serotonin (5-HT) in the aged mice was significantly increased by TJ-48 (6.38 ± 0.51 vs. 10.23 ± 0.96), but not by TJ-41 (6.38 ± 0.51 vs. 6.56 ± 0.93). The levels of dopamine (DA) and its metabolites in the aged mice showed a trend of increase by JT-48, but not by JT-41, and a significant increase was observed in 3-MT (0.44 ± 0.05 vs. 1.01 ± 0.25) (Table I).

(b) Hippocampus (Fig. 2). The content of NE and its metabolites in the aged mice was significantly increased by TJ-48 but not by TJ-41: NE (5.88 ± 0.67 vs. 8.17 ± 0.62), NM (0.76 ± 0.08 vs. 1.14 ± 0.12), and MHPG (1.10 ± 0.12 vs. 1.97 ± 0.17). The low level of serotonin in the aged mice was significantly enhanced by administration of TJ-48. The level of dopamine and its metabolites in the hippocampus was not influenced by either TJ-41 or TJ-48 (Table I).

(c) Hypothalamus (Fig. 3). The low levels of norepinepherine (NE) and its metabolites in the aged mice were significantly enhanced in the mice given TJ-48: NE (8.73 ± 0.84 vs. 12.84 ± 1.10), NM (0.37 ± 0.03 vs. 0.64 ± 0.04), and MHPG (1.69 ± 0.18 vs. 2.83 ± 0.16). Serotonin also showed a significant increase by TJ-48 (6.06 ± 0.83 vs. 10.68 ± 0.57), but not by TJ-41. The concentrations of DA and its metabolites were not influenced by either TJ-41 or TJ-48 (Table I). It is interesting to note that both TJ-41 and TJ-48 down-regulated the level of 5-HIAA (a metabolite of serotonin) in the cortex and the hippocampus of the young mice, although a statistically significant difference was observed in hippocampus and cortex by TJ-48 and in hippocampus by TJ-41 (Fig. 2a–c). Such an effect was not observed in aged mice.

DISCUSSION

Ageing is associated with a decrease of various physiological activities at variable degrees. Among these, the most important issue is the age-related decline of immunological and brain functions. Actually, immune system operates together with the endocrine and nervous systems, and there are many common mediators between these systems (8,9). Todorovic et al. (10) reported that a significant correlation was observed between behavioral and immune parameters, suggesting a senescent decline of a common neuroimmune regulatory mechanism.

We have been studying mechanism underlying the age-related decline of immune system (11) and

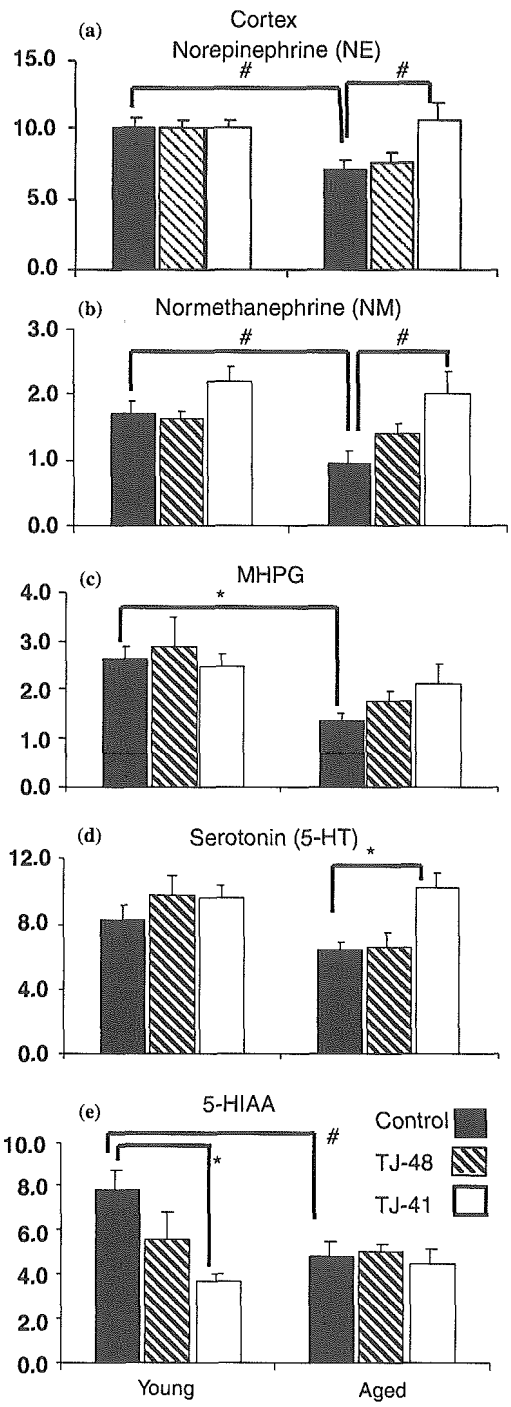

Fig. 1. Concentration of monoamines and their metabolites in the cortex of young and aged mice. The concentration is shown as the ratio to total protein assessed (pg/ng). MHPG: hydroxymethophenylglycol piperazinesalt. 5-HIAA: 5-hydroxyindole-3-acetic acid. The cortex was obtained from the frontal cortex. $*P < 0.01$. $\#P < 0.05$.

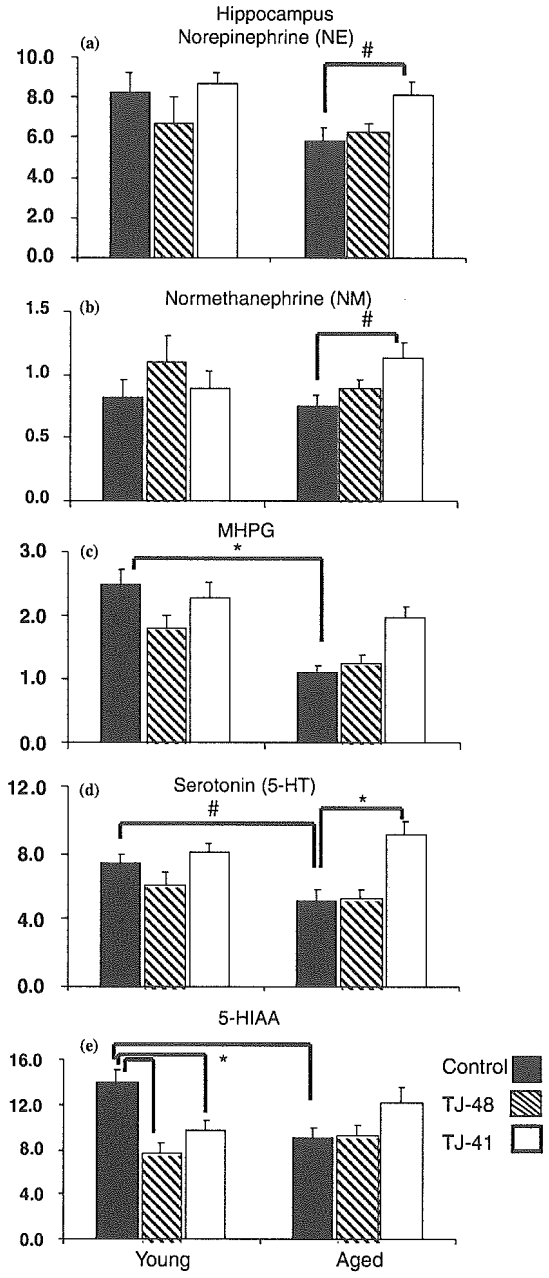

Fig. 2. Concentration of monoamines and their metabolites in the hippocampus of young and aged mice. The concentration is shown as the ratio to total protein assessed (pg/ng). Abbreviations, the same as above.

reported that the brain function is closely related with the immune function at the time of infection (4). In the meantime, we also searched for the methods improving the altered immune functions of the aged mice (2). Among various methods, we reported that Japanese herbal medicines (Kampo-hozai) were effective in the restoration of decreased immune functions of the aged mice (3). Because of close interrelationship between the immune system and brain, we expected that Kampo-hozai might improve not only the impaired immunological functions but also brain functions of aged mice.

