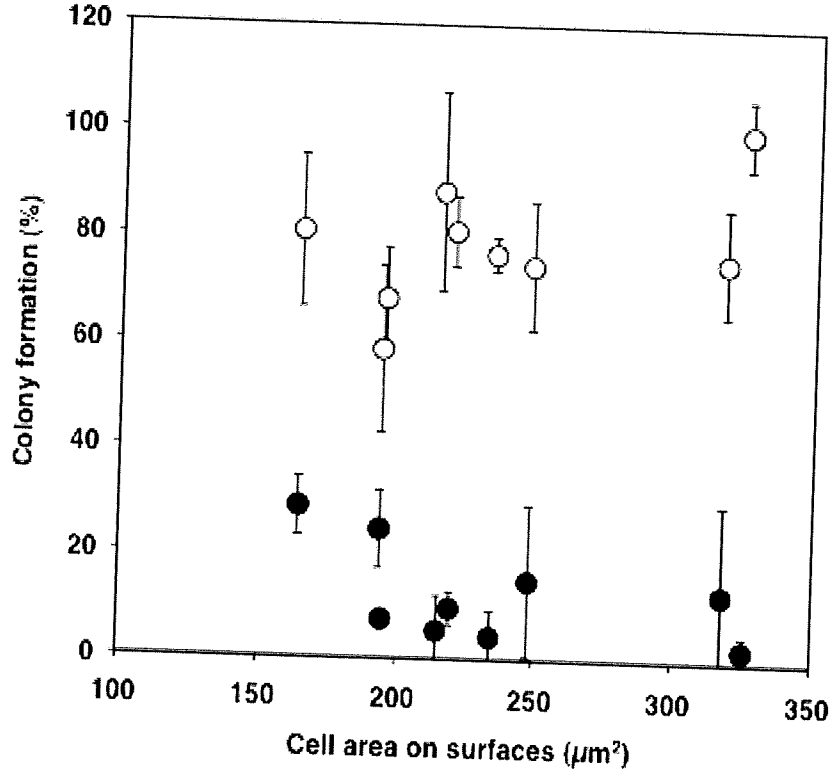
図６．メチル基と水酸基とからなるモデル表面の代謝協同阻害活性とそれらの表面上に接着した細胞の占有面積（１日後）との関連性
（○：細胞毒性。●：代謝協同阻害活性）



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研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Takashi Yamada, Ryusuke Nakaoka, Rumi Sawada, Atsuko Matsuoka, Toshie Tsuchiya	Effects of intracerebral microinjection of hydroxylated-[60]fullerene on brain monoamine concentrations and locomotor behavior in rats	J. Nanoscience Nanotechnol.	10	604-611	2010
Ryusuke Nakaoka, Yoko Yamakoshi, Kazuo Isama, Toshie Tsuchiya	Effects of surface chemistry prepared by self-assembled monolayers on osteoblast behavior	J. Biomed. Mater. Res. A			in press

III　研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍名	出版社名	ページ	出版年
土屋利江	フラーレンの毒性	ナノ材料のリスク評価と安全性対	フロンテイア出版	in press	
土屋利江	医療用ナノ材料の国際標準化動向	ナノ材料のリスク評価と安全性対	フロンテイア出版	in press	

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発表者名	論文タイトル	発表誌名	巻号	ページ	出版年
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Makoto Suto, Hiroyuki Kosukegawa, Kaoru Maruta, Makoto Ohta, Kazuyuki Tohji, Balachandran Jeyadevan	Heat diffusion characteristics of magnetic nanoparticles dispersed hydro-gel in alternating magnetic field	Journal of Magnetism and Magnetic Materials	321	3483-3487	2009
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Takashi Yamada, Ryusuke Nakaoka, Rumi Sawada, Atsuko Matsuoka, Toshie Tsuchiya	Effects of intracerebral microinjection of hydroxylated-[60] fullerene on brain monoamine concentrations and locomotor	J. Nanoscience Nanotechno	10	604-611	2010
石川 格, 澤田 留美, 加藤 幸夫, 辻 紘一郎, 邵 金昌, 山田 貴史, 加藤 玲子, 土	新無血清培地STK2のヒト間葉系幹細胞増殖における有用性について	YAKUGAKU ZASSHI	129(3)	381-384	2009
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IV　研究成果の刊行物・別刷

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Effects of Intracerebral Microinjection of Hydroxylated-[60]Fullerene on Brain Monoamine Concentrations and Locomotor Behavior in Rats

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Fullerenes are condensed ring aromatic compounds with extended π systems; they have unique cage structures. Current studies suggest that several fullerene derivatives have neuroprotective effects, and it is expected that fullerenes will be useful in drug delivery system and novel medical devices targeting the brain. However, little is known about the effects of fullerenes and its derivative on brain function. We examined the effect of fullerene$(OH)_{24}$ on the central nervous system in this study. In a V79 colony assay, the IC_{50} of fullerene$(OH)_{24}$ was 1.74 μg/ml. In an MTT assay, fullerene$(OH)_{24}$ reduced proliferation of normal human astrocytes obviously. In an vivo study, 0.25 mg/kg^{-1} of fullerene$(OH)_{24}$ was injected into the lateral ventricle of rat brains. The intracerebral injection of fullerene$(OH)_{24}$ remarkably decreased body weight and locomotor behavior of rats on day 1, but drastically increased locomotor behavior on day 7. The intracerebral injection of fullerene$(OH)_{24}$ changed the monoamine concentration greatly on day 1 and slightly on day 30 after the injection. These results suggest that intracerebral injection of fullerene$(OH)_{24}$ had strong and acute effects on the central nervous system, but that the effects were not permanent. In conclusion, we suggest that fullerene's derivative, fullerene$(OH)_{24}$ had toxic effects on brain cells and that intracerebral injection of fullerene$(OH)_{24}$ had acute harmful effects on brain monoamines neurotransmission and locomotor activity.

Keywords: Nanomaterials, Fullerene, Monoamine, Locomotor Activity, Neurotoxicity.

RESEARCH ARTICLE

1. INTRODUCTION

Recently, fullerenes have been used for medical devices such as carbon nanotubes because they have interesting properties that are useful in biomedical applications.[1] The biological properties of fullerenes include photocleavage,[2] antiapoptotic activity,[3] drug and gene delivery,[4,5] antioxidation,[6] chemotaxis,[7] antibacterial activity,[8] and enzyme inhibition.[9] In addition to these, there are some reports of neuroprotective effects of fullerenes. It was suggested that polyhydroxylated fullerene60 has neuroprotective potency associated with its ability to prevent hydrogen peroxide- and cumene hydroperoxide-caused damage in rat hippocampal slices.[10] Hexasulfobutylated C_{60} had a neuroprotective effect on focal cerebral is chemia in rats *in vivo*.[11] These results suggested that fullerenes have a neuroprotective effect in oxidative stress. However, the examinations of the effects of fullerenes in normal rats and normal condition *in vitro* are few. Moreover, several studies reported the risk and toxicity of fullerenes. Tsuchiya et al. estimated the effects of [60]fullerene (C_{60}) on normal mouse embryonic mid-brain cells *in vitro* and mouse embryos *in vivo*. They reported that intraperitoneal (i.p.) injection of C_{60} into pregnant mice at 50 mg/kg had a harmful effect on the embryo head region and tail.[12] Our previous study suggested that the effect of microinjection of C_{60} into the brain on brain functions was different from the effect of i.p. injection of C_{60} into the rat, and this difference involved the blood-brain barrier, which allows certain materials to pass from blood to brain and maintains homeostasis of the brain.[13] Thus, before fullerenes are used clinically, their effects on the brain should be examined carefully. In addition, it was suggested that different C_{60} derivatives have different biological actions and water solubilities. For example, it was suggested that hydroxylated-[60] fullerene [$C_{60}(OH)_{24}$], a fullerene with increased water solubility (Fig. 1), had nitric oxide-scavenging activity[14] and cardioprotective[15] and hepatoprotective effects.[16] On the other hand, $C_{60}(OH)_{24}$ had cytotoxicity and its toxicity differed from C_{60}.[17] Thus, we expect that different C_{60} derivatives have different neural cytotoxicity potentials

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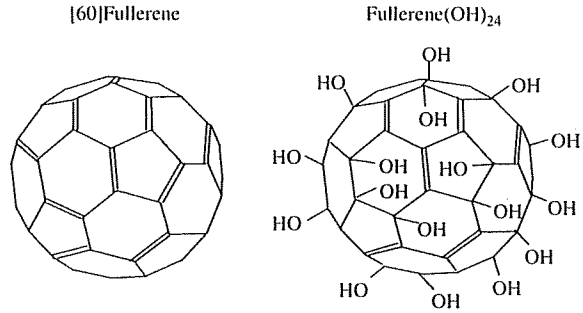

Fig. 1. Structures of [60]fullerene and fullerene$(OH)_{24}$.

depending on the chemical structure of the derivative, although all C_{60} derivatives have the same cage structure.

