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Original Article

Effects of in Utero and Lactational Exposure to Di(2-ethylhexyl)phthalate on Somatic and Physical Development in Rat Offspring

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Abstract: Di(2-ethylhexyl)phthalate (DEHP) has been reported to act as an antiandrogen and to affect the reproductive organs and accessory genital glands. Thus, to assess the reproductive toxicity of DEHP it is important to examine both its adverse effects on the development of offspring following maternal exposure and its effects on sexual function and fertility. In the present study, we examined whether in utero and lactational exposure to DEHP affects postnatal somatic growth of offspring in the rat. Pregnant females were orally administered various doses of DEHP (0, 25, 100 or 400 mg/kg body weight/day) from gestational day (GD) 6 through postnatal day (PND) 20. There were no significant changes in body weight, body length, tail length, or the weight of individual organs between the control and DEHP-treated groups. Somatic hormonal parameters were the same for all DEHP doses. These findings suggest that in utero and lactational exposure to various concentrations of DEHP has very little effect on postnatal development or endocrine and physical status of male and female rat offspring under the experimental conditions of the present study.

Key words: Di(2-ethylhexyl)phthalate, Postnatal development, In utero and lactational exposure, Offspring, Rat

Introduction

To date, several compounds have been suspected of exerting endocrine-disturbing effects even at ultra-low concentrations. Phthalates have been produced and used in the manufacture of chemically derived materials and products. Di(2-ethylhexyl)phthalate (DEHP) has been most widely used in polyvinyl chloride to impart structural flexibility, and it is used as a plasticizer in products such as food packaging, children's products (toys and crib bumpers) and medical devices. Significantly, DEHP has been detected in plasma samples[1]. Mono(2-ethylhexyl)phthalate (MEHP), which is an active and the predominant DEHP metabolite, is also considered as a testicular toxicant[2]. It has been estimated that mean DEHP intake is 8.2 μg/kg body weight per day for adults[3]. During recent years, DEHP has been excluded from many products to avoid consumer exposure. However, recent heightened public concerns about environmental exposure to high concentrations of DEHP have raised new questions about its possible occupational and medical health hazards.

Developmental toxicity studies of DEHP have been conducted in laboratory mice[4–8] and rats[8–10]. These reports suggest that in utero exposure to high doses of DEHP induces embryotoxicity and/or teratogenicity. Animal reproductive toxicity studies of DEHP have also been reported. In a study of adult male rats, testicular defects such as atrophy of the seminiferous tubules, loss of spermatogenesis and vacuolation of Sertoli cells were observed after 90 days of dietary exposure to DEHP at 500 and 5,000 ppm (equivalent to 37.6 and 375.2 mg/kg/day, respectively)[11]. Perinatal exposure to DEHP in rats from gestational day (GD) 14 through postnatal day (PND) 3 reduced anogenital distance, testis weight or the weight of androgen-dependent tissues[12].

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Dietary exposure of adult male rats given 0, 320, 1,250, 5,000, and 20,000 ppm DEHP (equivalent to 0, 17.5, 69.2, 284.1 and 1156.4 mg/kg/day, respectively) for 60 days, when mated with untreated adult females, did not affect the rate of neonatal death, initial pup weight or growth (up to PND 7), whereas the average litter size decreased in rats fed 20,000 ppm DEHP[13]. Inhalation exposure of adult male Wistar rats to 25 mg/m^3 for 6 h/day for 8 wk increased plasma testosterone level and seminal vesicle weight in a dose-dependent manner[14]. In a study of adult female rats, DEHP induced prolonged estrous cycles and suppressed plasma concentrations of estradiol and subsequent ovulation[15].

Several studies have shown that in utero and lactational exposure to DEHP leads to abnormalities in the hypothalamus-pituitary-testicular axis. Sprague-Dawley rats were orally dosed with DEHP (0–1,500 mg/kg/day) from GD 3 through PND 21, and dose-related effects in the male offspring included several parameters involved in sexual development[16]. Oral exposure of pregnant female Long-Evans rats to 100 mg/kg/day DEHP from GD 12–21 induced significantly increased levels of testosterone and luteinizing hormone in male offspring on PND 21 and PND 35, but by PND 90 the levels were comparable between treated and untreated animals[17], indicating that the magnitude of DEHP toxicity on reproductive function is influenced by the stage of development.

Thus, DEHP toxicity studies in laboratory animals have focused on embryotoxicity, teratogenicity and reproductive toxicological effects in addition to some developmental effects in the early postnatal period, yet extensive toxicity information for long-term development after DEHP exposure is still lacking. The purpose of the present study was to evaluate postnatal growth and physical development following in utero and lactational exposure to DEHP in male and female rat offspring until the post-pubertal period. We examined the effects of DEHP on pubertal development, and doses of DEHP were chosen based on the levels that caused no overt maternal toxicity. Additionally, the exposure period was extended to examine the effects of lactational exposure in addition to the effects of in utero exposure, to complement previous studies[4–10]. Thus, we administered several doses of DEHP orally by gavage to pregnant rats using an experimental schedule identical to one used previously[18], and we examined the effects on postnatal somatic and organ growth, as assessed by body weight, body length, tail length and main organ weights, including reproductive organs, in male and female offspring. In addition, to better assess physical status following DEHP exposure, we evaluated the levels of several plasma hormonal landmarks with regard to postnatal somatic growth.

Materials and Methods

Chemicals and experimental animals

DEHP (purity >99.9%, Cat# 289-10442) and corn oil were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. A total of 52 pregnant (GD 3) female rats (Crj: CD (SD) IGS strain, 9 wk of age) were purchased from Charles River Japan, Inc. (Tsukuba, Japan). The presence of a copulatory plug defined GD 0. They were acclimated on GD 3–6 and housed individually in plastic cages with sterilized wood chips (Soft chip, Japan Slc Inc., Shizuoka, Japan) for bedding and were maintained under controlled temperature (23 ± 1°C) and humidity (55 ± 5%) and with a 12-h light-dark cycle (08:00–20:00) throughout the study. A standard laboratory diet (CE-2, Clea Japan, Inc., Tokyo, Japan) and drinking water were available ad libitum.

Dose range-finding evaluation

Dams were randomly divided into five groups (four pregnant females per group). The DEHP-exposed groups were orally administered 500, 1,000, 1,500, or 2,000 mg DEHP/kg/day in corn oil vehicle (10 ml/kg of body weight); DEHP was given between 08:30 and 09:30 for five consecutive days each week (Monday–Friday) from GD 6 through GD 20, and the control group was given the same amount of corn oil during the same period. During the exposure period, we recorded maternal body weights and noted any clinical signs or abnormal behavior that may have resulted from toxic effects. These results were used to determine the range of the DEHP dose for the main study.

Main study

Dams were randomly divided into four groups (eight pregnant females per group) and weighed once daily from GD 3 through PND 20 (except for GD 4 and 5). The DEHP-exposed groups were orally administered 25, 100 or 400 mg DEHP/kg/day in corn oil vehicle (10 ml/kg of body weight); DEHP was given between 08:30 and 09:30 from GD 6 through PND 20, and the control group was given the same amount of corn oil during the same period. Maternal data were recorded as described above. For each dam, the gestational duration was recorded, and weight gain during gestation and lactation was measured. Dams were checked for birth until 10:00 on each day; the day on which pups were first observed was designated PND 0. The number of

live births and the weight of each live pup on PND 1 were recorded. The litter size was standardized to 10 (five males and five females when possible) between 10:00 and 11:00 on PND 7 (1 wk of age). Litters with a total of nine or fewer pups were not culled regardless of the sex ratio. Culled pups were used for the analysis at 1 wk of age. On PND 21, the remaining offspring were weaned, and thereafter males and females were housed in separate stainless steel cages by litter. Body weights were recorded with an electric balance (Shimadzu, Kyoto, Japan). Body length and tail length (millimeters) were measured with a digital caliper (Mitutoyo, Kanagawa, Japan). The nose-anus length was considered the body length. One male and one female offspring from each dam were dissected at 3 and 9 wk of age when possible. While the rat was under ether anesthesia, liver, kidneys and testes, prostate and seminal vesicles or ovaries and uterus were carefully removed and weighed.

Hormone determinations

For hormone determinations, blood samples were collected from the postcaval vein following euthanasia by ether inhalation at 9 wk of age. Plasma samples were obtained by centrifugation at 4°C and stored at –20°C until the analysis. Concentrations of the plasma thyroid hormones thyroxine (T_4) and tri-iodothyronine (T_3) were determined by a time-resolved fluoroimmunoassay (DELFIA T_4 Reagents and DELFIA T_3 Reagents, respectively, PerkinElmer Life and Analytical Sciences, Inc., MA, USA). Plasma growth hormone (GH) concentrations were determined by enzyme immunoassay (EIA) (Rat GH EIA Biotrak system, GE Healthcare Bio-Sciences Corp., NJ, USA). Plasma insulin-like growth factor-I (IGF-I) concentrations were also measured by EIA (ACTIVE mouse/rat IGF-I EIA kit, Diagnostic Systems Laboratories, Inc., TX, USA). Time-resolved fluorescence and absorbance were measured by a multilabel counter (VICTOR^2, PerkinElmer Life and Analytical Sciences, Inc.). All hormones were assayed according to the manufacturer's instructions.