As expected the results clearly indicated that a significant enhancement was observed in the levels of monoamines and their metabolites in the brain. An increased level of dopamine and norepinepherine in the frontal cortex is associated with enhanced locomotion activity (5). The present study clearly showed a significantly low level of norepinepherine (NE) in the cortex of the aged mice, and this is consistent with the age-related decline in locomotion activity in aged mice. The low level of NE in the cortex of aged mice was significantly enhanced in those given TJ-48. A similar enhancement of NE in aged mice by TJ-48 was observed in the hippocampus and hypothalamus. It is interesting to note that the enhancement of the NE level in the brain was observed in the aged mice given Kampo-hozai TJ-48, but not TJ-41, and not in the young mice.

The differential effect of TJ-41 and TJ-48 could be ascribed to the difference in the contents between them. Both TJ-41 and TJ-48 are composed of ten herbal plants. Five plants are common and five plants are different between them. Among them, Cinamoni Cortex in TJ-48 was reported to increase homovanillic acid and 5-hydroxyindoleacteic acid in mice (12). This plant could be one of candidates to influence brain monoamines. But the effect of herbal medicine is considered to be much complicated and caused by combination of several plants. Further study will be required to determine which elements of TJ-48 is actually operate for the up-regulation of brain monoamines. It is still not clear whether the observed changes in monoamines are due to a particular plant included in the mixture or whether the entire mix of medicinal plants is required to evoke the observed changes.

The level of dopamine (DA) detected was variable in the brain of the young mice and a statistically significant difference was not obtained in the present study. However, a trend of age-related decline was seen in the cortex, the hippocampus and the hypothalamus. In addition, a trend of enhancement was observed in the cortex of the aged mice given TJ-48, but not TJ-41.

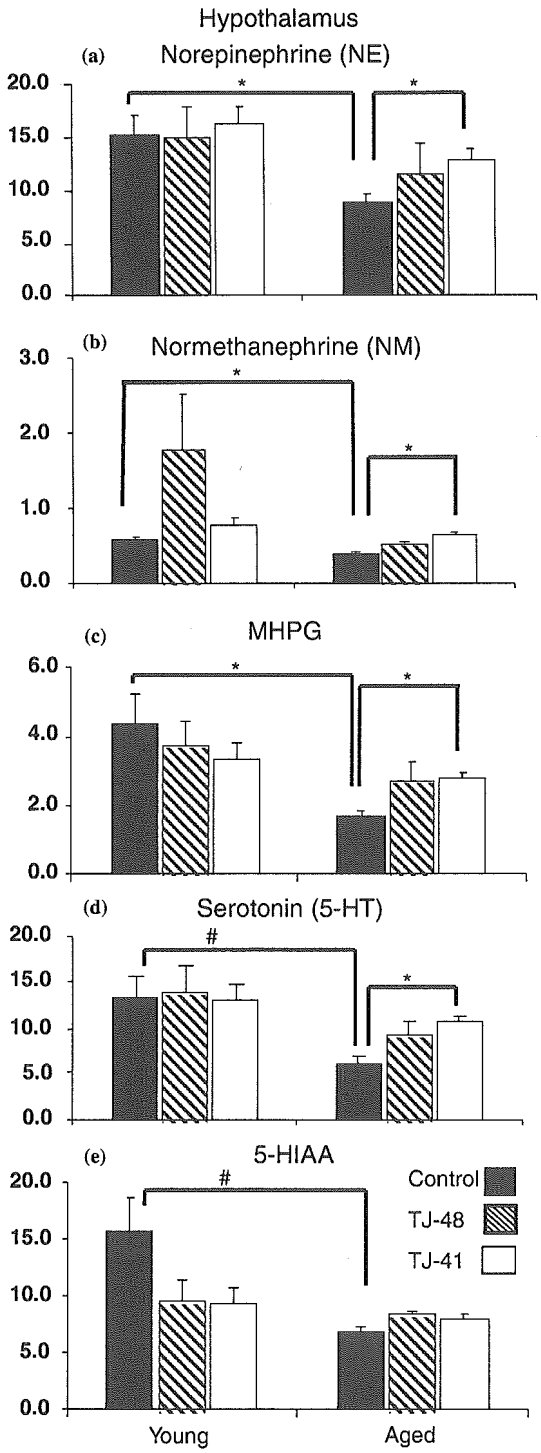

Fig. 3. Concentration of monoamines and their metabolites in the hypothalamus of young and aged brain. The concentration is shown as the ratio to total protein assessed (pg/ng). Abbreviations, the same as above.

Godefroy et al. (13) reported that there is no general age-related change in the level of monoamines and their metabolites in the brain showing a decrease in some areas and an increase in other areas. DA and HVA levels exhibit a pronounced decrease during aging (14). Dopamine release is significantly reduced by 20% in aged rats as compared to young rats (15). DA, 5-HT and 5-HIAA are decreased in the cerebral cortex of aged rats (16). DA and DOPAC were significantly lower in the striatum and mesolimbic areas of 24 months rats (17). Various monoamines are reduced with aging in the frontal cortex, hippocampus, striatum, amygdala and brain stem (18). Our findings about the age effect on brain monoamines were almost the same as those of Miguez et al. (18).

In a previous report (3), TJ-41 was effective in the restoration of immune function in aged mice, but not in young mice. The present study again showed that the improvement of brain monoamines was observed only in the aged mice by feeding of diet containing Japanese herbal medicines. These facts clearly indicate the importance of aged animals in animal experiments for observing the effects of medicines on the functions of organs and systems.

It is generally accepted that cytokine production changes from Th1 type to Th2 type with the advance of age (19). In our previous paper, we reported that production of IL-2 decreased while that of IFNγ increased in aged mice. In contrast, IL-4, a type of Th2 type cytokine, increased with age (20). Our preliminary study indicated that both TJ-41 and TJ-48 suppressed production of Th2 type cytokines in the aged mice, but not in the young mice (not reported in this study). Such a change in cytokine production may have influence on the content of brain monoamines.

There are accumulating data indicating that cytokine produced by immune cells can influence the brain function. Both TJ-41 and TJ-48 down-regulated production of Th-2 type cytokines, but only TJ-48 up-regulated the monoamine levels in old mice. The results taken together suggest that the rise of brain monoamines might be due to combination of the direct effect of some contents of TJ-48 and the indirect effect of cytokine change produced by immune cells.

In the present study, the period of administration of the herbal medicines was 5 months, since we obtained better results by 4 months rather than 2 weeks in the previous experiment to see the effect

of herbal medicine on the immune functions (3). However, further study would be necessary to see what would happen if the period of administration was decreased or increased.

In conclusion, the concentration of norepinepherine and serotonin and their metabolites was decreased in the cortex, hippocampus and hypothalamus of the aged mice. TJ-48, but not TJ-41, was effective in the enhancement of the levels of these monoamines and their metabolites in the brain of the aged mice.

These data have suggested that the aged mice are useful to see the effect of medicines on functions of organs and system.