Specific data on the safety of fullerene C_{60} and its derivatives for brain functions are needed before fullerenes are used as medical devises in the brain. Thus, we examined $C_{60}(OH)_{24}$, and demonstrated their effects on behavior and brain neurotransmitters in normal Wistar rats in this study.

2. MATERIALS AND METHODS

2.1. V79 Colony Assay

A cytotoxicity test was performed by colony assay. The colony assays using V79 cells followed "Guidelines for Basic Biological Tests of Medical Materials and Devices-Part III: Cytotoxicity test." Chinese hamster fibroblast V79 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). V79 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a 37° humidified atmosphere of 5% CO_2 and 95% air. In the assay, the medium was changed to Eagle's MEM supplemented with 5% FBS and 1% penicillin-streptomycin, and 50 cells/ml of V79 cell suspension were seeded on 24-well plates. After 24 hours incubation in a 37 °C humidified atmosphere of 5% CO_2 and 95% air, $C_{60}(OH)_{24}$ (ATR Co., Chiba Japan) or without fullerenes (control) was added to each well, and the cells were cultured for 7 days. The cell cultures were fixed with 10% formalin solution, and the colonies formed were stained with 5% Giemsa staining solution. The number of colonies in each well was counted, and the effect of the fullerene was calculated as a ratio of the number of colonies in the sample to that in the control. The data were expressed as averages of 5 wells, and the procedures were performed in triplicate.

2.2. NHA MTT Assay

The effects of C_{60} and $C_{60}(OH)_{24}$ on cell proliferation (including mitochondrial activity) of normal human astrocytes (NHA) were measured using a microtiter tetrazolium (MTT) assay. NHA cells were extracted from a human fetus at 18 wks gestation. The basic culture medium was ABM medium supplemented with 5% FBS and recombinant human epidermal growth factor, insulin, GA-1000, ascorbic acid, and L-glutamate (ANG bred kit). NHA were seeded into 24-well plates at a density of 1×10^4/well in ABM medium at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Stock solutions of C_{60} and $C_{60}(OH)_{24}$ were made directly in ABM medium. After 1 wk culture with 0.1, 1, 10, or 100 μg/ml of C_{60} or $C_{60}(OH)_{24}$, the medium in each well was replaced with 300 μl of fresh medium containing 6 μl TetraColor ONE reagent (Seikagaku Corp., Tokyo, Japan). After 2 hours, the absorbance at 450 nm/630 nm was measured using a plate reader. The data were expressed as averages of 5 wells, and the procedures were performed in triplicate.

2.3. Animals

Male Wistar rats (270 g weight; SLC, Shizuoka, Japan) were kept in individual wire cages in a temperature- and humidity-controlled room (24 °C and 55% relative humidity) under regular lightning conditions (12 h light:dark cycle), and given food and water *ad libitum*. This experiment was carried out in accordance with the guidelines for the care and use of laboratory animals of the National Institutes of Health Sciences (NIHS) that refer to the American Association for Laboratory Animals Science.

2.4. Intracerebral Injection of $C_{60}OH_{24}$

The rat was anesthetized and a hole was drilled in its skull using a stereotaxic high-speed drill (MUROMACHI KIKAI CO., LTD. Tokyo, Japan). A microliter syringe was stereotaxically inserted into the right lateral ventricle (0.6 mm posterior to the bregma, 1.6 mm lateral to the midline, and 3.5 mm ventral to the cortical surface) according to the atlas of Paxinos and Watson.[18] $C_{60}(OH)_{24}$ (0.25 mg/100 g^{-1}) dissolved in Ringer's solution was injected at the rate of 1 μl/min by a brain infusion stereotaxic injector (MUROMACHI KIKAI CO., LTD.). Control group rats were injected with the same volume of Ringer's solution.

2.5. Open Field Test

Motor activities (total locomotor distance, average locomotor speed, number of sections crossed, opening latency) of rats were measured by an open field test. The open field box size was $70 \times 70 \times 70$ cm. Tests were performed on day 1, day 7 and day 30 after intracerebral injection. The rat moved in the open field box for 10 minutes, and the behavior was recorded by a DV-Track video tracking system CompACT VAS/DV (MUROMACHI KIKAI CO., LTD.). The floor of the open field box was divided into 16 square sections, and a crossing counted when the rat stepped over one of the divisions.

2.6. Neurotransmitter Concentrations in Rat Brain Tissue

After the behavioral test, the rat was decapitated, and brain tissues were removed. Wet brain tissue was homogenized in two volumes of 0.2 M perchloric acid buffer (pH 2) and kept on the ice for 1 hour. Homogenates were centrifuged at 20,000 g for 15 min at 0 °C, then filtered through a 0.45 μm cellulose acetate membrane filter. Neurotransmitters were detected using an HPLC system under the following conditions: the mobile phase was 0.1 M sodium acetate citric acid buffer and 15% methanol containing EDTA-2Na and 1-octanesulfonic acid sodium salt. The HPLC system was equipped with a reversed-phase column (MA-5ODS, 4.6 × 150 mm, EICOM, Kyoto, Japan) and electro chemical detector (HTEC-500: EICOM). Recording of chromatograms and all calculations were performed using an integrator (EPC-500IS, EICOM). We measured dopamine (DA), serotonin (5-HT), and norepinephrine (NE), and the metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine hydrochloride (3MT), and 5-hydroxyindole-3-methoxyphenylacetic acid (5HIAA).

3. STATISTICAL ANALYSIS

In the *in vitro* study, data for an individual group is expressed as a mean, and one-way ANOVA was performed for statistical analysis. A Tukey-Kramer test was used to analyze differences between the control group and other groups in terms of cell viability in the V79 colony and NHA MTT assays. In the *in vivo* study, data for the individual groups are expressed as a mean, and one-way ANOVA was performed for statistical analysis. Student's *t*-test was used to assess differences between the two groups. In all cases, $P < 0.05$ was considered significant. Results are expressed as means ± SD.

4. RESULTS

4.1. V79 Colony Assay

Treatment with $C_{60}(OH)_{24}$ decreased V79 colony formation in a dose-dependent manner. The IC_{50} of $C_{60}(OH)_{24}$ on V79 cells was 1.74 μg/ml (Fig. 2). All data shown are an average of five samples.