Statistical analysis

The differences from the corresponding control group were statistically analyzed by an analysis of variance followed by Dunnett's test (significance at $p<0.05$).

Results

Dose range-finding evaluation

In the 1,000 mg/kg/day and higher DEHP groups, maternal toxicity was clearly manifested as greatly suppressed weight gain during gestation, which led us to discontinue subsequent dosing by GD 17 of this preliminary study. In the 500 mg/kg/day group, mean body weights decreased slightly at later stages of gestation compared with the control group (data not shown). Based on these observations, we set the highest dose at 400 mg/kg/day to exclude the influence of maternal toxicity and observe the effect of DEHP on the offspring. The lowest dose and the middle dose were set at 25 mg/kg/day and 100 mg/kg/day, respectively.

Main study

Dams

Table 1 shows the number of dams and their offspring used for examinations in each group. Weight gain did not differ between dams from the control group and the DEHP groups from GD 6 through GD 21. In the 400 mg/kg/day group, one dam was found dead on GD 23, and thus the dam was excluded from the analysis. No significant differences were observed between the control group and the DEHP groups with regard to gestational duration or the number of live births per litter on PND 1.

Figure 1 shows maternal body changes during gestation (left panel) and lactation (right panel). There were no statistically significant differences among groups with regard to maternal body weight during the gestation and lactation periods, although the 25 mg/kg/day group showed a transient but not significant weight reduction during early lactation.

Offspring

The number of offspring examined is shown in Table 2. In male and female offspring, there were no statistically significant differences in body weight, body length or tail length between the control and DEHP-exposed groups at 1, 3 or 9 wk of age (Figs. 2, 3 and 4). There were no statistically significant effects on liver or kidney weights in males or females at 1, 3 or 9 wk of age (Table 3, 4). In male offspring, testis weights did not differ among the control group and DEHP groups at 3 or 9 wk of age (Table 3). Prostate and seminal vesicle weights did not differ among the control group and DEHP groups at 9 wk of age (Table 3). In female offspring, ovary and uterus weights did not differ among the groups at 3 or 9 wk of age (Table 4).

Physical status of offspring

In male offspring, no statistically significant differences in plasma concentrations of T_4, T_3, GH or IGF-I were observed among the control group and the DEHP groups at 9 wk of age (Table 5). In female offspring, no statistically significant differences in plasma concentrations of T_4, T_3,

Table 1. Dams and litter data

	DEHP dose (mg/kg/day)			
	0	25	100	400
Females (n)	8	8	8	8
Pregnant females (n)	8	8	8	8
Dam weight gain (GD 6-21)	130 ± 7[a]	127 ± 5	135 ± 4	133 ± 5
Gestational period (days)	21.1 ± 0.1	21.4 ± 0.2	21.3 ± 0.2	21.3 ± 0.2
Live births/litter on PND 1	11.8 ± 0.7	13.6 ± 0.6	13.5 ± 0.5	11.7 ± 0.5 (7)[b]

[a]Values are mean ± SEM.

[b]The number in parentheses represents dams per dose group. One dam was found dead on GD 23, and thus the dam was excluded from the analysis.

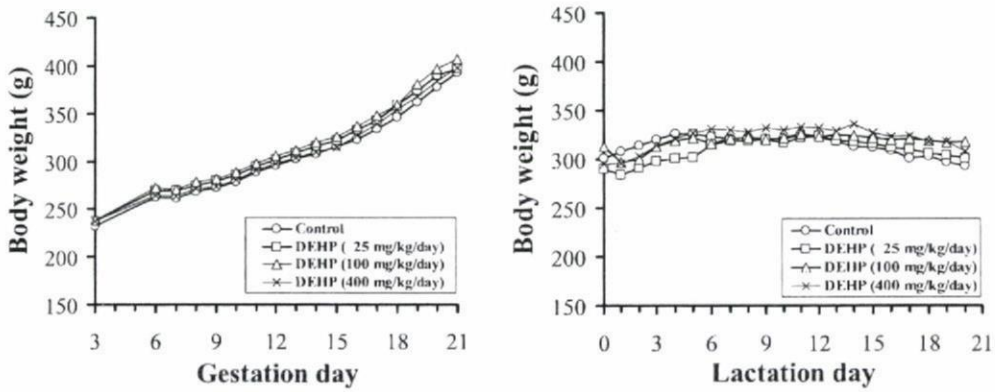

Fig. 1. Effects of exposure to di(2-ethylhexyl)phthalate (DEHP) on maternal body weight during gestation (left panel) and lactation (right panel).

Each point represents the mean.

GH or IGF-I were observed between the control group and the DEHP groups at 9 wk of age (Table 6).

Discussion

In recent years, the issue of endocrine-disrupting chemicals has been the topic of much discussion. Nagel *et al.*[19] and vom Saal *et al.*[20] reported that *in utero* exposure to low doses of bisphenol A (2 and/or 20 μg/kg/day) affects prostate and preputial gland weight and decreases daily sperm production efficiency in mouse offspring; moreover, their results indicated that exposure to low doses of xenoestrogens during a critical period can affect the reproductive organ systems of male offspring. On the other hand, other investigators have failed to find such effects in mouse offspring when using an identical experimental design[21, 22]. Thus, the issue of low-dose exposure to these potential endocrine-disrupting chemicals remains a matter of debate among investigators. Hence, as more refined analytical methods become available, risk assessment for previously characterized chemical substances should be repeated.

Table 2. Number of subjects examined

Group	DEHP dose (mg/kg/day)	No. of offspring examined			
		Age (wk)	1	3	9
Control	0	Male	8	8	8
		Female	6	8	8
DEHP	25	Male	10	7	7
		Female	11	7	7
DEHP	100	Male	13	8	7
		Female	9	8	8
DEHP	400	Male	9	7	6
		Female	7	7	7

Embryo-fetotoxicity and teratotoxicity of DEHP have been studied in mice[4-8] and rats[8-10]. These studies were conducted to elucidate whether in utero exposure to high doses of DEHP induces embryotoxicity and/or teratogenicity. The doses used in these previous studies were far in excess of human

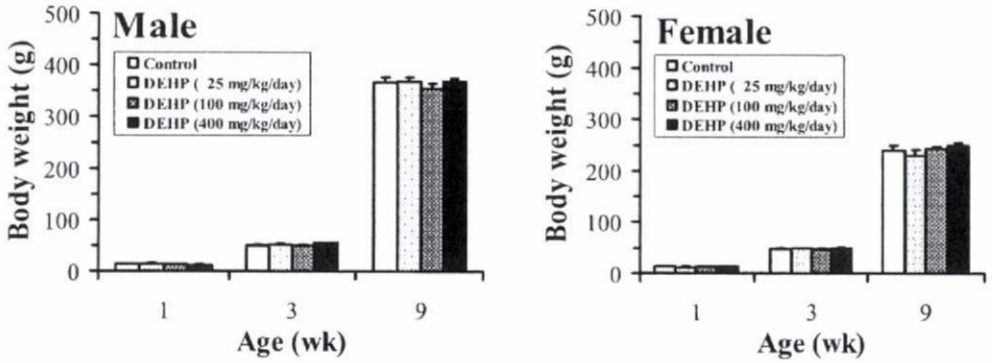

Fig. 2. Effects of maternal exposure to DEHP on postnatal body weight of offspring.
Body weights of male (left panel) and female (right panel) offspring are shown at 1, 3 and 9 wk of age. Each column and vertical bar represent the mean and SEM, respectively. There were no significant differences among groups.

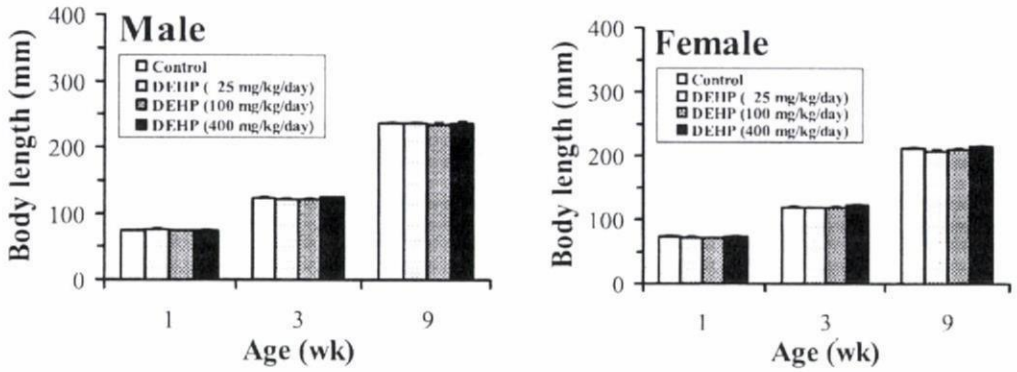

Fig. 3. Effects of maternal exposure to DEHP on postnatal body length of offspring.
Body lengths (nose to anus) of males (left panel) and females (right panel) are shown at 1, 3 and 9 wk of age. Each column and vertical bar represent the mean and SEM, respectively. There were no significant differences among groups.