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Increased Very Low Density Lipoprotein Secretion and Gonadal Fat Mass in Mice Overexpressing Liver DGAT1*

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Tomomi Yamazaki‡, Eriko Sasaki‡, Chihaya Kakinuma§, Takashi Yano§, Shinji Miura‡, and Osamu Ezaki‡¶

From the ‡Division of Clinical Nutrition, National Institute of Health and Nutrition, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8636, Japan and §Mochida Pharmaceutical Company Limited, 722 Jimba-aza-uenohara, Gotemba, Shizuoka 412-8524, Japan

Acyl-CoA:diacylglycerol acyltransferases (DGATs) catalyze the last step in triglyceride (TG) synthesis. The genes for two DGAT enzymes, DGAT1 and DGAT2, have been identified. To examine the roles of liver DGAT1 and DGAT2 in TG synthesis and very low density lipoprotein (VLDL) secretion, liver DGAT1- and DGAT2-overexpressing mice were created by adenovirus-mediated gene transfection. DGAT1-overexpressing mice had markedly increased DGAT activity in the presence of the permeabilizing agent alamethicin. This suggests that DGAT1 possesses latent DGAT activity on the lumen of the endoplasmic reticulum. DGAT1-overexpressing mice showed increased VLDL secretion, resulting in increased gonadal (epididymal or parametrial) fat mass but not subcutaneous fat mass. The VLDL-mediated increase in gonadal fat mass might be due to the 4-fold greater expression of the VLDL receptor protein in gonadal fat than in subcutaneous fat. DGAT2-overexpressing mice had increased liver TG content, but VLDL secretion was not affected. These results indicate that DGAT1 but not DGAT2 has a role in VLDL synthesis and that increased plasma VLDL concentrations may promote obesity, whereas increased DGAT2 activity has a role in steatosis.

Triglyceride (TG)[1] is the major energy storage form and is synthesized primarily in three tissues: liver, adipose, and small intestine. In the liver, synthesized TG is either stored in cytoplasmic droplets or secreted as very low density lipoprotein (VLDL) particles. Acyl-CoA:diacylglycerol acyltransferase (DGAT) is a membrane-bound enzyme that catalyzes the last step in the synthesis of TG. Classified by detergent sensitivity, two types of DGATs in microsomes have been proposed: the overt type (on the cytosol) catalyzes the synthesis of TG destined for cytoplasmic droplets, and latent type (on the lumen of the endoplasmic reticulum) catalyzes the synthesis of TG for VLDL formation (1). Because it has been well established that cytosolic droplet TG cannot be incorporated en bloc into VLDL (2), the relative activities of these two functions of DGAT may have a significant impact on the level of triglyceridemia as well as on the development of steatosis.

Regarding the molecular aspects of DGAT, the cDNAs of DGATs, *viz.* DGAT1 and DGAT2, have been recently cloned and sequenced (3, 4). DGAT1 and DGAT2 are unrelated proteins that exhibit DGAT activity. DGAT1 is expressed ubiquitously, with the highest expression levels in the small intestine (3), and the phenotypes of DGAT1-null mice have been extensively examined (5–9). DGAT1-null mice are viable, can still synthesize TG, and have normal 4-h fasted plasma TG levels (5). These mice have reduced adiposity and are resistant to diet-induced obesity through a mechanism that involves increased energy expenditure (5). The increased energy expenditure is due partly to the increased peripheral leptin sensitivity in DGAT1-deficient mice (6). DGAT2 is expressed ubiquitously, with high expression levels in the liver and white adipose tissue (WAT) (4). In contrast to DGAT1-deficient mice, DGAT2-deficient mice are lipopenic and die soon after birth from profound reductions in substrates for energy metabolism and from impaired permeability barrier function in the skin (10). It is evident that DGAT1 and DGAT2 have distinct roles in the whole body, but the roles of DGAT1 and DGAT2 in tissues such as liver, adipose, and small intestine that synthesize TG have not been well studied. In this work, to elucidate the roles of DGAT1 and DGAT2 in the liver, especially in overt and latent DGAT activities, we created liver DGAT1- and DGAT2-overexpressing mice by adenovirus-mediated gene transfection, and we investigated their phenotypes.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Adenoviruses—The full-length mouse *Dgat1* (GenBank™ accession number NM_010046) and *Dgat2* (GenBank™ accession number AF384160) coding sequences were amplified by PCR. The primers used for amplifying DGAT1 and DGAT2 cDNAs were tailed with either an XbaI site (forward) or a KpnI site (reverse). PCR products were subcloned into the XbaI/KpnI site of the pShuttle vector provided in the BD Adeno-X expression system (BD Biosciences). All sequences of DGATs were verified by DNA sequencing. An I-CeuI/PI-SceI restriction fragment from pShuttle containing the cytomegalovirus-IE promoter/enhancer 5′ to the DGAT cDNAs insert and the polyadenylation signal was ligated into an adenoviral DNA backbone that was also restricted with I-CeuI and PI-SecI. Following amplification and purification of recombinant viral DNA from bacteria, recombinant viral DNA was further amplified by transfecting PacI-linearized recombinant viral DNA into human embryonic kidney 293 cells via the use of Lipofectamine reagent (Invitrogen). The adenoviruses containing DGAT1 cDNA (Ad-DGAT1) and DGAT2 cDNA (Ad-DGAT2) were purified for injection into mice using the BD Adeno-X purification kit (BD Biosciences). BD Adeno-X enhanced green fluorescent protein (Ad-GFP;

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¶ To whom correspondence should be addressed. Tel.: 81-3-3203-5725; Fax: 81-3-3207-3520; E-mail: ezaki@nih.go.jp.

[1] The abbreviations used are: TG, triglyceride; VLDL, very low density lipoprotein; DGAT, acyl-CoA:diacylglycerol acyltransferase; WAT, white adipose tissue; Ad, adenovirus; GFP, green fluorescent protein; VLDLR, very low density lipoprotein receptor; HPLC, high performance liquid chromatography; BAT, brown adipose tissue; ER, endoplasmic reticulum; HDL, high density lipoprotein; LDL, low density lipoprotein.

BD Biosciences) was used as a control.

Each recombinant adenovirus (Ad-DGAT1, Ad-DGAT2, or Ad-GFP) was injected intravenously in a single dose (2×10^9 plaque-forming units in 200 μl) into a non-fasted mouse. The C57BL/6J mice (8 weeks of age) used in this experiment were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were fed standard rodent chow (CE2, CLEA, Tokyo). Animals were exposed to 12-h light/12-h dark cycles and maintained at a constant temperature of 22 °C.

Northern Blotting—The cDNA clones containing the coding sequences of mouse DGAT1 and DGAT2 were obtained by PCR as described above. cDNAs encoding the rat VLDL receptor (VLDLR) and mouse perilipin were kindly provided by Dr. T. Yamamoto (Tohoku University Gene Research Center) and Dr. C. Londos (National Institutes of Health), respectively. RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A portion of the RNA (20 μg/lane) was denatured with glyoxal and dimethyl sulfoxide and analyzed by electrophoresis on 1% agarose gels. After transfer to nylon membranes (PerkinElmer Life Sciences) and UV cross-linking, RNA blots were stained with methylene blue to locate 28 S and 18 S rRNAs (11). The stained 18 S rRNA is shown in Figs. 1 and 7 to indicate that the amount of loaded RNAs was similar in each group. cDNAs were labeled with [α-^{32}P]dCTP (Amersham Biosciences, Buckinghamshire, United Kingdom) using a random primer labeling kit (Rediprime II DNA labeling system, Amersham Biosciences). The amounts of mRNAs were quantitated with a BAS-2000 imaging analyzer (Fujifilm, Tokyo).