4.2. Proliferation of NHA Cells

Treatment with C_{60} significantly decreased NHA proliferation only at 100 μg/ml. Treatment with $C_{60}(OH)_{24}$ decreased NHA proliferations in a dose-dependent

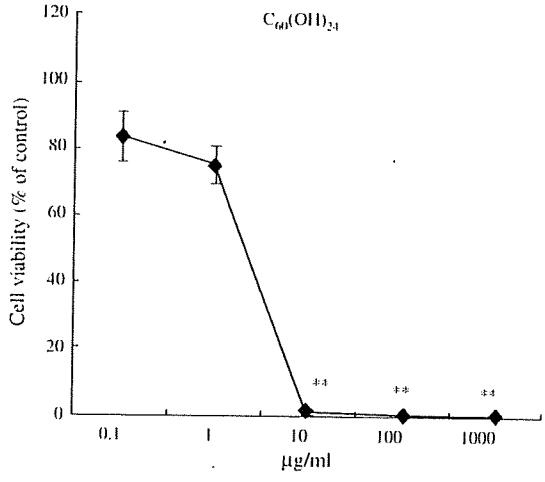

Fig. 2. V79 colony assay with $C_{60}(OH)_{24}$ treatment. V79 were treated with $C_{60}(OH)_{24}$ and cultured for 7 days. Data are expressed as means ± SD ($n = 5$). Experimental data showed significant differences from the control group (**; $p < 0.01$).

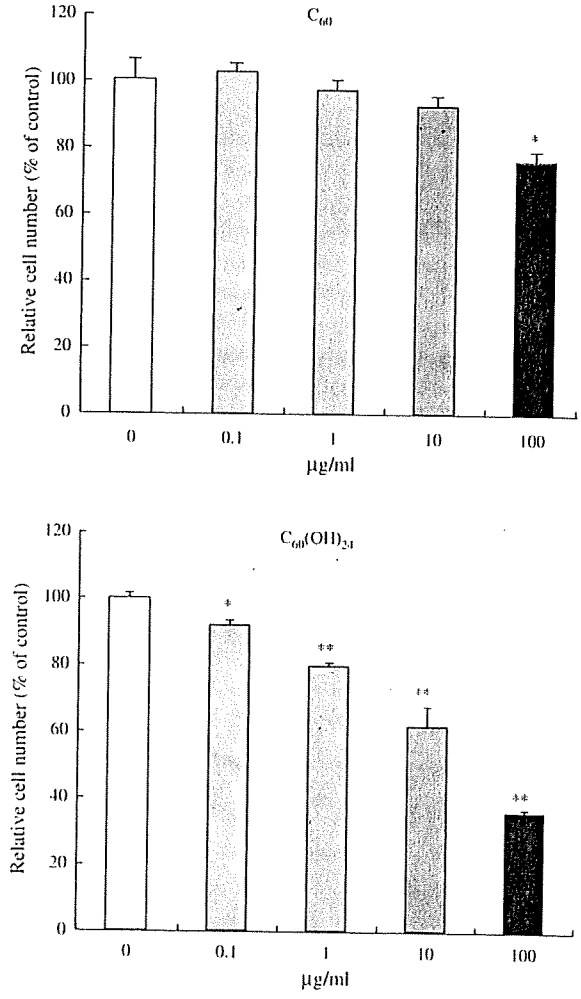

Fig. 3. MTT assay of normal human astrocytes with C_{60} and $C_{60}(OH)_{24}$ treatment. NHA was treated with C_{60} or $C_{60}(OH)_{24}$ and cultured for 7 days. Data are expressed as means ± SD ($n = 5$). Experimental data showed significant differences from the control group (*; $p < 0.05$. **; $p < 0.01$).

manner, and a significant decrease was found at concentrations over 0.1 μg/ml (Fig. 3). All date shown are an average of five samples.

4.3. Open Field Test

The intracerebral injection of $C_{60}(OH)_{24}$ (0.25 mg/100 g^{-1}) remarkably decreased postoperative body weight, but the loss of body weight was almost recovered after 30 days (Fig. 4). In the open field test, the group intracerebrally injected with $C_{60}(OH)_{24}$ ($n = 6$) showed seriously decreased locomotor distance and number of sections crossed on day 1, but they were sharply increased on day 7. The increases of locomotor distance and number of sections crossed tended to be suppressed but was statistically significant on day 30. The average locomotor speed was decreased and opening latency was increased significantly only on day 1 (Fig. 5).

4.4. Neurotransmitter Concentrations in Rat Brain Tissue

One day after the intracerebral injection of $C_{60}(OH)_{24}$ (0.25 mg/100 g^{-1}), the NE concentration was significantly changed in the hypothalamus, cerebral cortex, and striatum. Concentrations of serotonin and its metabolite 5HIAA were significantly changed in the hypothalamus, cerebral cortex, striatum, and hippocampus. Dopamine was not changed in any region, but its metabolite DOPAC and 3MT concentrations were significantly changed in the cerebral cortex and striatum. The turnover rates of serotonin were increased in the hypothalamus, cerebral cortex, striatum, and hippocampus. The turnover rates of dopamine were increased in the cerebral cortex and hippocampus (Table I). Thirty days after the intracerebral injection of $C_{60}(OH)_{24}$ (0.25 mg/100 g^{-1}), the NE concentration was significantly decreased in the hypothalamus and hippocampus, and serotonin and 5HIAA concentrations were significantly decreased in the hypothalamus, cerebral cortex, and hippocampus. Dopamine, DOPAC, HVA, and 3MT concentrations were not changed in any region. The turnover rates of serotonin were not changed in any region. The turnover rate of dopamine was increased only in the cerebral cortex (Table II).

5. DISCUSSION

Novel synthesized biomaterials are attractive candidates for medical devices. Recently nanomaterials have attracted attention because they can be developed in various forms for various functions, and have great potential for utilization as minimally invasive medical devices and drug delivery systems.[1,4,5] In addition, clinical brain and neural treatments have advanced rapidly, and implantation techniques and novel medical devices for brain are being developed every day. However, adverse events such as foreign-body reaction, inflammation, and tumor formation have been reported in clinical human and animal studies. Therefore, it is necessary to confirm the toxicity or safety of these materials. In particular, the brain performs various functions, and although the blood-brain barrier (BBB) usually protects the central nerve system (CNS) from harmful materials,[17] we considered that oral administration or i.p. injection studies and *in vitro* studies are insufficient to establish the effects of materials on brain function. Therefore, we need to investigate the direct effects of biomaterials on the CNS before application of novel materials as medical devices. Here, we showed that intracerebral and i.p. injection of C_{60} had different effects on rat brain functions.[13] Fullerenes have been developed in various

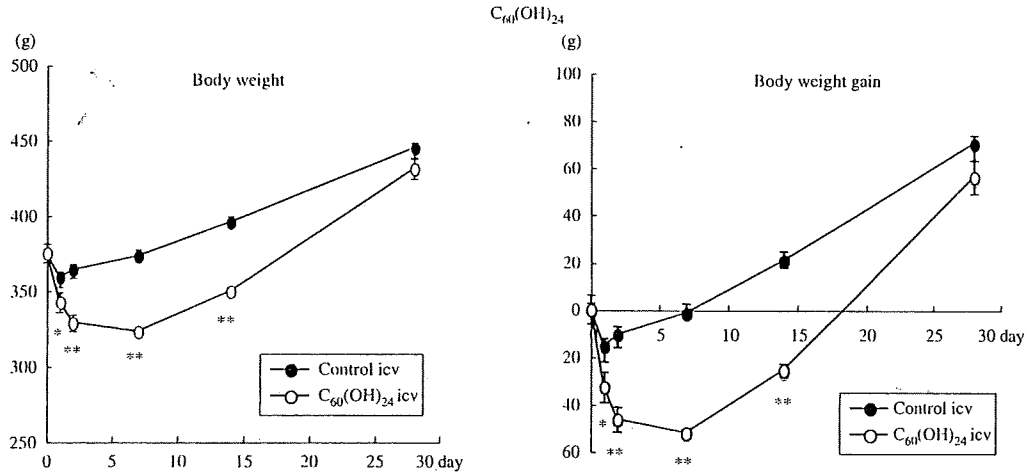

Fig. 4. Body weight changes after $C_{60}(OH)_{24}$ intracerebral injection. $C_{60}(OH)_{24}$ (0.25 mg/100 g^{-1}) was injected into the right lateral ventricle. Control rats were injected with the same amount of Ringer's solution. Data are expressed as means ± SD ($n = 6$). Experimental data showed significant differences from the control group (*; $p < 0.05$, **; $p < 0.01$).