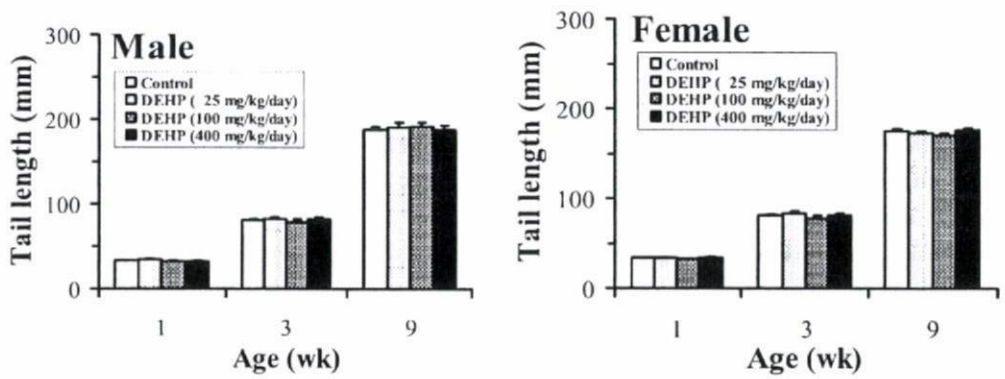

Fig. 4. Effects of maternal exposure to DEHP on postnatal tail length of offspring.
Tail lengths of males (left panel) and females (right panel) are shown at 1, 3 and 9 wk of age. Each column and vertical bar represent the mean and SEM, respectively. There were no significant differences among groups.

Table 3. Organ weights in male offspring

Organ	Group	DEHP dose (mg/kg/day)	Age (wk)		
			1	3	9
Liver (g)	Control	0	0.372 ± 0.011[a]	1.974 ± 0.090	15.55 ± 0.439
	DEHP	25	0.367 ± 0.024	1.984 ± 0.156	16.73 ± 0.560
	DEHP	100	0.334 ± 0.016	1.936 ± 0.138	14.78 ± 0.735
	DEHP	400	0.372 ± 0.037	2.276 ± 0.122	15.83 ± 0.691
Kidneys (g)	Control	0	0.191 ± 0.004	0.618 ± 0.018	2.951 ± 0.093
	DEHP	25	0.188 ± 0.008	0.585 ± 0.037	3.049 ± 0.124
	DEHP	100	0.164 ± 0.007	0.582 ± 0.042	2.842 ± 0.078
	DEHP	400	0.163 ± 0.015	0.632 ± 0.024	3.071 ± 0.092
Testes (g)	Control	0	-[b]	0.222 ± 0.009	3.065 ± 0.095
	DEHP	25	-	0.225 ± 0.014	2.999 ± 0.102
	DEHP	100	-	0.213 ± 0.011	2.834 ± 0.050
	DEHP	400	-	0.241 ± 0.012	3.070 ± 0.092
Prostate (g)	Control	0	-	-	0.443 ± 0.026
	DEHP	25	-	-	0.428 ± 0.033
	DEHP	100	-	-	0.372 ± 0.032
	DEHP	400	-	-	0.358 ± 0.026
Seminal vesicles (g)	Control	0	-	-	1.109 ± 0.057
	DEHP	25	-	-	1.064 ± 0.060
	DEHP	100	-	-	0.979 ± 0.034
	DEHP	400	-	-	1.014 ± 0.096

[a]Values are mean ± SEM. [b]-, not examined.

environmental exposure, and the duration of dosing was limited to the period of gestation. The present study was thus designed to investigate whether in utero and lactational exposure to DEHP affects the development of the next generation. For the main study, we set the highest dose at 400 mg/kg/day to avoid the influence of maternal toxicity and observe the effect of DEHP on the offspring. The exposure period was prolonged to examine the effects of lactational exposure in addition to the effects of gestational exposure. The offspring of dams in which no overt toxicity was observed (0, 25, 100 and 400 mg/kg/day), as determined by body weight and general behavior during gestation and lactation, were used in our study.

In recent years, certain studies have focused on the effects of DEHP and its antiandrogenic action on the hypothalamus-pituitary-gonadal axis[16, 17, 29]; very few studies, however, have reported the effect of DEHP on longer term postnatal development. Hence, it is important to examine the developmental toxicity of DEHP from birth until puberty. In this regard, our study was performed to evaluate the effects of in utero and lactational exposure to DEHP in rat offspring with a special focus on postnatal growth and physical status. We found that somatic and tissue growth and related endocrine landmarks were not affected by DEHP exposure.

Liver weights were slightly increased in the 400 mg/kg/day group for both male and female offspring at 3 wk of age, but no significant differences were observed among treatment groups. DEHP and other phthalates, such as di(2-ethylhexyl) adipate (DEHA) and butylbenzyl phthalate, are peroxisome proliferators that activate peroxisome proliferator–activated receptors and cause liver enlargement[23]. Induction of peroxisome proliferator–activated receptors could result in liver enlargement following DEHP exposure (Table 3, 4). This phenomenon could be an adaptive response following consecutive exposures to DEHP. However, this trend was no longer apparent at 9 wk of age. Since the DEHP groups were not exposed to the compound after 3 wk of age, body burden might be decreased because of metabolic clearance.

In a study of reproductive and accessory organ development following DEHP exposure, dose-dependent reductions in ventral, dorsolateral and/or anterior prostate weight were reported in rat offspring on PND 21 and PND 63 in response to oral administration of DEHP (0, 375, 750 and 1,500 mg/kg/day, GD3–PND21)[16]. This study also showed that DEHP significantly reduced testis weight on PND 21 and PND 63 in a dose-dependent manner. In the present study, on the other hand, testis weights were not

Table 4. Organ weights in female offspring

Organ	Group	DEHP dose (mg/kg/day)	Age (wk)		
			1	3	9
Liver (g)	Control	0	0.338 ± 0.007[a]	1.899 ± 0.117	9.665 ± 0.573
	DEHP	25	0.322 ± 0.015	1.886 ± 0.103	9.279 ± 0.511
	DEHP	100	0.349 ± 0.014	1.808 ± 0.105	9.760 ± 0.505
	DEHP	400	0.367 ± 0.030	2.046 ± 0.092	9.643 ± 0.441
Kidneys (g)	Control	0	0.176 ± 0.006	0.605 ± 0.026	2.039 ± 0.078
	DEHP	25	0.177 ± 0.006	0.593 ± 0.025	1.849 ± 0.091
	DEHP	100	0.179 ± 0.007	0.583 ± 0.023	1.983 ± 0.055
	DEHP	400	0.171 ± 0.007	0.583 ± 0.020	1.959 ± 0.039
Ovaries (mg)	Control	0	-[b]	18.95 ± 0.76	79.57 ± 4.08
	DEHP	25	-	17.80 ± 1.98	74.28 ± 8.14
	DEHP	100	-	14.83 ± 1.83	71.00 ± 4.26
	DEHP	400	-	16.67 ± 0.82	73.42 ± 3.29
Uterus (mg)	Control	0	-	26.03 ± 1.91	327.4 ± 25.3
	DEHP	25	-	30.72 ± 3.95	300.7 ± 14.2
	DEHP	100	-	31.96 ± 2.37	376.3 ± 30.9
	DEHP	400	-	27.82 ± 2.15	340.5 ± 16.1

[a]Values are mean ± SEM. [b] -, not examined.

Table 5. Hormone determinations in male offspring at 9 wk of age

Parameter		DEHP dose (mg/kg/day)			
		0	25	100	400
T_4	(ng/ml)	83.1 ± 6.9[a]	74.1 ± 3.7	73.2 ± 4.7	81.2 ± 7.5
T_3	(ng/ml)	1.74 ± 0.05	1.70 ± 0.06	1.63 ± 0.07	1.81 ± 0.09
GH	(ng/ml)	140.0 ± 35.3	137.3 ± 30.2	130.5 ± 16.3	96.5 ± 19.5
IGF-I	(ng/ml)	669.6 ± 49.0	641.7 ± 57.8	758.6 ± 49.6	743.5 ± 23.8

[a]Values are mean ± SEM.

Table 6. Hormone determinations in female offspring at 9 wk of age

Parameter		DEHP dose (mg/kg/day)			
		0	25	100	400
T_4	(ng/ml)	70.0 ± 7.4[a]	70.7 ± 5.4	67.7 ± 4.8	69.1 ± 6.4
T_3	(ng/ml)	1.88 ± 0.11	1.91 ± 0.06	1.76 ± 0.06	1.79 ± 0.10
GH	(ng/ml)	98.4 ± 9.6	99.5 ± 19.6	121.3 ± 22.4	109.4 ± 19.4
IGF-I	(ng/ml)	499.0 ± 34.4	574.0 ± 34.6	528.6 ± 42.5	632.6 ± 66.0

[a]Values are mean ± SEM.

significantly different between the control and DEHP groups. No significant differences in prostate weights were observed among the groups, although they were reduced in a dose-dependent manner (Table 3). The outcomes of the present study at the highest dose (400 mg/kg/day) were in accordance with those of Moore *et al.*, who conducted a study that used 375 mg/kg/day as the lowest dose[16]. The magnitude of DEHP effects in the present study was much smaller than that found in the study by Moore *et al.*[16]; this discrepancy could be explained by the large difference in dosage range.