Liver Homogenization and Permeabilization of Microsomes for DGAT Assay—To examine the effects of homogenization methods on the recovery and intactness of microsomes, liver tissues (0.2 g) were homogenized in three different types of tissue grinders: Dounce-type (Kimble/Kontes, Vineland, NJ), type B pestle, 30 strokes; motor-driven Potter-type (Iuchi, Osaka, Japan), 200, 400, 600, 1000, 2000, and 4000 rpm for 3 × 10 s; and Polytron (PT10/35, Kinematica AG, Lucerne, Switzerland), setting 4 for 3 × 10 s. All homogenization steps were carried out in 2 ml of ice-cold medium containing 300 mM sucrose, 1 mM EGTA, and 5 mM Tris-HCl (pH 7.4), followed by centrifugation at 10,000 × *g* for 15 min at 4 °C. The resultant supernatant was recentrifuged at 100,000 × *g* for 70 min at 4 °C to obtain the microsomes. The microsomes were resuspended to a final volume of 1 ml and separated into aliquots, which were snap-frozen in liquid N_2 and stored at −80 °C. The recovery of microsomes was compared by measuring the total mannose-6-phosphatase activity in microsomal fractions. The intactness of microsomes was estimated by measuring the mannose-6-phosphatase activities in microsomal fractions in the presence and absence of permeabilizing agents. Potter-type homogenization at 200 or 400 rpm for 3 × 10 s, a relatively mild method, resulted in a poor recovery of total microsomes (32–48% relative to those obtained by Polytron homogenizer) but a good recovery of intact microsomes (81–84%), whereas Polytron homogenization, a powerful method, resulted in a relatively good recovery of total microsomes but a poor recovery of intact microsomes (54%). Because the intactness of microsomes was more important than the recovery of microsomes in measuring overt and latent DGAT activities of microsomes, we chose Potter-type homogenization at 400 rpm for 3 × 10 s in the following experiment.

To examine the effects of permeabilizing agents on DGAT activity, a portion of the microsomes (2 mg/ml protein) was permeabilized by preincubation on ice for 30 min with either the channel-forming peptide alamethicin (final concentration of 0.06 mg/ml in 0.38% ethanol; Sigma) or deoxycholate (final concentration of 2 mg/ml; Sigma) (1, 12, 13). The permeabilizing efficiency estimated by mannose-6-phosphatase activity was not different between these two agents, but DGAT activity in the presence of deoxycholate was 76% lower than in the presence of alamethicin (data not shown). It was shown previously that taurocholate inhibits DGAT activity by 30%, but alamethicin does not (1). Thus, in the study, we used alamethicin as a permeabilizing agent of microsomes.

Assay of DGAT Activity—DGAT activity was determined by measuring the incorporation of the [^{14}C]oleoyl moiety into trioleoylglycerol with [^{14}C]oleoyl-CoA (acyl donor) and *sn*-1,2-dioleoylglycerol (acyl acceptor) as described previously (14, 15). The acyl acceptors were introduced into the reaction mixture by liposomes prepared with phosphatidylcholine (molar ratio of acyl acceptor to phosphatidylcholine of 1:5). The reaction mixture (200 μl) contained 100 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1.25 mg/ml fatty acid-free bovine serum albumin (Serologicals Corp., Kankakee, IL), 200 mM sucrose, 25 μM [^{14}C]oleoyl-CoA (PerkinElmer Life Sciences), 200 μM acyl acceptors, and 10 μg of either permeabilized or non-permeabilized liver microsomal protein. After a 10-min incubation at 37 °C, lipids were extracted with 2:1(v/v) chloroform/methanol. After centrifugation to remove debris, aliquots of the organic phase-containing lipids were dried and separated on Silica Gel 60 TLC plates (Merck, Darmstadt, Germany) with 80:20:1 (v/v/v) hexane/ethyl ether/acetic acid. Individual lipid moieties were identified by standards with exposure to I_2 vapor. The TLC plates were exposed to an Fujifilm imaging plate to assess the formation of ^{14}C-labeled lipid products. Imaging signals were visualized and quantitated with a BAS-2000 imaging analyzer.

To estimate overt and latent DGAT activities, mannose-6-phosphatase activity, a marker of latent enzyme in microsomes, was used for correction of membrane damage and inside-out sealing of microsome in overt DGAT activity (1). Thus, the following equations were used to calculate overt and latent DGAT activities: overt DGAT = $D_0 - ((D_t - D_0)M_0/M_t)$, and latent DGAT = $(D_t - D_0)M_t/(M_t - M_0)$, where D_0 and D_t represent DGAT activity before and after treatment with alamethicin, respectively, and M_0 and M_t represent mannose-6-phosphatase activity before and after treatment with alamethicin, respectively (1, 16).

Electron Microscopy—Diced mouse liver samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h. After washing with phosphate buffer, glutaraldehyde-fixed blocks were placed in 2% osmium tetroxide in phosphate buffer for 2 h at 4 °C, dehydrated, infiltrated with Epon 812, and polymerized for 3 days. Sections were cut out with Ultratome III (LKB Bromma, Stockholm, Sweden) and stained with saturated uranyl acetate and lead acetate. Thin sections were examined with a Hitachi H-300 electron microscope.

Serum Lipoprotein Analysis—Serum obtained from the retro-orbital plexus of an individual mouse was subjected to gel filtration HPLC on two tandemly connected TSK-Gel Lipopropak XL columns (300 × 7.8 mm; Tosoh Corp., Tokyo) as described (17). TG and cholesterol were measured simultaneously using an on-line dual detection system. The particle size of lipoproteins was estimated by the elution time of the column and appropriate size markers.

Liver and Microsomal Lipid Analysis—Lipids in the liver were extracted quantitatively with ice-cold 2:1 (v/v) chloroform/methanol by the method of Folch *et al.* (18). Total cholesterol and TG concentrations in the liver homogenates and microsomal fractions were measured by enzymatic colorimetric methods using the cholesterol and TG E tests (Wako Pure Chemicals, Osaka), respectively.

Measurement of in Vivo VLDL Secretion Rates—Hepatic production of VLDL TG was measured in Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected mice. On day 12 after adenovirus injection, Triton WR-1339 (Sigma) was intravenously administered after a 4-h fast (19). Mice were bled prior to injection and at 1, 2, 3, and 4 h post-injection. Increases in serum TG concentrations over 4 h in Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected mice after intravenous injection of Triton WR-1339 were measured by enzymatic colorimetric methods using the TG E test.

Immunoblotting—Pooled membrane fractions of either isolated epididymal or subcutaneous adipocytes from three mice of each group were prepared by collagenase digestion and differential centrifugation as described by Kono *et al.* (20). Ten μg of membrane protein obtained by centrifugation at 175,000 × *g* for 60 min at 4 °C was applied to 12.5% SDS-polyacrylamide gel and transferred to Clear Blot Membrane-P (Atto Corp., Tokyo). Immunoblot analysis was performed using the enhanced chemiluminescence Western blotting detection system kit (Amersham Biosciences). Membrane sheets were first incubated with antibody against the VLDLR (sc-18824, Santa Cruz Biotechnology, Inc.) for 1 h at 22 °C, washed three times, and incubated with horseradish peroxidase-conjugated anti-mouse IgG according to the protocol recommended by the manufacturer. The amount of VLDLR was quantitated using NIH Image Version 1.6.3.

Statistical Methods—Statistical comparisons of groups were made by one-way analysis of variance, and the VLDL secretion curve in Fig. 4 was compared by repeated measure analysis (StatView Version 5.0, Abacus Concepts, Inc., Berkeley, CA). When statistically significant, each group was compared with the others by Fisher's protected least significant difference test.