RESEARCH ARTICLE

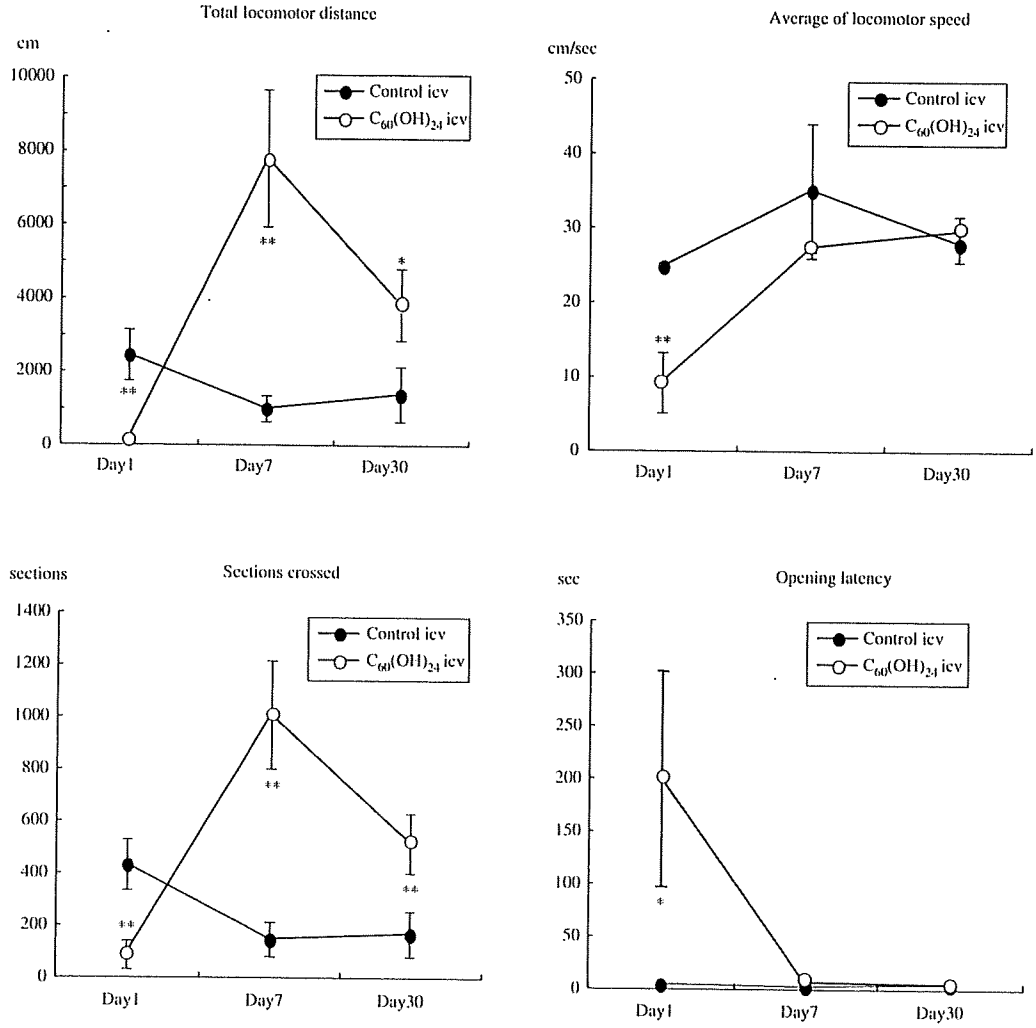

Fig. 5. Effects of $C_{60}(OH)_{24}$ intracerebral injection on locomotor activity in rats. $C_{60}(OH)_{24}$ (0.25 mg/100 g^{-1}) was injected into the right lateral ventricle. Control group rats were injected same amount of Ringer's solution. Open field tests were performed 1, 7, and 30 days after fullerene injection. Data were expressed as means ± SD ($n = 6$). Experimental data showed significant differences from the control group (*; $p < 0.05$, **; $p < 0.01$).

forms to fulfill various functions. C_{60} derivatives have different biological actions and water solubility. This implies that these fullerene materials might have various different effects. Some reports have shown the benefits of fullerene and its derivatives, such as antioxidative effect, neuroprotective effects, and the others.[6, 10, 15, 16] These results suggest that several fullerenes will be applied to drug delivery system and novel medical materials targeting the brain. However, there are a few reports questioning the safety of fullerenes, in particular *in vivo* study data. We consider that comparative *in vivo* methods are effective to characterize the safety of fullerene and its derivatives for brain functions, and we focused on the water solubility of fullerenes and investigated the effects of the more water soluble fullerene $C_{60}(OH)_{24}$ on rat brain functions.

First, we examined the cytotoxicity of $C_{60}(OH)_{24}$ by *in vitro* studies. In the V79 colony assay, the IC_{50} of $C_{60}(OH)_{24}$ in the V79 cells was 1.74 μg/ml. On the other hand, we had shown that the IC_{50} of C_{60} in the V79 cells was 1620 μg/ml.[13] In the MTT assay, 100 μg/ml C_{60} significantly decreased NHA cell proliferation to 80% of the control. Concentration over 0.1 μg/ml $C_{60}(OH)_{24}$ significantly decreased NHA cell proliferation, and 100 μg/ml $C_{60}(OH)_{24}$ decreased it to 40% of the control. These results suggested that $C_{60}(OH)_{24}$ was more harmful to cell viability than C_{60}, and that the cytotoxicity of C_{60} on cells was weak. However, the mechanism and reason for the difference were not clear. We observed the forms of C_{60} and $C_{60}(OH)_{24}$ suspended in water using an electron microscope and cells incubated with fullerene containing media by a stereoscopic microscope in this study. The particles of $C_{60}(OH)_{24}$ in the solution were finer and smoother than those of C_{60}, and both fullerenes adhered to the cells (data not shown). Therefore, we hypothesized that the physical

Table I. Effects of $C_{60}(OH)_{24}$ intracerebral injection on brain monoamine neurotransmitters at 1 day.

		NE	5HT	5HIAA	DA	DOPAC	HVA	3MT	5HIAA/5HT	(DOPAC+HVA (+3MT))/DA
Hypothalamus	Control	17.8±0.81	10.0±0.44	8.61±0.61	4.01±0.16	1.66±0.05	0.31±0.03		0.85±0.03	0.54±0.02
	$C_{60}(OH)_{24}$	10.4±1.70*	7.69±0.22*	12.1±1.10	3.89±0.18	1.54±0.11	0.41±0.10		1.58±0.11*	0.53±0.04
Cerebral cortex	Control	32.0±1.15	27.3±0.80	35.6±1.68	2.09±0.09	1.58±0.07	1.39±0.15		1.30±0.03	1.43±0.13
	$C_{60}(OH)_{24}$	23.1±2.08*	20.0±2.80*	56.7±7.30*	2.67±0.56	3.04±1.02*	3.31±1.23		3.08±0.58*	2.20±0.28*
Striatum	Control	0.99±0.06	3.33±0.18	6.64±0.42	66.4±4.38	12.6±0.83	5.32±0.29	2.45±0.10	1.99±0.03	0.32±0.02
	$C_{60}(OH)_{24}$	1.38±0.19*	2.99±0.19	11.0±2.18*	67.3±4.89	15.6±3.08	8.20±2.56	1.83±0.18*	3.71±0.78*	0.37±0.06
Hippocampus	Control	7.41±0.81	8.14±0.94	10.9±1.20	0.44±0.20	0.31±0.20	0.48±0.23		1.35±0.04	1.66±0.20
	$C_{60}(OH)_{24}$	6.20±1.02	6.00±0.86	20.2±1.44*	0.39±0.13	0.46±0.24	0.63±0.23		3.78±0.82*	2.72±0.38*
										ng/mg tissue

$C_{60}(OH)_{24}$ (0.25 mg/100 g^{-1}) was injected into the right lateral ventricle, and rats were decapitated 1 day later. Control group rats were injected with the same amount of Ringer's solution. Data are expressed as means ± SD ($n = 6$). Experimental data showed significant differences from the control group (*; $p < 0.05$).

stimulus of fullerenes was involved in the V79 colony formation and astrocyte proliferation, and that $C_{60}(OH)_{24}$ had greater effect than C_{60} because $C_{60}(OH)_{24}$ could approach a cell membrane more closely than C_{60} could.