Thyroid hormones play pivotal roles in normal growth, neuronal development and metabolism in animals. Endocrine disturbance following chemical exposure is suspected to occur at the embryonic and/or neonatal stage rather than at

the adult stage. An epidemiological study has suggested that toxicants such as polychlorinated biphenyls and dioxins, which are persistent and cumulative compounds in the environment, may affect growth and development through thyroid impairment[24]. Animal studies have reported that 2,3,7,8-tetracholorodibenzo-*p*-dioxin disrupts thyroid homeostasis[25] and causes developmental defects[26] and bone growth deficits[27]. Thyroid hormones are hormonal regulators of bone growth. The principal hormonal regulators during postnatal development are GH and IGF-I, and these hormones, which are regulated by thyroid hormones, are considered biomarkers for longitudinal somatic growth[28]. In the present study, hormonal parameters regarding developmental somatic growth were determined in the offspring to better assess the physical status following DEHP exposure. There were no significant differences in any parameters in male and female rat offspring (Table 5, 6). The fact that normal hormonal parameters were observed in rat offspring following exposure of dams to DEHP (even at high doses) leads us to conclude that postnatal development remains intact in the offspring.

The level of DEHP exposure used in the present study was much greater (~1,000-fold higher) than the estimated intake due to either medical exposure or consumer exposure in adult humans[3]. It was recently suggested that the magnitude of testicular toxicity after DEHP exposure is associated with the duration and/or the route of exposure[14, 29]. Inhalation of DEHP caused an elevation of plasma testosterone without affecting gonadotropin and several steroid enzymes that are involved in testosterone synthesis in male prepubertal rats[14]. These findings suggest that levels of DEHP that cause hormonal disturbance when inhaled may not have the same effect if consumed orally.

In conclusion, our results suggest that prenatal and postnatal exposure to DEHP does not affect postnatal somatic growth or endocrine and physical status of either males or females under the experimental conditions we used. The effects of DEHP exposure, however, remain uncertain and must be clarified using a wider dosage range, an extended exposure period, a side-by-side comparison of different exposure routes and a larger number of animals.

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臨床免疫・アレルギー科，51(1)：60-65，2009

総　説

Review

ダイオキシンによる免疫異常*

石丸直澄**　林　良夫**

Key Words：2,3,7,8-tetrachlorodibenzo-p-dioxin(TCDD), aryl hydrocarbon receptor(AhR), autoimmune disease, T cell

はじめに

環境ホルモンの一つとして知られるダイオキシン(図1)は免疫系，生殖系，神経系などの生物反応に重大な影響を及ぼすことが報告されてきた[1)~3)]．その中で免疫系への影響に関しては動物モデルを用いた研究が中心に行われ，免疫細胞の中で，T細胞やB細胞にダイオキシンの直接的な作用と間接的な影響に関して報告されてきた[4)~7)]．その中で，ダイオキシンのレセプターであるaryl hydrocarbon receptor(AhR)を介した分子シグナルの詳細が明らかにされようとしている．最近，ヘルパーT(Th)細胞の中でTh17細胞への分化をダイオキシンが調節することが判明した[8)]．さらに，Th17細胞が原因とされる自己免疫疾患の一つである多発性硬化症のモデルを用いた病態発症機序にダイオキシンが大きく影響を及ぼすことが明らかとされている[9)]．本稿ではこれまでのダイオキシンと免疫異常に関係する文献的知見を踏まえ，筆者らが明らかにしている自己免疫疾患に対するダイオキシンの影響に関する新知見を解説する．

免疫細胞へのダイオキシンの影響

正常マウスにダイオキシンを投与すると，胸腺が萎縮することが知られている[10)]．胸腺細胞の正負の選択に関連したアポトーシスにダイオキシンが影響を及ぼしている可能性や，胸腺間質細胞のFasLの発現にダイオキシンが調節因子として働きFasを発現した胸腺細胞のアポトーシスを制御しうるといったことが報告されているものの，明確な分子機序は不明である[11)]．さらに，ダイオキシン投与により，末梢のT細胞の機能低下が観察され，遅延型接触過敏反応やT細胞の細胞障害性活性の低下がみられる一方で，ダイオキシンによって各種刺激に対するT細胞の増殖反応やIL-2などのサイトカインの分泌は上昇することも知られている[12)13)]．また，卵白抗原(OVA)特異的なT細胞の反応性は初期の活性化には大きな影響は認められないかわりに，OVAに対するT細胞の増殖反応はダイオキシンによって亢進する[14)]．つまり，ダイオキシンの作用はT細胞の活性化ではなく生存に関係する分子群に影響を及ぼしている可能性がある．さらに，ダイオキシン投与により末梢での$CD25^+CD4^+$調節性T細胞(regulatory T cell；Treg cell)を誘導可能であるというユニークな報告もある[8)]．

一方で，ダイオキシンのB細胞への影響として，ヒツジ赤血球抗原の免疫に対する抗体産生はダイオキシンの投与により抑制され，さらに，lipopolysaccaride(LPS)あるいはIgM抗体などによる刺激でB細胞の増殖反応がダイオキシン添加により阻害されることも報告されている[15)]．B細胞の最終分化段階である形質細胞への分化をダイオキシンが阻害する結果も知られている[16)]．加えて，ダイオキシン投与マウスへのインフル

* Immune disorder by dioxin.

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エンザウイルスの感染実験では，T細胞，B細胞の機能低下とともにインフルエンザウイルスに対する抗体の産生も劇的に抑制されることが判明した[17]．

また，LPSの腹腔内誘導によるマクロファージの活性化をダイオキシンがTNF-αの産生上昇を介して亢進させる働きがある[18]．さらに，樹状細胞へのダイオキシンの影響については，抗原の取り込みや活性化に関してはダイオキシンが阻害的効果を有しているものの，T細胞への抗原提示能を上昇させる作用があることも報告されている[19]．

ダイオキシンは免疫細胞の種類やそれらの細胞の種々の機能に対して幅広い影響が認められるが，免疫細胞の機能に対して抑制的な効果が目立つ．表1にそれぞれの免疫細胞におけるダイオキシンの影響についてまとめる．

ダイオキシンによる細胞内分子シグナル

細胞内に入ったダイオキシンは細胞質に存在するそのレセプターであるAhRと結合する(図2)．AhRはヘリックス-ループ-ヘリックス(helix-loop-helix；HLH)ファミリーに属する転写因子として知られている．ダイオキシンと結合して活性化したAhRはAhR nuclear translocator(ARNT)とヘテロダイマーを形成し，核内に移行した後，さまざまな遺伝子上に存在するdioxin responsive element(DRE)として知られるxenobiotic response element(XRE)に結合することによりその遺伝子の転写が調節される[1)20)21)]．AhR複合体の標的遺伝子として，もっとも知られているのがcytochrome P-450 1A1(CYP1A1)である．CYP1A1は増殖・アポトーシスなどの細胞の生死を中心に重要な役割を果たしており，AhRを介したダイオキシンの細胞内における分子機序を解析するのに有効なメルクマールとして広く知られている[22)-24)]．また，AhRはnuclear factor-κB(NF-κB)のサブユニットの一つであるRelBとも結合することにより，免疫反応に重要な転写因子であるNF-κBの制御に影響を及ぼしていることも報告されている[25)]．AhRを中心としたダイオキシンの分子シグナルの解析にはAhRノックアウトマウスを用いることで明確な現象を観察することが可能となる．また，AhRノックアウトマウスを用いた免疫細胞への実験に関しても機能解析を中心に多くの報告がなされてきた[1)10)26)]．

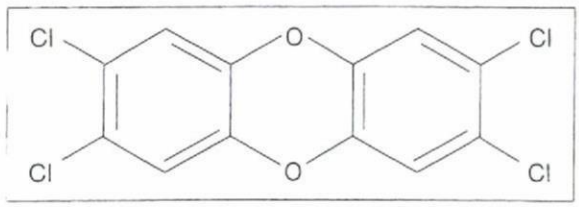

図1 ダイオキシン〔2,3,7,8-tetrachlorodibenzo-p-dioxin(TCDD)〕の化学式

ダイオキシンは，AhRを起点として，CYP1A1やNF-κBを介した多彩な免疫細胞機能に対して複雑に影響を及ぼしている．しかし，AhRのリガンドはダイオキシンだけでなくさまざまな生体物質あるいは非生体物質があげられることに加えて，ダイオキシン自体が内分泌かく乱物質としてエストロジェンレセプターと相互作用することにより，本来性ホルモンで制御されている生体機能のホメオスターシスの維持を破綻させる複雑な分子機序を有していることから，実際の生体内で起こっているダイオキシンの詳細な動態，正確な分子シグナルに関しては多くの謎が残されている．