RESULTS

Overexpression of DGAT1 or DGAT2 in Mouse Liver—To gain insight into the physiological roles of DGAT1 and DGAT2 in the liver, either DGAT1 or DGAT2 was overexpressed in the liver by adenovirus-mediated gene transfection. We injected 2×10^9 plaque-forming units of recombinant virus containing DGAT1 cDNA, DGAT2 cDNA, or GFP cDNA (control) into mice. This dose has been found to cause expression of the foreign gene in the majority of hepatocytes (21). On day 12 after administration of the recombinant viruses, the expression lev-

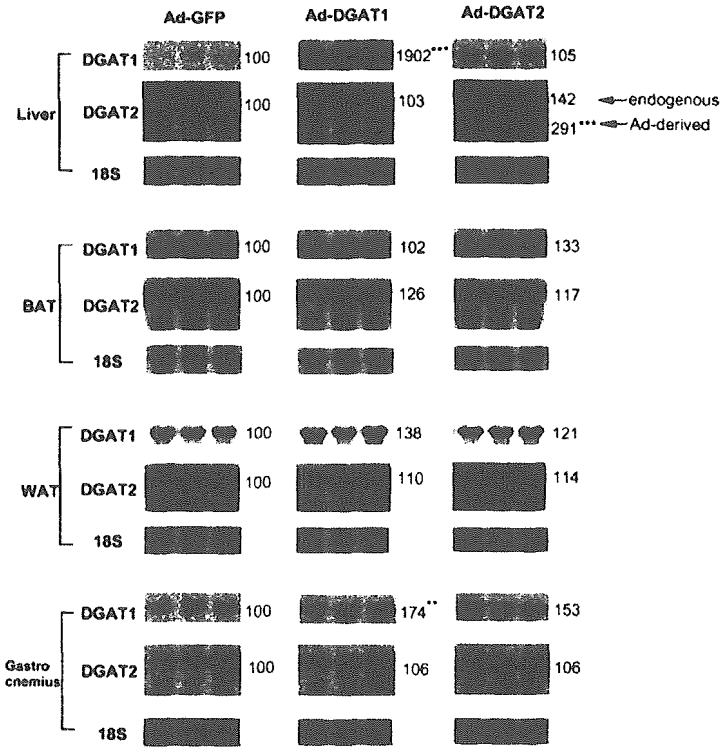

FIG. 1. **Northern blot analysis of DGAT1 and DGAT2 expression in the liver, BAT, epididymal WAT, and the gastrocnemius in Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected mice.** Total RNA was isolated from the liver, BAT, epididymal WAT, and the gastrocnemius of non-fasted Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected male mice on day 12 after injection of each adenovirus construct, and 20 μg of RNA was subjected to electrophoresis, transferred to a nylon membrane, and hybridized with the indicated ^{32}P-labeled cDNA probes. Each lane represents a sample from an individual mouse. The molecular sizes of DGAT1, endogenous DGAT2, and Ad-derived DGAT2 mRNAs are 1.8, 2.5, and 1.7 kb, respectively. 18 S rRNA was used as a loading control. Quantification of DGAT1 and DGAT2 mRNAs is expressed relative to Ad-GFP-injected mice. **, $p < 0.01$ *versus* Ad-GFP-injected mice; ***, $p < 0.001$ *versus* Ad-GFP-injected mice.

els of DGAT1 and DGAT2 were measured by Northern blotting. Because the molecular sizes of DGAT1 mRNAs derived from the endogenous *Dgat1* gene and the Ad-DGAT1 construct were almost the same, they could not be distinguished by Northern blotting, whereas because the molecular size of DGAT2 mRNA derived from the Ad-DGAT2 construct was smaller than that of DGAT2 mRNA derived from the endogenous *Dgat2* gene, they could be distinguished (Fig. 1).

The DGAT1 mRNA levels in Ad-DGAT1-injected mice were increased by 19-fold in the liver and by 1.7-fold in the gastrocnemius compared with endogenous DGAT1 mRNA levels in control Ad-GFP-injected mice, but no changes were seen in brown adipose tissue (BAT) and epididymal WAT. DGAT2 expression in Ad-DGAT1-injected mice was not affected in all of these tissues. The DGAT2 mRNA levels in Ad-DGAT2-injected mice were increased by 4-fold in the liver compared with endogenous DGAT2 mRNA levels in control Ad-GFP-injected mice, but were not altered in BAT, WAT, and the gastrocnemius. The DGAT1 expression levels in Ad-DGAT2-injected mice were not affected in any of these tissues. Because DGAT1 expression in the gastrocnemius was increased slightly in both Ad-DGAT1-injected (significantly) and Ad-DGAT2-injected (insignificantly) mice, an increase in gastrocnemius DGAT1 expression might be due to metabolic changes in the whole body, secondary to liver DGAT overexpression. Because, as estimated by Northern blotting, the expression level of DGAT1 in normal liver was very low compared with that of DGAT2, when expressed as the -fold increase relative to endogenous DGAT1 mRNA levels, the increased DGAT1 mRNA levels in Ad-DGAT1-injected mice were much higher than the increased DGAT2 mRNA levels in Ad-DGAT2-injected mice. Plasma aspartate aminotransferase and alanine aminotransferase levels were not elevated after gene transfection, and liver damage did not occur (data not shown).

DGAT1 Possesses Latent DGAT Activity—To examine whether overexpressed liver DGAT1 and DGAT2 are functional, the overt and latent DGAT activities in liver microsomes from Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected mice were estimated (Fig. 2). DGAT activity was assessed by the formation of ^{14}C-labeled TG from [^{14}C]oleoyl-CoA (Fig. 2*A*). Overt DGAT activity in microsomes is defined as enzyme activity on the cytosol involved in the synthesis of cytosolic droplet TG, whereas latent DGAT activity is defined as enzyme activity on the endoplasmic reticulum (ER) lumen involved in VLDL synthesis (1). Overt and latent DGAT activities were estimated by measuring DGAT and mannose-6-phosphatase activities in the absence and presence of alamethicin as described under "Experimental Procedures."

DGAT activity in the absence of alamethicin was significantly increased in both DGAT1- and DGAT2-overexpressing mice compared with control Ad-GFP-injected mice, whereas DGAT activity in the presence of alamethicin (total DGAT activity in microsomes) was markedly increased by 3.2-fold in DGAT1-overexpressing mice, but did not differ in DGAT2-overexpressing mice (Fig. 2*B*). When latent and overt DGAT activities were estimated by measuring the mannose-6-phosphatase activity (22), latent DGAT activity was increased by 4.2-fold in DGAT1-overexpressing mice, and overt DGAT activity was increased by 2.5-fold in DGAT2-overexpressing mice (Fig. 2*C*).

Because DGAT protein concentrations in microsomal fractions were not measured because of the lack of appropriate antibodies to DGAT1 and DGAT2, comparison of DGAT activity on a protein basis was not made between DGAT1- and DGAT2-overexpressing mice. However, if we assume that alamethicin did not affect the DGAT1 and DGAT2 molecules, this indicates that the latent/overt DGAT activity was much larger in DGAT1-overexpressing mice than in DGAT2-overexpressing mice; the latent/overt DGAT activity in Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected mice was 2.4, 14.1, and 0.35, respectively. Thus, these data indicate that DGAT1 but not DGAT2 possesses marked latent DGAT activity, whereas DGAT2 might possess overt DGAT activity.

DGAT1-overexpressing Mice Have Increased Serum VLDL Concentrations and Particle Size—Because latent DGAT activ-