In the next study, we examined the effects of $C_{60}(OH)_{24}$ in rats by $C_{60}(OH)_{24}$ injection into lateral ventricle. Gharbi et al. showed that 500 mg/100 g^{-1} $C_{60}(OH)_{24}$ i.p. injection did not show acute toxicity in mice.[19] Injac et al. showed that 10 mg/100 g^{-1} $C_{60}(OH)_{24}$ i.p. injection prevents doxorubicin-induced hepatotoxicity in rats,[16] and Trajkovic et al. showed that 10 mg/100 g^{-1} $C_{60}(OH)_{24}$ i.p. injection had tissue protective effects from ionizing radiation.[20] On the other hands, Ueng et al. showed that the LD50 value of fullerenol-1, a polyhydroxylated fullerene, was estimated to be 1.2 g/kg^{-1} in mice.[21] Then intracerebral injection of 0.25 mg/100 g^{-1} C_{60} did not show lethal and acute toxicity in our previous study.[13] Therefore, we injected 0.25 mg/100 g^{-1} $C_{60}(OH)_{24}$ into the rat brain, and compared the effects of $C_{60}(OH)_{24}$ with that of C_{60}. Intracerebral injection of $C_{60}(OH)_{24}$ considerably decreased postoperative body weight and locomotor activities of rats on the day after operation. This result suggested that $C_{60}(OH)_{24}$ had acute toxicity. However, locomotor activities of these rats were remarkably increased on day 7, and the locomotor distance of $C_{60}(OH)_{24}$-injected rats was approximately 8 times that of the control, and the body weight of $C_{60}(OH)_{24}$-injected rats remained low. However, on day 30, the body weight loss of these rats was recovered and was no different from that of control rats. The locomotor activities of $C_{60}(OH)_{24}$-injected rats also tended to approximate those of C_{60}-injected rats. On the other hand, our previous study showed that the intracerebral injection of C_{60} did not significant effects on the postoperative body weight of rats. In the open field test, the total locomotor distance, locomotor speed, and number of sections crossed were significantly increased by C_{60} on the day after operation. In addition, the body weight gain, total locomotor distance, and number of sections crossed of C_{60}-injected rats tended to increase more than those of control rats on day 30.[13] On the other hand, several substances, for example, the glutamate receptor agonist kainic acid,[22] the gamma-aminobutyric acid (GABA) receptor antagonist strychnine,[23] and other neurotoxins[24] expressed neurotoxicity in doses quite a bit lower than the fullerene concentrations used in this study and cause vital body weight loss and locomotor difficulty or death. From these results, we

Table II. Effects of $C_{60}(OH)_{24}$ intracerebral injection on brain monoamine neurotransmitters at 30 days.

		NE	5HT	5HIAA	DA	DOPAC	HVA	3MT	5HIAA/5HT	(DOPAC+HVA (+3MT))/DA
Hypothalamus	Control	31.93±2.08	14.07±0.89	12.09±1.72	5.74±0.75	3.58±0.23	0.97±0.18		1.23±0.10	0.83±0.10
	$C_{60}(OH)_{24}$	22.56±0.90*	10.03±0.44*	8.09±0.31	4.57±0.20	2.46±0.19	0.87±0.06		1.43±0.07	1.07±0.05
Cerebral cortex	Control	2.60±0.19	1.21±0.06	1.70±0.16	0.31±0.02	0.17±0.01	0.07±0.01		1.15±0.07	0.65±0.08
	$C_{60}(OH)_{24}$	2.18±0.22	0.95±0.06*	1.52±0.10	0.32±0.06	0.34±0.11	0.09±0.01		1.29±0.15	1.68±0.44*
Striatum	Control	0.41±0.14	0.58±0.05	1.31±0.07	16.5±0.58	8.10±0.31	1.64±0.06	0.76±0.03	1.86±0.09	0.52±0.03
	$C_{60}(OH)_{24}$	0.39±0.02	0.69±0.06	1.47±0.08	15.7±0.37	7.21±0.72	1.64±0.05	0.76±0.04	1.80±0.04	0.60±0.05
Hippocampus	Control	2.09±0.17	1.16±0.11	2.28±0.21	0.10±0.03	0.08±0.01	0.19±0.01		1.61±0.10	2.62±0.37
	$C_{60}(OH)_{24}$	1.48±0.06*	0.83±0.04*	1.53±0.05*	0.08±0.00	0.06±0.00	0.17±0.01		2.00±0.15	3.35±0.37
										ng/mg tissue

$C_{60}(OH)_{24}$ (0.25 mg/100 g^{-1}) was injected into the right lateral ventricle, and rats were decapitated 30 days later. Control group rats were injected with the same amount of Ringer's solution. Data are expressed as means ± SD ($n = 6$). Experimental data showed significant differences from the control group (*; $p < 0.05$).

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suggest that $C_{60}(OH)_{24}$ had higher acute toxicity than C_{60}, but neither fullerene is necessarily lethal. The toxic patterns of these fullerenes were different, and the toxicity of $C_{60}(OH)_{24}$ might be not permanent.