表1 免疫細胞におけるダイオキシンの影響

免疫細胞	TCDDによる影響	文献番号
胸腺細胞	アポトーシス亢進	10)11)
T細胞	細胞障害性低下，増殖反応低下，Th17分化	8)9)12)～14)
調節性T細胞	誘導	8)
B細胞	抗体産生低下，増殖反応低下	15)～17)
マクロファージ	活性化亢進	18)
樹状細胞	活性化低下，抗原提示能亢進	19)

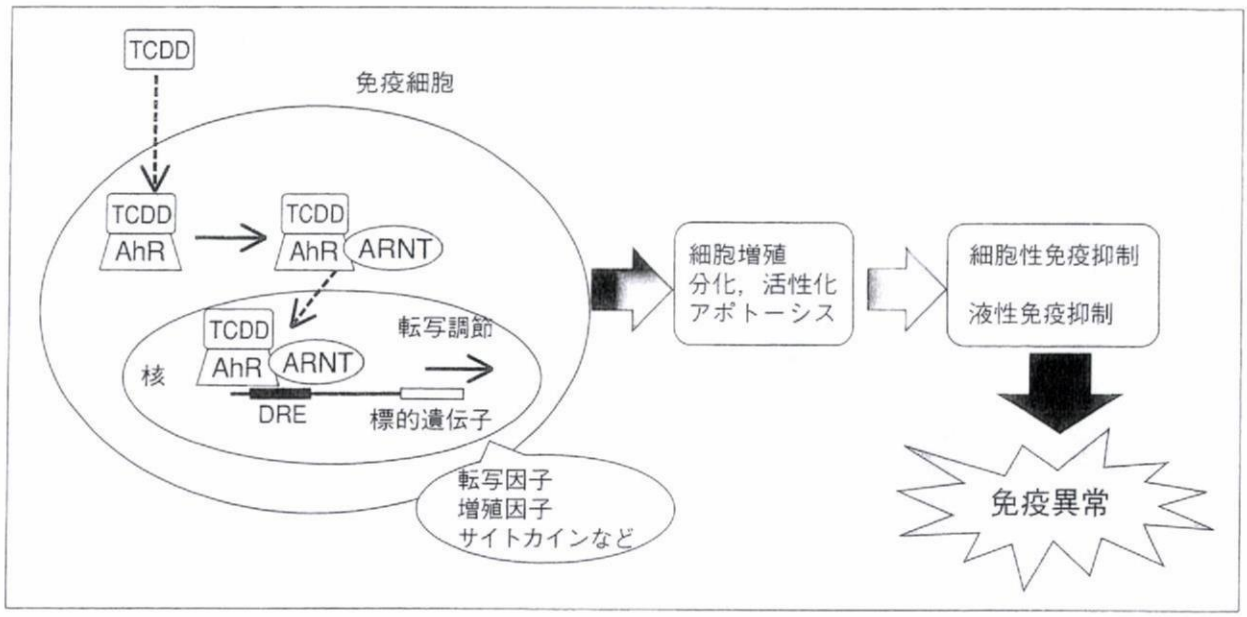

図2　免疫細胞におけるAhRを中心とした分子シグナル

ダイオキシンとTh17細胞

最近，Nature誌の同号にAhRとT細胞分化あるいは自己免疫疾患との関係を決定づける2本の論文が発表された[8)9)]．ダイオキシン(TCDD)の刺激で活性化されたAhRを介してTreg細胞においてもっとも重要とされる転写因子Foxp3の発現が亢進されることによって，Treg細胞の細胞数が増加することが判明した．さらに，多発性硬化症のモデルマウス〔実験的自己免疫性脳脊髄炎(experimental autoimmune encephalomyelitis；EAE)〕にダイオキシンを投与することによりTreg細胞の増加を介する病態の抑制効果があることを見出した．しかしながら，AhRの内因性リガンドの一つである6-formylindolo[3,2-b]carbazole(FICZ)をEAEモデルマウスに投与すると病態は増悪した．FICZはT細胞のIL-22およびIL-17の産生を上昇させることにより，EAEの病態形成にきわめて重要なTh17細胞の分化を促進させていることが明らかとなった．TCDDの投与で増加していたTreg細胞に関してはFICZ投与では影響がなかった．さらに，AhRの別のリガンドであるβ-naphthoflavoneを用いた実験においても，FICZと同様の効果が認められた．AhRは複数のリガンドと結合するため，リガンド依存性の転写制御機構が存在するものと考えられている．Th17細胞は従来知られていたIFN-γやIL-2などのサイトカインを分泌するTh1細胞とIL-4やIL-10などを分泌するTh2細胞とは異なるT細胞セブセットとして同定され，多発性硬化症などの自己免疫疾患の病態発症に重要な役割を果たしているという多くの報告がなされている．Nature誌に報告された2本の論文では共通してFICZは健常人の皮膚に存在し，紫外線によって活性型となりAhRと結合することが知られている．ダイオキシンや他のリガンドとAhRとの結合様式や親和性などいくつかの相違点があるものの，AhRの活性化機構に関しては不明な点が多い．また，Treg細胞におけるダイオキシンによるFoxp3の発現亢進の分子機序に関しても議論の余地を残している．

ダイオキシンと自己免疫疾患

上述のEAEの発症にFICZの投与によってAhRを介したT細胞異常に起因した自己免疫疾患の悪化効果があることが判明したものの，ダイオキシンが自己免疫疾患に影響するか否かは不明のままである．筆者らはこれまでに，唾液腺，涙腺を標的臓器とする自己免疫疾患であるシェーグレン症候群(Sjögren's syndrome；SS)のモデルマウスを確立し，その病態に関し研究を進めてきた[27)~29)]．SSの臨床病態は閉経期以降の女性に発症ピークを有し，ドライアイ，ドライマウスなどの乾燥症候群を呈し，血清自己抗体として抗SSAあるいは抗SSB抗体が検出され，小唾液腺の口唇生検により導管周囲性のリンパ球浸潤が

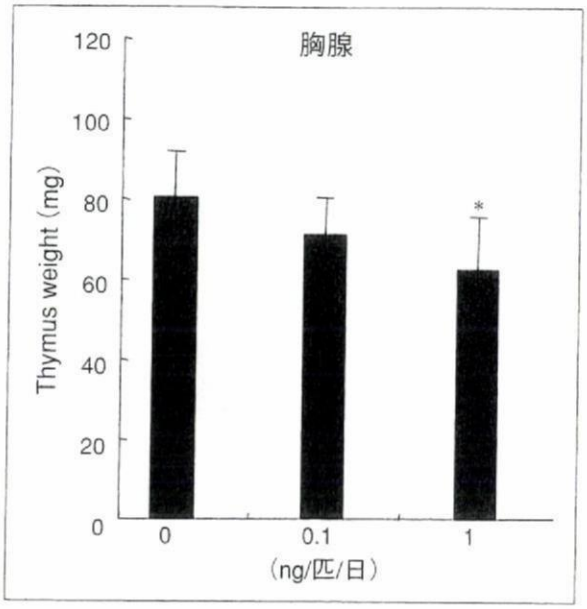

図３　新生仔期にTCDDを投与した２か月齢での胸腺の臓器重量

観察される[30]．筆者らが確立したSSのモデルマウスは舌下腺の分化異常をきたすことが知られているNFS/*sld*マウスに生後３日目においてＴ細胞の教育の場所である胸腺を外科的に切除することによって若齢期から高率に唾液腺，涙腺に限局する自己免疫性病変が観察される[27]．ダイオキシンによって胸腺細胞のアポトーシスが亢進するという報告に着目して，本マウスの新生仔期に胸腺を摘出する代わりにダイオキシンを投与することによって自己免疫病変が誘導されるか否かを検討した．ダイオキシンを投与されたマウスでは２か月齢において胸腺の臓器重量が対照群に比較して有意に減少していた(図３)．ダイオキシン投与により唾液腺には２か月齢より本来のモデルマウスで観察される自己免疫病変に類似した炎症性病変が観察された(図４)．病態誘導の詳細な分子機構に関しては不明であるが，新生仔期にダイオキシンに曝露されることにより胸腺の分化や成熟に異常が発生し，自己，非自己を区別する中枢性免疫寛容システムが破綻することにより，自己免疫疾患が発症したものと想定される．このことはヒトの新生児期や若齢期にダイオキシンが仮に曝露されたとすると，将来的に自己免疫疾患の発症リスクが上昇してしまう可能性を示唆している．しかし，自己免疫疾患は一つの因子で発症が決定づけられるわけではなく，遺伝因子や環境因子などが複雑に絡み合って中枢性および末梢性免疫寛容の破綻に結びついていくものと理解されているので，ダイオキシンそのものが自己免疫疾患の発症を直接的に左右しているとは言いがたい．そのレセプターであるAhRを起点とした分子シグナルの複雑さを考慮すると，ダイオキシンによる自己免疫疾患の発症に及ぼす影響には，病態に関与する免疫細胞および標的臓器細胞などへのAhRを介した分子機序に内在性のAhRリガンド，さらにホルモンなどのダイオキシンとの相互作用などさまざまな因子を考慮する必要がある．