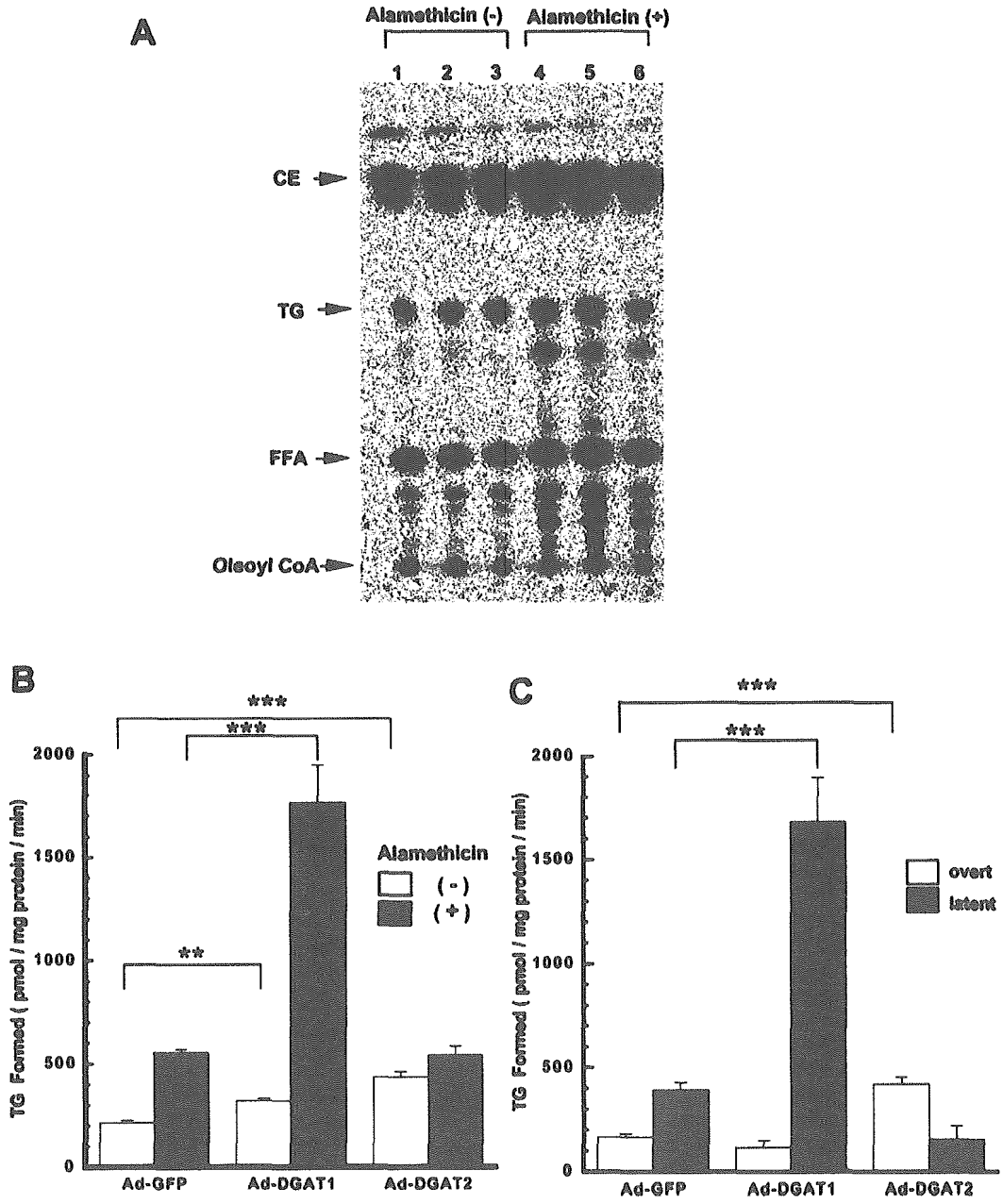

FIG. 2. **Estimated overt and latent DGAT activities in Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected mouse livers homogenized using a Potter-type homogenizer.** *A*, representative autoradiogram of DGAT activity in Ad-GFP-injected (*lanes 1* and *4*), Ad-DGAT1-injected (*lanes 2* and *5*), and Ad-DGAT2-injected (*lanes 3* and *6*) male mouse liver microsomes prepared using a Potter-type homogenizer in the absence and presence of alamethicin. Each lane represents a sample from an individual mouse. Mice were killed on day 12 after adenovirus administration. The amount of TG corresponds to DGAT activity. *CE*, cholesteryl ester; *FFA*, free fatty acid. Additional bands between TG and FFA and between FFA and oleoyl-CoA were observed when both microsomes and alamethicin were added to the incubation mixture, but their molecules have not been identified. *B*, quantification of DGAT activities in *A*. *C*, overt and latent DGAT activities in liver microsomes isolated from Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected mice estimated by the activity of mannose-6-phosphatase, a marker of latent enzyme in microsomes, as described under "Experimental Procedures." The mannose-6-phosphatase activities in microsomal preparations before and after alamethicin treatment were found to be 23.8 and 167 nmol of P_i formed per min/mg of protein, respectively. Data represent means ± S.E. ($n = 4$). **, $p < 0.01$ *versus* Ad-GFP-injected mice; ***, $p < 0.001$ *versus* Ad-GFP-injected mice.

ity was increased in DGAT1-overexpressing mice, we expected that blood VLDL concentrations might be increased in DGAT1-overexpressing mice. Thus, we measured serum lipoprotein profiles in DGAT1- and DGAT2-overexpressing mice by HPLC (Fig. 3). As expected, compared with the control Ad-GFP-injected mice, VLDL TG concentrations were increased by 1.6-fold in DGAT1-overexpressing mice, but were not changed in DGAT2-overexpressing mice (Fig. 3*A* and Table I). High density lipoprotein (HDL) TG concentrations were very low in each group of mice. VLDL cholesterol concentrations were also increased in DGAT1-overexpressing mice (Fig. 3*B* and Table I). However, low density lipoprotein (LDL) and HDL cholesterol concentrations did not differ among the different groups of mice. The particle size of lipoproteins was estimated by the elution time of TSK-Gel Lipopropak XL columns. VLDL particle size was significantly enlarged in DGAT1-overexpressing

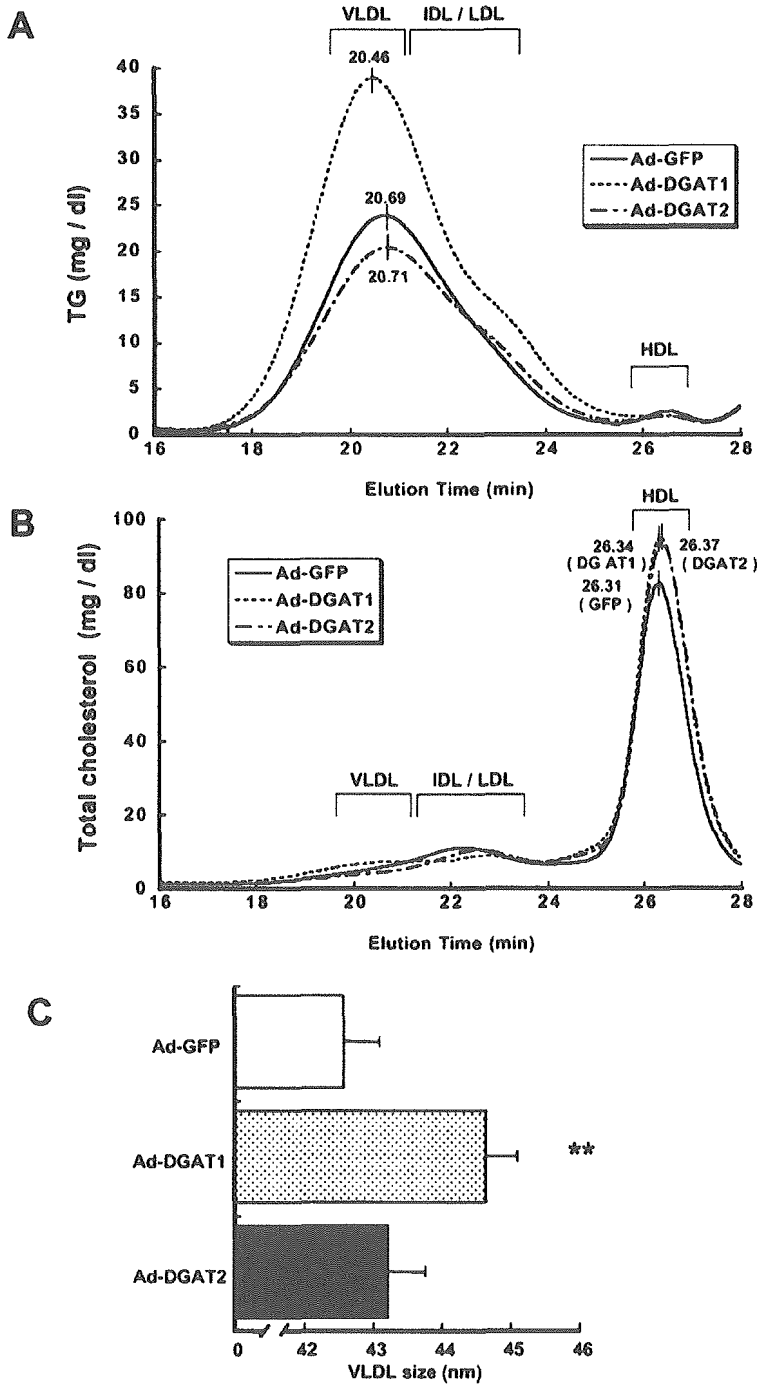

FIG. 3. **Increased serum VLDL concentration and particle size in DGAT1-overexpressing mice.** Serum from each group of mice on day 12 after injection of Ad-GFP, Ad-DGAT1, or Ad-DGAT2 was subjected to gel filtration HPLC, and the TG and cholesterol content of each fraction was measured as described under "Experimental Procedures." Representative data from individual mice are shown in *A* (TG) and *B* (cholesterol). The mean of VLDL particle size is shown in *C*. Data represent means ± S.E. (n = 10–12). *IDL*, intermediate density lipoprotein. **, $p < 0.01$ *versus* Ad-GFP-injected mice.