From the results of the behavioral tests, we speculated that intracerebral injection of fullerenes affected neurotransmission relating to locomotor or feeding behavior. Thus, we measured the concentration of serotonin (5HT) and norepinephrine (NE), major neurotransmitters related feeding behavior, emotional behavior, stress, and hormone secretion, and the metabolite 5HIAA. 5HT and NE are found in abundance in the hypothalamus, and the main neurotransmission occurs there. Dysbolisms of these neurotransmitters cause cibophobia, hyperphagia, dystrophy, and depressive behavior.[25,26] Previously we had shown that, the concentrations of 5-HT and 5HIAA were changed in the hypothalamus, cerebral cortex, striatum, and hippocampus, and 5HT turnover rates were increased in these four regions at 30 days after intracerebral injection of C_{60}. NE concentrations also changed in the cerebral cortex and hippocampus.[13] It was known that 5HT and NE neurotransmissions are enhanced when homeostasis is in disorder due to, for example, stress or inflammation and a variety of other changes in peripheral conditions.[25,26] This result suggested that C_{60} injected chronically might work as a stressor. In addition to this, the intracerebral injection of C_{60} also increased dopamine turnover rates in the hypothalamus, cerebral cortex, and striatum at 30 days, and we suggested that intracerebral injection of C_{60} increased-dopamine neurotransmission in might induce locomotor hyperactivity.[13] Dopamine is an important neurotransmitter in the brain striatum for behavior, and dopamine neurotransmission has been shown to promote ambulation or decrease anxiety in the rat.[27,28] Dopamine dysbolism is relate to depression[29] and depressive behavior of rats, and administration of the kappa-opioid receptor agonist salvinorin A reduced the extracellular concentration of dopamine within the nucleus accumbens.[30] In contrast, an excess of dopamine neurotransmission induces locomotor hyperactivity.[31,32] Dopaminergic neurotransmission is closely involved in motor activity and emotional behavior. In this study, NE concentrations in the hypothalamus and hippocampus were reduced, but 5HT turnover rates were unchanged in all regions at 30 days after intracerebral injection of $C_{60}(OH)_{24}$. In addition, concentration of dopamine and its metabolites were unchanged in all regions, and its turnover rate was increased only in the cerebral cortex. Accordingly, $C_{60}(OH)_{24}$ affected monoamine transmission weakly compared with C_{60}, but locomotor activity of these rats was significantly increased. The result was inconsistent with the result induced by C_{60}. We expected that the changes of monoamines on day 1, and the substantial decline of locomotor activity of rats observed from day 7 and day 30 might be involved in this inconsistency. However, to explain this point, we must demonstrate such an effect of $C_{60}(OH)_{24}$ on the brain monoamines 7 days after injection and the others, and we will attempt this in a further study. It was confirmed that $C_{60}(OH)_{24}$ affected brain monoamine concentrations and their metabolism in various brain regions in the day after operation but not 30 days after. The locomotor activity of $C_{60}(OH)_{24}$-injected rats was greatly changed on day 1 and 7 day after injection, but the fluctuation was abated on day 30. This indicated that $C_{60}(OH)_{24}$ affected brain monoamine metabolism and locomotor activity of rats acutely not but chronically. We hypothesized that the temporal difference between the effects of C_{60} and $C_{60}(OH)_{24}$ involved the blood-brain barrier function. The BBB protects and regulates the homeostasis of the brain, and materials are carried into or out of the brain by active transport or passive diffusion.[33] Our previous study suggested that C_{60} did not cross the BBB because i.p. injection of C_{60} did not affect brain neurotransmitters, unlike intracerebral injection of C_{60}, the effect of which was long-lasting.[13] On the other hand, it was suggested that the effects of intracerebral injection of $C_{60}(OH)_{24}$ on brain monoamines tended to have disappeared by 30 days in this study. $C_{60}(OH)_{24}$ is more water soluble than C_{60} and is supposed to diffuse as a nanometer of homogeneous diffusate in the solution. Therefore, we expected that $C_{60}(OH)_{24}$ could cross the BBB and that $C_{60}(OH)_{24}$ injected into the rat brain might have been eliminated from the brain by degrees. However, we have no confirmation of this hypothesis at present. Recently, much research encompasses central nervous system disorders and brain drug delivery,[34,35] and nanomaterials are utilized as nanocarriers[36] or materials of medical devices.[37] On the other hand, a recent report suggested that multi-wall carbon nanotubes, which are made of fullerenes, had harmful effects such as inducing mesothelioma in rats due to fibrous or rod-shaped micrometer particulates of fullerene.[38,39] A close examination of whether fullerenes and its derivatives function safely in the workplace is an urgent necessity.

In conclusion, we investigated the neurotoxicity of intracerebral injection of C_{60} and $C_{60}(OH)_{24}$. In the *in vitro* study, we showed cytotoxicity of $C_{60}(OH)_{24}$ at 1.74 μg/ml (1.74 ppm) treatment for V79 cells. In the *in vivo* study, intracerebral injection of 0.25 mg/ml $C_{60}(OH)_{24}$ elicited acute high neurotoxicity. Acute exposure to $C_{60}(OH)_{24}$ changed concentrations of some neurotransmitters and its metabolism in various brain regions, but chronic exposure to $C_{60}(OH)_{24}$ did not. We suggest that acute exposure to $C_{60}(OH)_{24}$ had harmful effects on brain functions and behavior compared with that of C_{60}, but the damage was not permanent. Some reports show that fullerenes have a cytoprotective effect from some toxicants and oxidative stresses *in vitro* and *in vivo*.[14–16] However, we suggest that fullerenes have neurotoxicity, and each has an individual character. We need to demonstrate the risks of fullerenes and their diversity quickly. Recent medical progress makes

novel medical devices targeting the brain. We assert the necessity of reexamine materials used for medical devices targeting the brain.

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Letter

Higher susceptibility of NOG mice to xenotransplanted tumors

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ABSTRACT — The purpose of tumorigenicity testing, as applied not only to cell substrates used for viral vaccine manufacture but also stem cells used for cell-based therapy, is to discriminate between cells that have the capacity to form tumors and cells that do not. Therefore, tumorigenicity testing is essential in assessing the safety of these biological materials. Recently developed NOD/Shi-*scid* IL2Rg^{null} (NOG) mice have been shown to be superior to NOD/Shi-*scid* (SCID) mice for xenotransplantation of both normal and cancerous cells. To select a suitable mouse strain as a xenogenic host for tumorigenicity testing, we compared the susceptibility of NOG (T, B, and NK cell-defective), SCID (T and B cell-defective), and the traditionally used nude (T cell-defective) mice to tumor formation from xenotransplanted HeLa S3 cells. When 10^4 HeLa S3 cells were subcutaneously inoculated into the flanks of these mice, the tumor incidence on day 22 was 10/10 (100%) in NOG, 2/10 (20%) in SCID, and 0/10 (0%) in nude mice. The subcutaneous tumors formed reproducibly and semiquantitatively in a dose-dependent manner. Unexpectedly, half of the NOG mice (5/10) that had been inoculated with a mere 10^1 HeLa S3 cells formed progressively growing subcutaneous tumors on day 78. We confirmed that the engrafted tumors originated from inoculated HeLa S3 cells by immunohistochemical staining with anti-HLA antibodies. These data suggest that NOG mice may be the best choice as a suitable strain for testing tumorigenicity.

Key words: HeLa S3, NOD/Shi-*scid* IL2Rg^{null} (NOG), Tumorigenicity testing, Xenograft

INTRODUCTION

One of the greatest contributions of the cultured cell to health care is the production of biologicals involving viral vaccines (Balducci *et al.*, 1962; Robinson *et al.*, 1966) and humoral factors (Delzer *et al.*, 1985; Sambrook *et al.*, 1986). Recently developed cell culture technology provides us with a new application known as "stem cell therapy" in the field of regenerative medicine (Badayan and Cudkowicz, 2008; Dalbello-Haas *et al.*, 2008). Stem cell-based therapy has received attention as a possible alternative to organ transplantation, owing to the ability of stem cells to repopulate and differentiate at the engrafted site. In particular, induced pluripotent stem (iPS) cells (Okita *et al.*, 2007; Takahashi *et al.*, 2007) would be the best source of patient-specific cell therapy, and thus enable us to autologously transplant without an immune rejection because of immunological incompatibility between patient and donor. In contrast to cell substrates for producing biologicals, in cell-based therapies, the therapeutic cells must be transplanted into the patient directly. The anticipated benefit of patient-specific cell therapies has always been tempered by inherent worries concerning the undesirable growth of contaminated undifferentiated stem cells in the recipient. Although many factors (e.g., cell migration, differentiation, phenotype expression, tumorigenicity) have been involved, the transplantable therapeutic cell has always been considered a safety risk. The purpose of tumorigenicity testing, as applied not only to cell substrates used for viral vaccine manufacture but also differentiated stem cells (e.g., derived from embryonic, mesenchymal, hematopoietic stem, and iPS) used for cell-based therapy, is to discriminate between cells that have the capacity to form tumors and cells that do not.

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Recently, we developed NOD/Shi-*scid* IL2Rg^{null} (NOG) mice by crossing IL-2 receptor gamma chain-deficient (IL2Rg^{null}) mice (Ohbo *et al.*, 1996) with NOD/Shi-*scid* mice (SCID) (Koyanagi *et al.*, 1997). NOG mice lack T and B lymphocytes and natural killer (NK) cells, and demonstrate impaired dendritic cell function (Ito *et al.*, 2002, 2008; Yahata *et al.*, 2002). Because of their severe immunodeficient state, NOG mice have been used as an *in vivo* model to study human cells and tissues (Ito *et al.*, 2002; Yahata *et al.*, 2002; Matsuura-Sawada *et al.*, 2005; Masuda *et al.*, 2007; Suemizu *et al.*, 2008). NOG mice also showed superiority in cancer xenotransplantation systems compared to SCID (Nakamura and Suemizu, 2008). To select a suitable mouse strain as a xenogenic host for testing tumorigenicity, we compared the susceptibility of NOG (T, B, and NK cell-defective), SCID (T and B cell-defective), and the traditionally used nude (T cell-defective) mice for tumor formation from xenotransplanted HeLa S3 cells.

