HDL cholesterol (≤40 mg/dl), history of CHD, hypertension, diabetes, history of cancer, depressive symptoms, impaired cognitive function, smoking habits/history, PA, use of nonsteroidal anti-inflammatory drugs (NSAIDs), statin drugs, aspirin, angiotensin-converting enzyme inhibitors and n-3 PUFA intake levels (the consumption of n-3 PUFA per 2,000 kcal of energy intake categorized in tertiles) in all subjects or in subjects who had a very low serum CRP concentration (<1.0 mg/l). All p values for linear trend across the tertile of CRP and CRP >1.0 mg/l group were calculated by using the median of each CRP group. Tukey post-hoc analysis also was conducted. The interactions were assessed by testing the interaction term added to the adjusted model as a covariate. Furthermore, multiple linear regression analysis was used to establish the relationship between log-transformed CRP levels, treated as a continuous variable and physical performance after adjustment for the same covari-