TABLE I
Serum lipoprotein profiles in Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected mice

Serum lipoproteins in male mice were assayed on day 12 after adenovirus injection as described in the legend to Fig. 3. Data are means ± S.E. (n = 10–12).

	Ad-GFP	Ad-DGAT1	Ad-DGAT2
Total TG (mg/dl)	87.3 ± 12.8	132.8 ± 10.8[a]	105.1 ± 14.8
VLDL TG (mg/dl)	66.4 ± 10.0	103.8 ± 8.7[a]	76.1 ± 11.2
LDL TG (mg/dl)	18.5 ± 2.8	25.5 ± 2.4	26.1 ± 4.0
HDL TG (mg/dl)	1.9 ± 0.4	2.4 ± 0.4	2.2 ± 0.3
Total cholesterol (mg/dl)	87.9 ± 4.5	86.9 ± 5.7	87.2 ± 2.9
VLDL cholesterol (mg/dl)	7.5 ± 0.8	9.4 ± 0.7[a]	6.3 ± 0.4
LDL cholesterol (mg/dl)	12.2 ± 0.6	11.2 ± 0.6	11.2 ± 0.4
HDL cholesterol (mg/dl)	68.1 ± 3.9	56.3 ± 5.1	69.6 ± 2.5

[a] $p < 0.05$ *versus* Ad-GFP-injected mice.

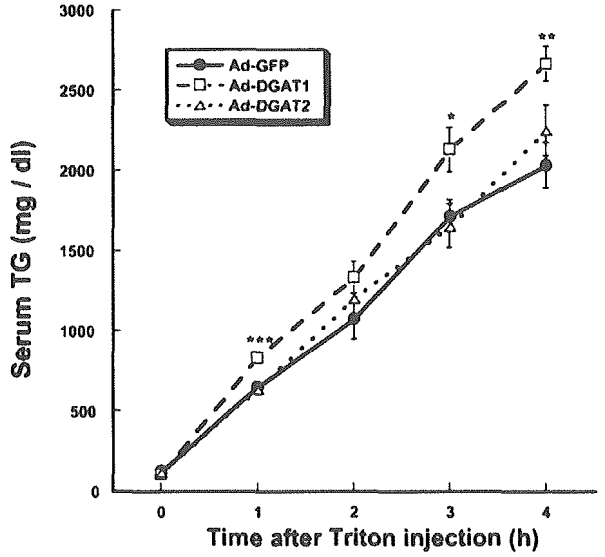

FIG. 4. **Increased VLDL secretion in DGAT1-overexpressing mice.** Serum TG concentrations were measured prior to and 1, 2, 3, and 4 h after Triton WR-1339 injection in male mice on day 12 after injection of Ad-GFP, Ad-DGAT1, or Ad-DGAT2. Data represent means ± S.E. (n = 8). *, $p < 0.05$ *versus* Ad-GFP-injected mice; **, $p < 0.01$ *versus* Ad-GFP-injected mice; ***, $p < 0.001$ *versus* Ad-GFP-injected mice.

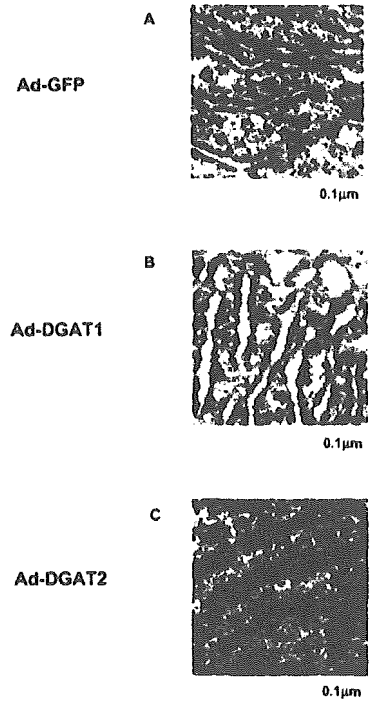

FIG. 5. **Enlargement of the rough ER in DGAT1-overexpressing mice.** The ultrastructure of hepatocytes from Ad-GFP- (*A*), Ad-DGAT1- (*B*), and Ad-DGAT2-injected (*C*) male mice (day 12) was observed by electron microscopy. The lumen of the ER in Ad-DGAT1-injected mice was markedly enlarged compared with control Ad-GFP- and Ad-DGAT2-injected mice and contained many lipid-like particles. Magnification is ×10,000.

mice (Fig. 3*C*), but there were no changes in HDL particle size among the different groups of mice (data not shown).

Increased VLDL Secretion in DGAT1-overexpressing Mice—The increase in the VLDL concentration in DGAT1-overexpressing mice may be due to an increase in VLDL secretion or/and inhibition of VLDL clearance. To determine the cause, the rate of hepatic VLDL secretion was estimated by monitoring serum TG concentrations in the presence of Triton WR-1339 (Fig. 4), which blocks lipolysis of VLDL in peripheral tissues. The TG concentration significantly increased in DGAT1-overexpressing mice compared with control mice following Triton WR-1339 administration (by repeated analysis of variance, $p < 0.01$). An increase in the TG concentration in DGAT2-overexpressing mice was not observed. Thus, the increase in the blood TG concentration in DGAT1-overexpressing mice was due, at least in part, to increased VLDL secretion.

Electron Microscopy Study of the ER in Livers from DGAT-overexpressing Mice—Next, to examine whether the increased rate of VLDL secretion observed in DGAT1-overexpressing mice is due to increased VLDL synthesis in the liver ER, an electron microscopy study was conducted (Fig. 5). The VLDL secretion pathway in the liver has been elucidated (23–25). TG is synthesized in the smooth ER, and this lipid particle then moves to the smooth surface ends of rough ER cisternae. At the junction between the smooth ER and the rough ER, apolipoprotein B is bound to the lipid particle to form VLDL. Alternatively, in the rough ER, TG and apolipoprotein B are synthesized and assembled into VLDL with the microsomal TG transfer protein MTP (26). Electron microscopy examination of the liver revealed that the rough ER from DGAT1-overexpressing mice was markedly dilated and contained small particles, possibly synthesized TG particles in the lumen of the rough ER, but these changes were not observed in control and DGAT2-overexpressing mice. The diameter of these small particles was 46 ± 2 nm (means ± S.E., n = 21), similar to the size of VLDL in blood (Fig. 3*C*). Indeed, the TG concentration in the microsomal fractions from DGAT1-overexpressing mice was 2.1-fold larger than in control mice (microsomal TG concentrations in Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected mice of 95 ± 15, 206 ± 28, and 117 ± 21 μg/mg of protein, respectively (n = 4); $p < 0.01$). Even when expressed as wet liver weight, the microsomal TG amount in DGAT1-overexpressing mice was 2.2-fold larger than in control mice (microsomal TG amounts in Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected mice of 3.6 ± 0.5, 7.8 ± 1.1, and 5.3 ± 1.2 mg/g of liver, respectively (n = 4); $p < 0.05$). This finding supports the hypothesis that increased latent DGAT activity, which occurs in the lumen of the rough ER, leads to increased VLDL secretion in DGAT1-overexpressing mice.