MATERIALS AND METHODS

The protocol of the present study was reviewed beforehand and approved by the Animal Ethics Committee of the Central Institute for Experimental Animals (CIEA, Kanagawa, Japan), and all animal experiments were performed according to the Ethical Guidelines for Animal Experimentation from the CIEA.

Cells

HeLa S3 (human cervical cancer cell line) cells were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). The cells were maintained in F-12 (HAM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), incubated in a humidified (37°C, 5% CO_2) incubator, and passaged on reaching 80% confluence.

Detection of the tumorigenic cells *in vivo*

We used BALB/cA *nu/nu* (nude; Clea Japan Inc., Tokyo, Japan) and SCID (Clea Japan Inc.), and NOG mice aged 6-8 weeks. For direct comparison of susceptibility to cancer cell engraftment, various concentrations (10^1 to 10^6 cells/head) of HeLa S3 cells suspended in 0.1 ml of serum-free medium were subcutaneously inoculated into ten mice (5 males and 5 females) of each strain. The mice were surveyed daily and tumors were measured with calipers. The tumor volume (TV) was calculated using the formula $TV = 1/2 \times A \times B^2$ (A: length (mm); B: width (mm)). The criteria for successive engraftment were as follows: progressive nodule growth at the site of injection and TV values exceeding 10 mm^3.

Histology and immunohistochemistry

The engrafted tumors were fixed with 4% (v/v) phosphate-buffered formalin, and paraffin-embedded sections were stained using hematoxylin and eosin (H&E). Some sections were autoclaved for 10 min in target retrieval solution (0.1 M citrate buffer, pH 6.0; 1 mM EDTA, pH 9.0) and then placed at room temperature for 20 min. Monoclonal mouse anti-HLA class I-A, B, C (clone EMR8-5; Hokudo, Sapporo, Japan), mouse antihuman CK8/18 (clone 5D3; Novocastra Laboratories, Newcastle, UK), and mouse monoclonal antihuman Ki67 antigen (clone MIB-1; Dako A/S, Glostrup, Denmark) were the primary antibodies used. The antibodies for mouse Ig were visualized using amino acid polymer/peroxidase complex-labeled antibodies (Histofine Simple Stain Mouse MAX PO (M); Nichirei Bioscience, Tokyo, Japan) and diaminobenzidine (DAB) (Dojindo Laboratories, Kumamoto, Japan) substrate (0.2 mg/ml 3,3'-diaminobenzidine tetrahydrochloride, 0.05 M Tris-HCl (pH 7.6), and 0.005% H_2O_2). Sections were counterstained with hematoxylin.

Statistical analyses

The differences in incidence of each engraftment were tested by Fisher's direct probability method with $p < 0.05$ as the cutoff for significance. All statistical analyses were performed using Prism 5 software (GraphPad Software, La Jolla, CA, USA).

RESULTS AND DISCUSSION

In previous reports, we demonstrated a high rate of liver metastasis in NOG mice inoculated with small numbers of pancreatic cancer cells (as few as 100 cells) and higher levels of liver metastasis in NOG mice than in SCID mice (Suemizu *et al.*, 2007). Therefore, NOG mice are expected to be better subjects for testing tumorigenicity in assessing the safety of cell substrates and therapeutic cells. The purpose of this study was to quantify the advantage of NOG mice over SCID and nude mice in terms of transplantability of human tumor cells. HeLa S3 was examined for its ability to engraft in the subcutaneous spaces of these mice (Table 1). No statistically significant differences were found in the tumorigenic incidence between male and female mice in all strains. Therefore, tumorigenic incidence was directly compared between each strain. The athymic nude mice, traditional standards for tumorigenicity testing, showed no tumor formation when HeLa S3 cells were seeded at a density

Table 1. Comparative growth of HeLa S3 cells among BALB/cA *nu/nu*, SCID, and NOG mice

Cell dose (cells/head)	Sex	Number of mice with tumors (% engraftment)[a]		
		BALB/cA *nu/nu*	SCID	NOG
$1x10^2$	Male	NT	0/5 (0%)	3/5 (60%)
	Female	NT	0/5 (0%)	3/5 (60%)
	Total	NT	0/10 (0%)	6/10 (60%) *
$1x10^3$	Male	0/5 (0%)	0/5 (0%)	3/5 (60%)
	Female	0/5 (0%)	0/5 (0%)	3/5 (60%)
	Total	0/10 (0%)	0/10 (0%)	6/10 (60%) #
$1x10^4$	Male	0/5 (0%)	2/5 (40%)	5/5 (100%) **
	Female	0/5 (0%)	0/5 (0%)	5/5 (100%) ##
	Total	0/10 (0%)	2/10 (20%)	10/10 (100%) ##
$1x10^5$	Male	5/5 (100%)	5/5 (100%)	NT
	Female	3/5 (60%)	4/5 (80%)	NT
	Total	8/10 (80%)	9/10 (90%)	NT

[a] Engraftment was evaluated 22 days after inoculation by 1 x 10^3, 10^4, and 10^5 cancer cells, and 43 days after inoculation by 1 x 10^2 cancer cells. Fisher's exact test was performed in statistical analysis. * $P < 0.05$ compared to the SCID strain. ** $P < 0.01$ compared to the BALB/cA *nu/nu* strain. # $P < 0.05$ compared to the BALB/cA *nu/nu* and SCID strain. ## $P < 0.01$ compared to the BALB/cA *nu/nu* and SCID strain. NT: not tested.

of up to 10^5 cells. The SCID mice developed tumors with a lower cell inoculation dosage (10^4 cells) compared to nude mice. This result supports the report stating that the implantation rate of human xenografts in subcutaneous tissue is significantly higher in SCID mice than in nude mice (Taghian *et al.*, 1993). SCID mice inoculated with 10^4 HeLa S3 cells formed tumors within 22 days postinoculation, but at a rate of only 20% (2/10). In contrast to nude and SCID mice, all NOG mice (10/10; 100%) inoculated with 10^4 HeLa S3 cells formed tumors within 22 days. Furthermore, 60% (6/10) of the NOG mice formed subcutaneous solid tumors within 22 days of inoculation with only 10^3 HeLa S3 cells, and all NOG mice (10/10; 100%) formed tumors within 43 days (data not shown). On the other hand, no tumor formation was observed in SCID mice within 22 days of inoculation with 10^3 HeLa S3 cells, while 60% (6/10) formed subcutaneous solid tumors within 43 days (data not shown). Earlier, we demonstrated that just 10^2 AsPC-1, MIA PaCa-2, and PANC-1 cells (human pancreatic cancer cell lines) were needed for engraftment in NOG mice livers by intrasplenic inoculation within 56 days (Suemizu *et al.*, 2007). In this study, we examined the tumor-forming potential of subcutaneous tissue in NOG mice when inoculated with 10^2 HeLa S3 cells. Although the inoculation site differed from that of previous studies, the NOG mice showed a high engraftment rate (6/10; 60%) within 43 days of inoculation with 10^2 HeLa S3 cells, but not within 22 days (0/10; 0%, data not shown). These results demonstrate that the NOG mouse model is more sensitive for detecting tumorigenic-

ity of HeLa S3 cells, and is faster than traditional animal models using nude or SCID mice.