おわりに

ダイオキシンの生体への影響に関してはその濃度が重要であることが知られている．動物実験では比較的高濃度での研究が進められているが，低濃度のダイオキシン曝露により晩発性の影響(low dose late effect)がすでに知られている．発癌，免疫異常，代謝異常など年齢という因子によって発症がある程度左右される疾患に関し

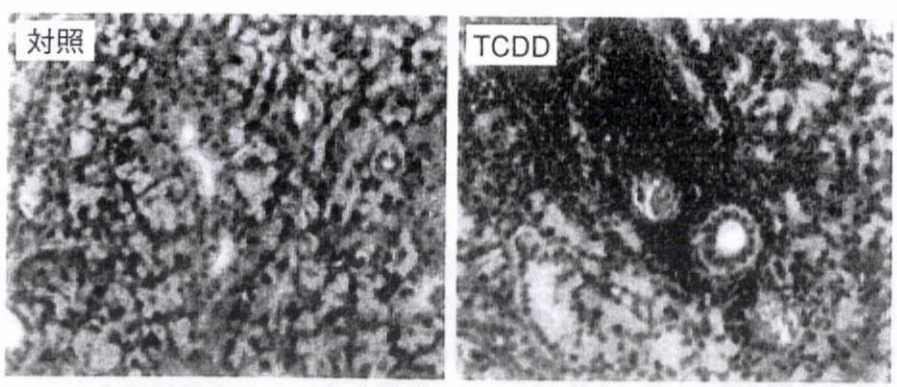

図４　新生仔期にTCDDを投与したマウスの唾液腺組織(Hematoxylin & Eosin染色)

てはダイオキシンの晩発性の影響は小さくないものと考えられる．たとえば，幼少期にダイオキシンに低濃度で曝露される環境にあれば，免疫疾患の好発年齢でより発症するリスクは高くなるのかもしれない．内分泌かく乱物質はダイオキシンだけではなく，われわれが生活する中で数多くの物質が生体内に入ってくる可能性があり，その中で内分泌かく乱物質として生体の恒常性を破綻してしまうものも現在知られているもの以外に存在する恐れもある．十年以上前に動植物のメス化とダイオキシンを代表とする内分泌かく乱物質の関係がクローズアップされてから，さまざまな角度から明らかにされてきたダイオキシンの分子メカニズムに関する研究は今後起こりうる人類に向けられた予言的な警告であると考えられる．生体システムにおいていまだ全容解明にまで至っていないさまざまな化学物質による“かく乱”の分子機構が今後明らかにされる必要がある．

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SHORT COMMUNICATION

Atelocollagen-mediated local and systemic applications of myostatin-targeting siRNA increase skeletal muscle mass

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RNA interference (RNAi) offers a novel therapeutic strategy based on the highly specific and efficient silencing of a target gene. Since it relies on small interfering RNAs (siRNAs), a major issue is the delivery of therapeutically active siRNAs into the target tissue/target cells in vivo. *For safety reasons, strategies based on vector delivery may be of only limited clinical use. The more desirable approach is to directly apply active siRNAs* in vivo. *Here, we report the effectiveness of* in vivo *siRNA delivery into skeletal muscles of normal or diseased mice through nanoparticle formation of chemically unmodified siRNAs with atelocollagen (ATCOL). ATCOL-mediated local application of siRNA targeting myostatin, a negative regulator of skeletal muscle growth, in mouse skeletal muscles or intravenously, caused a marked increase in the muscle mass within a few weeks after application. These results imply that ATCOL-mediated application of siRNAs is a powerful tool for future therapeutic use for diseases including muscular atrophy.*

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Keywords: *myostatin; RNA interference; atelocollagen; muscle; mouse; muscular dystrophy*

RNA interference (RNAi) is the process of sequence-specific, posttranscriptional gene silencing in plants and animals from flatworms to human,[1] which is mediated by ~22-nucleotide small interfering RNAs (siRNAs) generated from longer double-stranded RNA. Since it was demonstrated that siRNAs can intervene gene silencing in mammalian cells without induction of interferon synthesis or nonspecific gene suppression,[2] an increasing number of remedies utilizing highly specific siRNAs targeted against disease-causing or disease-promoting genes have been developed.[3] Effective delivery of active siRNAs to target organs or tissues is therefore the key to the development of RNAi as a broad therapeutic platform. For this purpose, different strategies have been used to deliver and achieve RNAi-mediated gene silencing *in vivo*;[3] for example, polymers represent a class of materials that meet the needs of a particular siRNA delivery system, condensing siRNAs into nano-sized particles taken up by cells.[4] However, some of the synthetic polymers, which have been used for delivery of nucleic acids, may trigger cell death in a variety of cell lines and thus suffer from limitations for its application in siRNA delivery *in vivo*.[4] On the other hand, atelocollagen (ATCOL), a pepsin-treated type I collagen lacking in telopeptides in N and C terminals that confer its antigenicity, has been shown to elicit an efficient delivery of chemically unmodified siRNAs to metastatic tumors *in vivo*.[5–7] In this study, we sought to examine the effectiveness of siRNA-ATCOL therapy for a nontumorous systemic disease, targeted against myostatin (growth/differentiation factor 8, GDF8), a negative regulator of skeletal muscle growth.[8]

Skeletal muscles are the crucial morphofunctional organs, and their atrophy causes severe conditions for life such as muscular dystrophies. Duchenne muscular dystrophy (DMD), for instance, is a severe muscle wasting disorder affecting 1 out of 3500 male birth.[9] There is currently no effective treatment, but gene therapy approaches are offering viable avenues for treatment development.[10] As one of therapeutic approaches, inhibition of myostatin by using anti-myostatin-blocking antibodies has been employed to increase muscle mass.[11] However, generating antibodies against recombinant target proteins is time consuming and requires a lot of efforts. Recently, we demonstrated that inhibition of myostatin by overexpression of the myostatin prodomain[12] prevented muscular atrophy and

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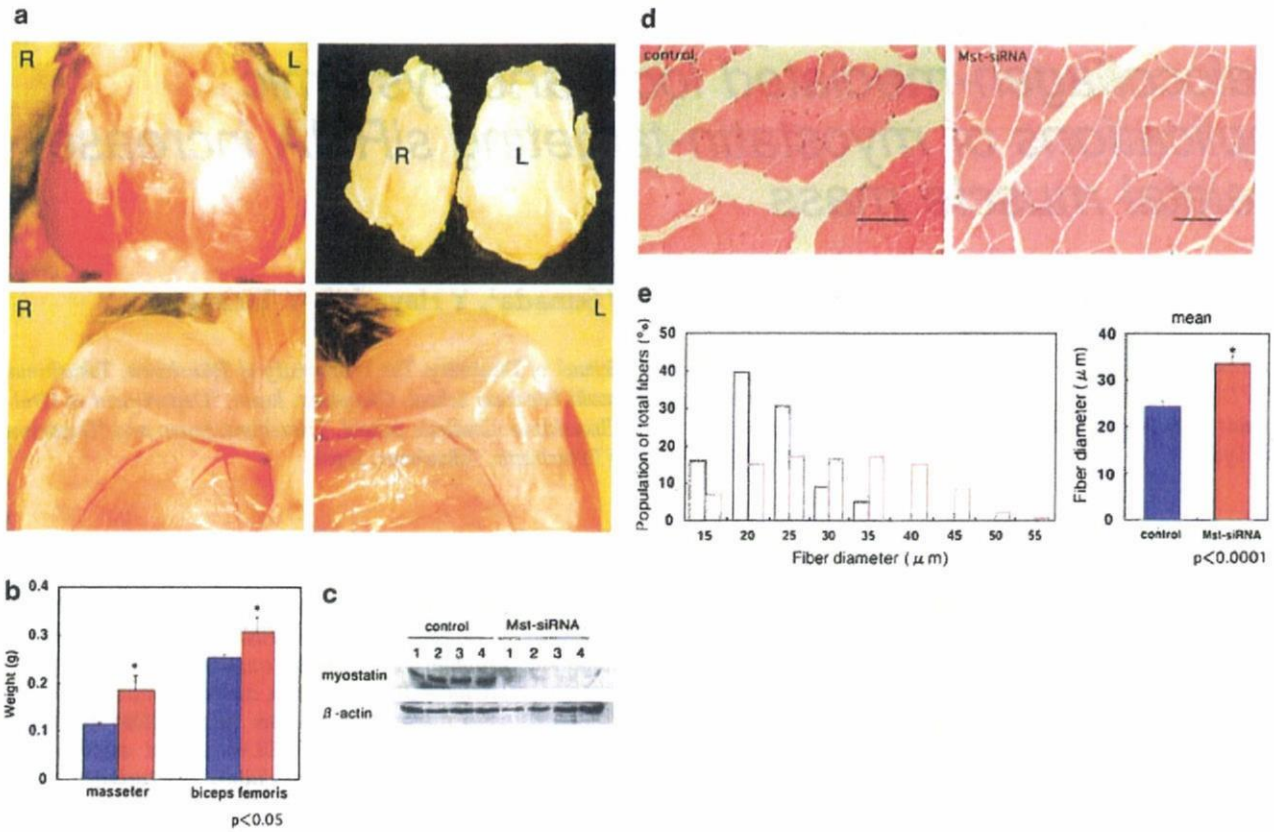