In cell-based therapies, huge numbers of cells are transplanted at the site of the lesion; most problematic among the risks is their potential neoplastic transformation or contamination of transformable undifferentiated stem cells. Therefore, susceptibility to xenotransplanted tumor cells has to be as high as possible. We examined the transplantability of NOG mice in response to a much lower (10^1) dose of HeLa S3 cells. Unexpectedly, half of the NOG mice (5/10) inoculated with this dosage showed tumor formation in their subcutaneous spaces at 78 days after transplantation. Fig. 1 shows the growth curve of engrafted HeLa S3 cells in NOG mice. The tumors grew progressively and formed a large spheroid mass, although it was localized at the inoculation site and did not invade surrounding areas. A necrotic core was usually observed in advanced tumors (Fig. 2A). Almost all tumor cells, except for the necrotic tumor tissue, were positive for the cell-cycle-regulated nuclear protein, Ki67 antigen, which is widely used as an operational marker of proliferation (Fig. 2B). To determine the origin of the engrafted tumors in the NOG mice, serial sections from formalin-fixed, paraffin-embedded tumors were treated with an anti-HLA monoclonal antibody using the immunoperoxidase staining method. The immunohistochemical reactivity of the anti-HLA monoclonal antibody with the tissue sections demonstrated that the engrafted tumors originated from a human source (Fig. 2C). HeLa S3 cells are immortalized epithelial cells obtained from a human cervical carcinoma (Masters, 2002) and retain the original characteristics of those cells. Moll *et al.* (1982) reported that the antibodies for cytokeratin component 18 strongly stained a variety of tumors of epithelial origin. Therefore, we checked the expression of cytokeratin 8 and 18 intermediate filament proteins (CK8/18) as an epithelial marker. Fig. 2D illustrates the immunohistochemical reactivity of the anti-CK8/18 monoclonal antibody with a tissue section from subcutaneous tumors. Tumor cells were strongly stained, but cells in the stroma were negative. These results demonstrate that the engrafted tumors were progressively growing, originated from human HeLa S3 cells, and were not spontaneously generated by mouse cells.

Manufacturers and regulatory agencies have been developing scientifically based guidelines for the use of cell substrates for biologicals (2006, Center for Biologics Evaluation and Research, Food and Drug Administration (CBER/FDA)). The use of an animal model known to be susceptible to tumor formation by tumorigenic cells has been recommended. Due to their immunodeficiency (T-cell deficient), athymic nude mice have been the animals most commonly used for tumorigenicity testing. In this study, we demonstrated that NOG mice are more susceptible to tumor formation than the nude mice traditionally used. This suggests that NOG mice may be the best choice when identification of a weakly tumorigenic phenotype or a small contamination of transformable undifferentiated cells is important.

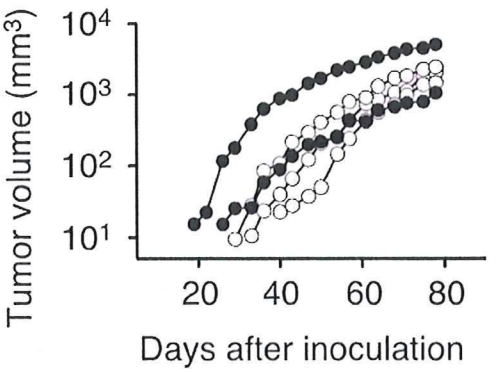

Fig. 1. Growth curve of subcutaneous tumors in NOG mice formed by inoculation with 10^1 HeLa S3 cells. Closed and open circles indicate male (n = 2) and female (n = 3) mice, respectively.

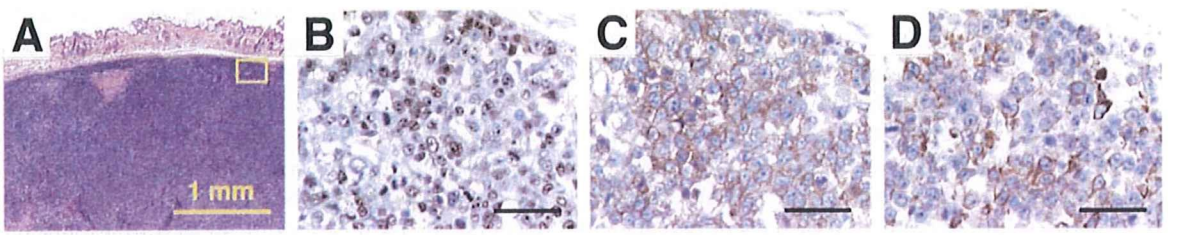

Fig. 2. Histology and immunohistochemistry of subcutaneous tumors in NOG mice formed by inoculation with 10^1 HeLa S3 cells. Serial sections were stained with H&E (A), Ki67 (B), HLA (C) and h-CK8/18 (D). B to D shows the boxed area in A at higher magnification. Scale bar, 50 μm.

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特集 安全・安心と高分子

医療機器とその材料の生物学的安全性と生体適合性

Evaluation of Safety and Biocompatibility of Medical Devices and Their Materials

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健康維持のために使用される医療機器には高分子材料も汎用されているが，人体に適用するためその材料の安全性を事前に担保する必要がある。本稿では，医療機器用材料の生物学的安全性評価について概説し，その安全性および生体適合性に関連した研究を紹介する。また，医療機器の生物学的安全性評価に関する国内ガイドラインについて簡単に述べる。

Abstract: Before utilizing medical devices, evaluating their safety is indispensable. When polymers are utilized for the medical devices, not only themselves but also their components such as monomers, catalysts and additives must be evaluated to estimate their safety as well as their biocompatibility by appropriate methods. Various studies on cell-polymer interaction provide valuable information to develop the safety and biocompatibility of materials utilized for medical devices.
Keywords: Medical Devices / Safety / Biocompatibility / Cell-Polymer Interaction / Cytotoxicity / Genotoxicity / ISO

1. はじめに

現在，医療機器への使用を目的として研究が行われている材料は，その多くが最終的に間接的あるいは直接的に人体と接触することになる。実際にそれらの材料を人体に使用する場合，その安全性，すなわち致命的な毒物反応やその他の障害を引き起こす可能性の有無をあらかじめ確認しておく必要がある。材料の生物学的安全性を評価するためには，事前に動物実験による安全性評価を行うことが適当と思われるものの，現在，動物愛護の観点から動物実験を可能な限り減らすことが求められている。また，費用，簡便性などの点からも，動物実験による材料の安全性評価は必要最小限に留めることが望ましい。このような背景から，生体に対する材料の安全性評価を行うにあたっては，細胞を用いた評価手法を採用することが第一選択肢になる。細胞を利用した安全性評価手法は，医療機器に用いる材料選択のみならず，新しい医療機器によって生じ得る不具合の原因究明にも応用できるため，非常に有効な方法となってきている。本稿では，医療機器に使用する材料，とくに高分子材料の安全性に関する研究状況を紹介するとともに，安全性とともに重要な因子となる生体適合性，および我が国の安全性評価ガイドラインと国際調和状況について概説する。

2. 生物学的安全性

材料が人体に障害を引き起こす原因は，材料と接触した血液や組織液に溶出してきた化学物質の毒性に由来することが多い。たとえば，金属材料の場合，遊離金属イオンが生体にアレルギー反応を惹起することがよく知られている。高分子材料の場合，通常その毒性は材料自身から溶出するオリゴマー，未反応モノマー，添加物，残留触媒，分解産物などに起因する。よって，高分子材料の安全性を確保するためには，原材料の選択と合成した高分子の精製度合とが重要な因子となる。その他，材料自体と組織との接触により，何らかの障害が誘導される可能性も考慮する必要がある。

材料によって生体が受ける障害は，炎症，アレルギー，発熱，組織壊死，腫瘍などの形で出現するため，各毒性反応を検出しうる生物学的試験を用いてそれらの安全性を評価することになる。その具体的な手法例は，後述するガイドラインに示されている[1,2]。たとえば，細胞毒性試験は，材料抽出液存在下で細胞を培養し，一定時間後に形成されるコロニー数を基準に細胞毒性を定量的に解析する手法で，比較的簡便な試験法の一つである。図1に，同試験で材料の細胞毒性を評価した実例を示した。これは，過去に不具合事例が数件報告された高分子製吸収性止血材や癒着防止膜から調製した抽出液の細胞毒性と不具合の相関性を考察


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