Figure 1 Local administration of the Mst-siRNA/atelocollagen (ATCOL) complex increases skeletal muscle mass and fiber size in wild-type mice through inhibition of myostatin expression. For the experiments depicted in (**a**–**e**) Mst-siRNAs (final concentration, 10 μM) were mixed with ATCOL (final concentration for local administration, 0.5%.[5]) (AteloGene, Kohken, Tokyo, Japan) according to the manufacturer's instructions. After anesthesia of mice (20-week-old male C57BL/6) by Nembutal (25 mg/kg, i.p.), the Mst-siRNA/ATCOL complex was injected into the masseter and biceps femoris muscles on the left side. As a control, scrambled siRNA/ATCOL complex was injected into the contralateral (right) muscles. After 2 weeks, the muscles on both sides were harvested and processed for analysis. (**a**) Photographs of muscles. Increased muscle mass were observed in the Mst-siRNA/ATCOL-treated (L) masseter (upper panels) and biceps femoris (lower panels), but not in the contralateral muscles (R). (**b**) Muscle weight. Mst-siRNA/ATCOL-treated muscles had an increased weight significantly compared to those with control siRNA/ATCOL (masseter, 0.185 ± 0.041 versus 0.115 ± 0.019 g; biceps, 0.307 ± 0.040 versus 0.232 ± 0.039 g; $n = 4$; $P < 0.05$). Student's *t*-test was used for determining statistical significance. Graphical representation of data uses the following convention: mean ± s.d.; treated muscles or mice in red; control muscles or mice in blue. (**c**) Western blot analysis of myostatin (52 kDa) in the control and Mst-siRNA/ATCOL-treated masseter muscles, assessed at 2 weeks after single injection. Total 80 μg of masseter muscle homogenates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes for immunoblotting. After a blocking reaction (5% nonfat milk/1% bovine serum albumin in phosphate-buffered saline (PBS) and 0.05% Triton X-100), the blots were incubated for 1 h at room temperature with mouse monoclonal anti-myostatin antibody (1:500; R&D Systems, Minneapolis, MN, USA) or anti-β-actin. After incubation with a secondary antibody (1:10000; horseradish peroxidase-conjugated anti-rat antibody; Biosource International, Camarillo, CA, USA), the blots were developed using the ECL Plus kit (Amersham, Buckinghamshire, UK). We used a purified myostatin protein and proteins extracted from cells transformed with a myostatin cDNA to confirm that the bands are due to 52 kDa myostatin. (**d**) Hematoxylin and eosin staining of the control and Mst-siRNA/ATCOL-treated masseter muscle. Muscles were fixed in 4% paraformaldehyde/PBS at 4 °C overnight, dehydrated and paraffin-embedded. Serial sections (5 μm thickness) were cut at mid-belly of muscle and stained. Scale bar, 50 μm. (**e**) Distribution of myofibril sizes of the control (blue bars) and Mst-siRNA/ATCOL-treated (red bars) muscles. The right panel shows the average myofibril size (33.6 ± 1.5 versus 24.4 ± 1.1 μm; $n = 200$; $P < 0.0001$). NIH Image (NIH, USA) software was used for morphometric measurements.

normalized intracellular myostatin signaling in the model mice for limb-girdle muscular dystrophy 1C.[13] On the other hand, Magee *et al.*[14] demonstrated that downregulation of myostatin expression by transduction of a plasmid expressing a short-hairpin interfering RNA (shRNA) against myostatin using electroporation can increase local skeletal muscle mass. For safety reasons, however, strategies based on vector delivery may be of only limited clinical use. The more desirable approach is to directly apply active siRNAs *in vivo*. As one of the practical platforms for siRNA delivery, we sought to employ an ATCOL-mediated oligonucleotide delivery system to apply myostatin-targeting siRNA into muscles.

We utilized the siRNA sequences reported previously[14] (GDF8 siRNA26, 5′-AAGATGACGATTAT CACGCTA-3′, position 426–446). It has been noted that this sequence can target myostatin mRNA not only of mouse but also human, rat, rabbit, cow, macaque and baboon, based on Blast search (National Center for Biotechnology Information).[14] To confirm the silencing effect of this siRNA, we constructed a plasmid of pSilencer 2.1-U6 neo containing the target sequence and transfected the plasmid into a mouse myoblast cell line, C2C12 cells, which had been made forced to stably express myostatin. We confirmed that the RNAi construct could effectively downregulate the expression

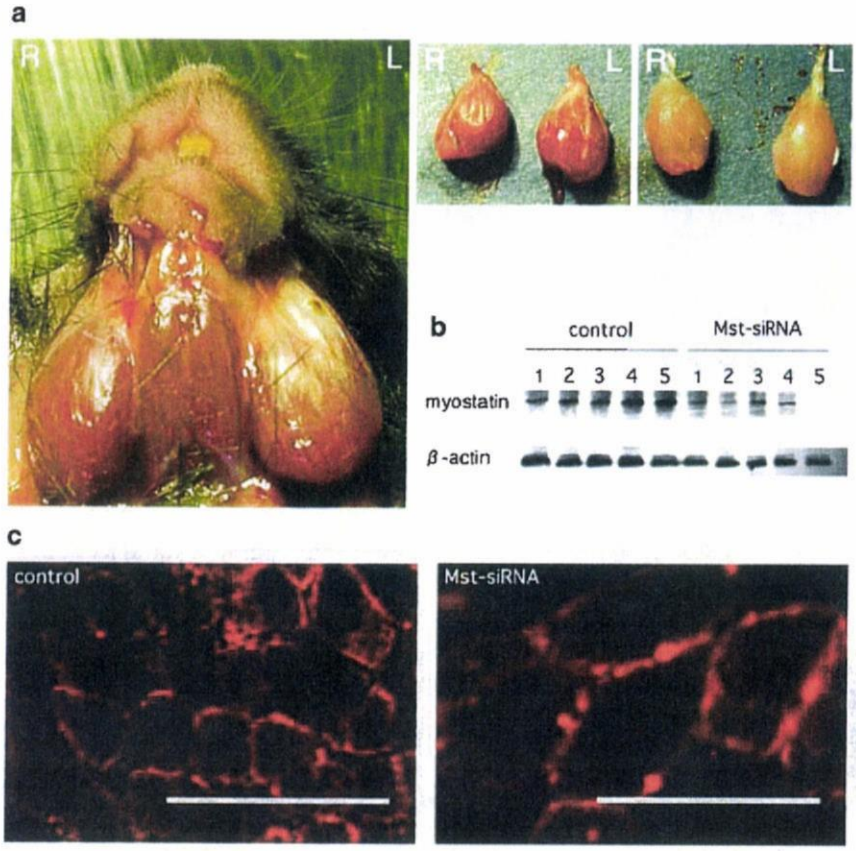

Figure 2 Mst-siRNA/atelocollagen (ATCOL) treatment improves myofibril size in *mdx* mice. (**a**) Photographs of muscles. The leftward masseter (left and middle panels) and tibial (right panel) muscles injected with the Mst-siRNA/ATCOL complex intramuscularly show a marked increased muscle mass in 20-week-old *mdx* male mice. (**b**) Western blot analysis of the control and Mst-siRNA/ATCOL-treated masseter muscles, assessed at 2 weeks after single injection. Myostatin protein levels in the muscles injected with the Mst-siRNA/ATCOL complex are markedly decreased, but not in the contralateral muscles injected with the control-siRNA/ATCOL. (**c**) Immunohistochemical analysis of the cross-sectional myofiber area of the masseter muscle, with the anti-laminin α2 antibody (4H8-2, Sigma, St Louis, MO, USA), showing increased fiber size in the Mst-siRNA/ATCOL-treated (right panel) muscle, compared to that of control (left panel). Alexafluor 594-conjugated anti-rat immunoglobulin G antibodies (A-11007, Invitrogen, Carlsbad, CA, USA) were used for immunohistochemistry. Scale bar, 100 μm.

of myostatin in the C2C12 cells[15] (Supplementary Figure S1).

We prepared the nanoparticle complex containing the GDF8 siRNA26 (10 μM) and ATCOL. Then, we injected the GDF8 siRNA26-ATCOL (Mst-siRNA/ATCOL) complex into the masseter and biceps femoris muscles of 20-week-old C57BL/6 mice. As a control, we injected control-scrambled siRNAs/ATCOL complex in the contralateral muscles. We observed gross morphology of the muscles and dissected the muscle tissues 2 weeks after injection. After injection of the Mst-siRNA/ATCOL complex, both muscles (on the left side) were enlarged, while no significant change was observed on the contralateral side (Figure 1a). We also measured the muscle weight, finding that the Mst-siRNA/ATCOL-treated muscles weighed significantly more than those on the control side (Figure 1b). The Mst-siRNA/ATCOL-treated muscles were further examined by a western blot analysis for myostatin (52 kDa), showing the decreased expression of myostatin on the treated side (Figure 1c). We quantified each result as a ratio to the internal control and statistically analyzed a difference between control (average ratio 0.90 ± 0.07) and treated (average ratio 0.44 ± 0.22) muscles. This difference is significant ($P < 0.01$, Student's *t*-test, $n = 4$). Histological analysis showed that the myofibril sizes of the masseter muscles treated with the Mst-siRNA/ATCOL complex were larger than those of the control (Figure 1d). Examining the sizes of 200 myofibers per group, the population of myofibril sizes indicated a shift from smaller to larger fibers in the Mst-siRNA/ATCOL-treated muscle (Figure 1e). The average myofibril size of the muscle treated with Mst-siRNA/ATCOL gained approximately 1.3 times more than that of control (Figure 1e). No obvious morphological change was observed in other tissues than the treated masseter muscles. In the meanwhile, we did not observe any general sign of ill health and deaths during the period of experiment. These results indicate that the increase of the Mst-siRNA/ATCOL-treated muscle mass is caused by their hypertrophy and that the siRNA complex gives no obvious adverse effects.

We next questioned whether this effect of hypertrophy after local injection of the Mst-siRNA/ATCOL complex observed in normal mice was relevant to dystrophin-deficient *mdx* mouse, an animal model for DMD.[16] We intramuscularly injected the same Mst-siRNA/ATCOL complex into the masseter and tibial muscles on the left side of 20-week-old *mdx* male mice. Within 2 weeks after the single injection, a dramatically increased muscle

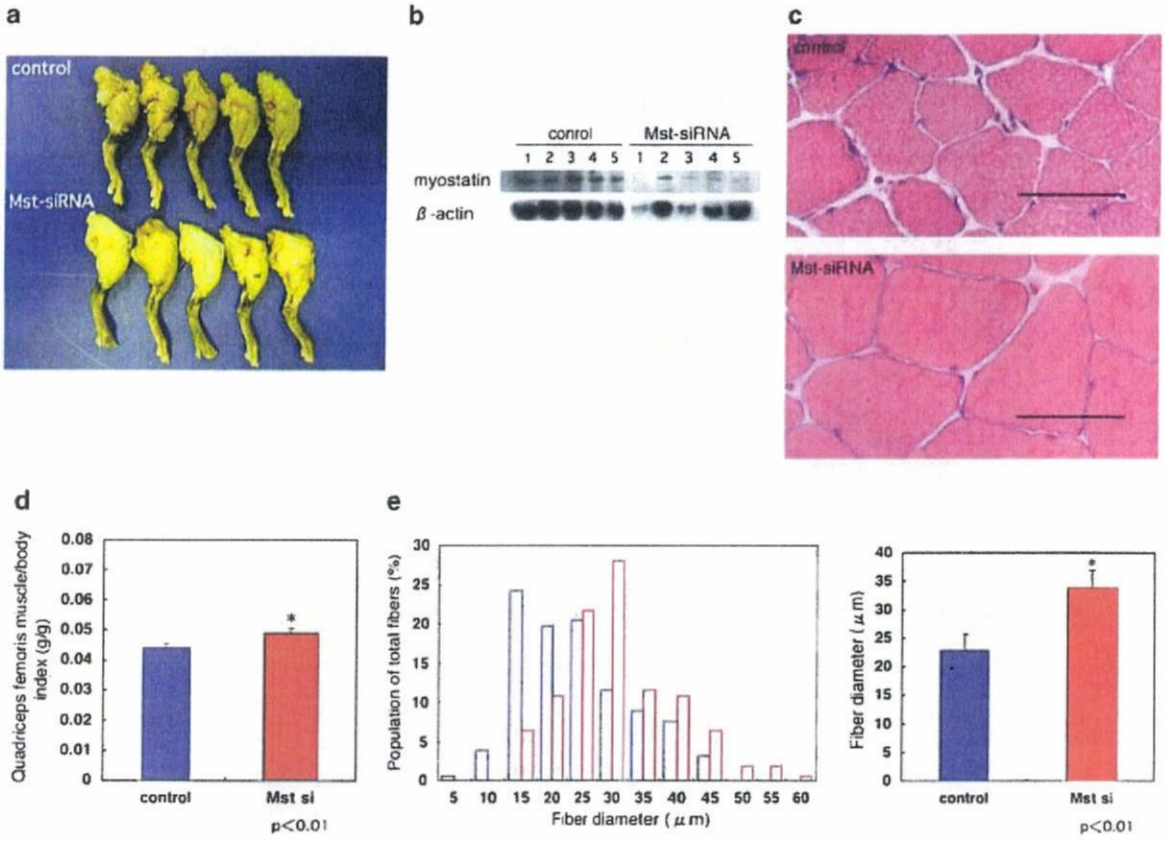

Figure 3 Systemic administration of the Mst-siRNA/atelocollagen (ATCOL) complex induces muscle enlargement in the mouse through inhibition of myostatin expression. For systemic administration, the siRNA (final concentration, 40 μM)/ATCOL (final concentration, 0.05%[6] complex, 200 μl) was introduced intravenously via orbital veins at 4, 7 and 14 days after the first application ($n=5$). As a control, control-scrambled siRNAs were injected into wild-type male mice (20 weeks, $n=5$). After 3 weeks, the quadriceps muscles on both sides were harvested and processed for analysis. **(a)** Photographs of lower limbs from control (upper panel) and Mst-siRNA/ATCOL-treated (lower panel) mice. **(b)** Western blot analysis of the control and Mst-siRNA/ATCOL-treated muscles (quadriceps femoris), assessed at 3 weeks after triple injection. **(c)** Hematoxylin and eosin staining of the control (upper panel) and Mst-siRNA/ATCOL-treated quadriceps muscle (lower panel). Scale bar, 50 μm. **(d)** Comparison of muscle weight/body weight index between the Mst-siRNA/ATCOL and control-siRNA/ATCOL-treated mice (0.048 ± 0.002 versus 0.043 ± 0.001 $n=5$; $P<0.01$). **(e)** Distribution of myofibril sizes of the control and Mst-siRNA/ATCOL-treated quadriceps muscles. The right panel shows the average myofibril size (33.92 ± 2.91 versus 22.95 ± 1.54 μm, $n=156$; $P<0.01$).

mass was observed in the Mst-siRNA/ATCOL-treated muscle (Figure 2a). Western blot analysis showed that the protein levels of myostatin in the muscles treated with the Mst-siRNA/ATCOL complex were significantly decreased (average ratio 0.55 ± 0.03), but not in the contralateral muscles treated with control siRNAs/ATCOL complex (average ratio 0.83 ± 0.01) (Figure 2b; $P<0.05$, $n=5$). Furthermore, immunohistochemical analysis on the masseter using an anti-laminin α2 antibody showed increase in the mean myofiber size of the Mst-siRNA/ATCOL-treated muscle (Figure 2c), as is the case for the wild-type (not shown). On the basis of these results, it seems that myostatin maintains satellite cells or muscle stem cells in a quiescent state. Reduced myostatin activity would lead to activation of these cells and fusion into existing fibers (Supplementary Figure S1e and f), resulting in fiber hypertrophy as proposed previously.[14]

We further examined whether systemic administration of the Mst-siRNA/ATCOL complex would have an effect on silencing the myostatin expression and lead to muscle enlargement. The Mst- or control siRNA/ATCOL complex was applied intravenously into normal mice four times in 3 weeks. Strikingly, we observed an obvious enlargement of skeletal muscles of lower limbs (Figure 3a), masseters and other muscles. Since change in the muscles of lower limbs is much larger than others, we used them for further analyses. We confirmed reduction of myostatin proteins in the muscles treated with the Mst-siRNA/ATCOL complex (average ratio 0.67 ± 0.11) (Figure 3b; $P<0.01$, $n=5$; average ratio for control 0.87 ± 0.03). We observed that the treated lower limbs are much larger than the controls, although the average body weights were 26.7 ± 0.7 and 25.8 ± 0.4 g for controls and treated mice, respectively. No increase in the body weight of the treated mouse was observed, probably because increase in the muscle weight compensated for reduction of fat accumulation.[17] To show increase in muscle weights, we used the muscle weight/body weight ratio (Figure 3d), in case the body weight exhibited variation. Significant increase in muscle fiber size (Figures 3c and e) was also observed after 3 weeks. These results indicate that siRNAs targeting against myostatin, intravenously administered with ATCOL, can specifically repress the expression of myostatin, inducing muscle hypertrophy in normal mice.

We present evidence that local and systemic applications of siRNA against myostatin coupled with ATCOL markedly stimulate muscle growth *in vivo* within a few