DGAT2 Overexpression Increases Liver TG Concentrations—As hepatic overexpression of DGAT1 and DGAT2 increased DGAT activity (Fig. 2), we examined whether the liver TG concentration was increased in these DGAT-overexpressing mice on day 12 after administration of the recombinant viruses (Fig. 6*A*). Accumulation of TG in DGAT1-overexpressing mice was not expected because of lower overt DGAT activity and/or an increase in latent DGAT activity, which promotes the excretion of TG from the liver to the blood circulation as VLDL. However, either DGAT1- or DGAT2-overexpressing mice increased liver TG concentrations; DGAT1- and DGAT2-overexpressing mice showed 1.9- and 3.1-fold increases in liver TG concentrations, respectively, compared with control mice. Similar increases in TG concentrations were noted on day 6 after administration of the recombinant viruses (data not shown). Although an ~2-fold increase in the TG concentration in microsomes was observed in DGAT1-overexpressing mice, the contribution of the TG amount in microsomes to that in the total liver was <10% (estimated by the average amount of TG in microsomal fractions and homogenates); the 1.9-fold increase in the TG amount in the total liver in DGAT1-overexpressing mice was not due to the TG increase in the microsomes. Thus, both DGAT1 and DGAT2 contributed to cytosolic

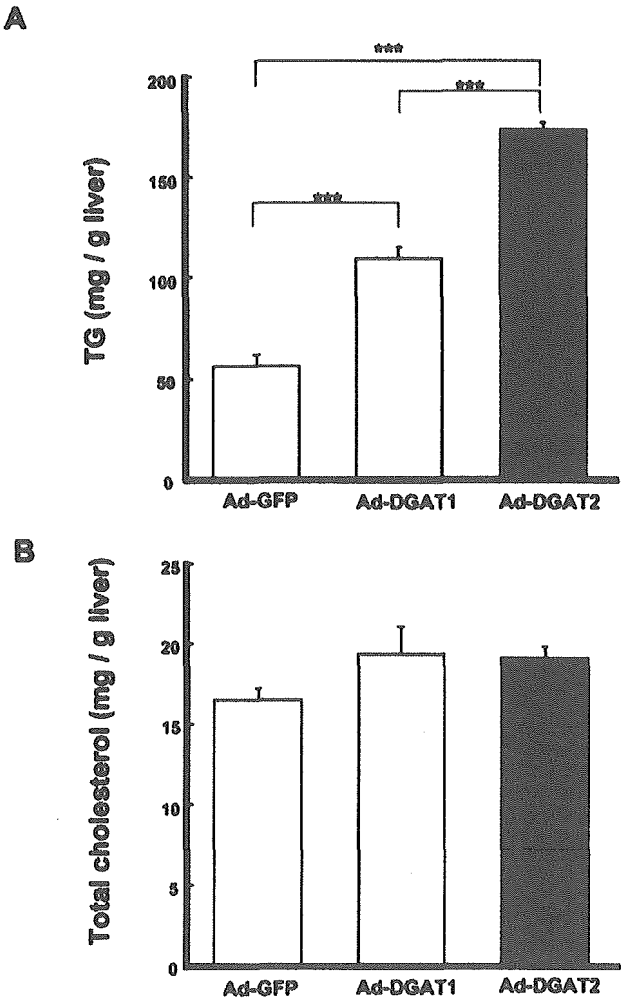

FIG. 6. **Liver TG and cholesterol concentrations in Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected mice.** Male mice were killed on day 12 after adenovirus injection. Liver TG (*A*) and cholesterol (*B*) concentrations were measured. Data represent means ± S.E. (n = 5). ***, $p < 0.001$ (significantly different).

triglyceride levels. The larger increase in TG accumulation in DGAT2-overexpressing mice indicated that DGAT2 possessed potent activity to synthesize TG in the liver cytosol. The liver cholesterol concentration did not differ among the three groups of mice (Fig. 6*B*).

DGAT1-overexpressing Mice Have Increased Epididymal WAT Weight—To examine the effects of increased VLDL secretion in DGAT1-overexpressing mice on the whole body, the body and tissue weights of Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected mice were measured on day 12 after administration of the recombinant viruses (Table II). There were no changes in total body weights among these three groups of mice. However, epididymal WAT weight was significantly increased by 1.2-fold in DGAT1-overexpressing mice, but abdominal subcutaneous WAT weight was not altered. No increase in epididymal WAT weight was observed in DGAT2-overexpressing mice. In female mice, DGAT1 overexpression also caused a 1.5-fold increase in parametrial WAT weight ($p < 0.01$), but did not alter subcutaneous WAT weight (data not shown). These results suggest that TG transfer from the liver to WAT might lead to increased gonadal WAT weight. Because the observation periods were short (12 days), increases in WAT weight might not cause apparent obesity. Although its mechanism was not clear, liver weight was slightly decreased in DGAT1-overexpressing mice irrespective of the increased lipid accumulation (Fig. 6*A*). However, we speculated that increased DGAT1 expression over a longer time period might lead to increased liver weight with a gradual accumulation of TG. The glucose and insulin tolerance curves on day 12 were not significantly different among these groups (data not shown).

Gonadal WAT Expresses a Large Amount of VLDLR—To elucidate the mechanism for the higher sensitivity of VLDL in gonadal WAT, the expression levels of VLDLR in subcutaneous WAT and epididymal WAT were examined in normal mice (Fig. 7). The expression level of VLDLR mRNA was 6-fold larger in epididymal WAT than in abdominal subcutaneous WAT (Fig. 7, *A* and *B*). Expression of perilipin (a fat droplet marker) was also increased by 2-fold in epididymal WAT, but its increase was much lower than the VLDLR mRNA increase. In female mice, a 4-fold increase in VLDLR mRNA was also observed in parametrial WAT ($p < 0.01$) (data not shown). Immunoblot analysis of the VLDLR also confirmed that the VLDLR protein was increased by ~4-fold in adipocytes from epididymal WAT compared with those from abdominal subcutaneous WAT (Fig. 7*C*). Furthermore, overexpression of liver DGAT1 or DGAT2 did not regulate the expression levels of VLDLR mRNA in epididymal WAT (Ad-GFP-injected mice, 100 ± 16%; Ad-DGAT1-injected mice, 116 ± 7%; and Ad-DGAT2-injected mice, 119 ± 13%; n = 3) and subcutaneous WAT (Ad-GFP-injected mice, 100 ± 6%; Ad-DGAT1-injected mice, 81 ± 5%; and Ad-DGAT2-injected mice, 89 ± 5%; n = 3). These data indicate that the preferential increase in gonadal WAT mass observed in DGAT1-overexpressing mice might be due to high VLDLR expression levels in gonadal WAT and increased VLDL secretion.

DISCUSSION

In this study, to examine the roles of liver DGAT1 and DGAT2, we investigated the effects of overexpression of liver DGAT1 and DGAT2 by adenovirus-mediated gene transfection on DGAT activity, TG synthesis, and VLDL secretion. DGAT1-overexpressing mice had increased latent DGAT activity and a dilated ER, whereas these changes were not observed in DGAT2-overexpressing mice (Figs. 2 and 5). As expected from the increased latent DGAT activity in DGAT1-overexpressing mice, VLDL secretion and particle size were increased (Figs. 3 and 4), resulting in increased gonadal fat mass expressing a large amount of VLDLR (Fig. 7 and Table II).

Our *in vivo* evidence that DGAT1 is located in the lumen of the ER and promotes VLDL secretion is in a good agreement with recent *in vitro* studies. Overexpression of human DGAT1 in McArdle rat hepatoma cells (RH7777) results in increased TG-rich VLDL secretion (27). When DGAT1 and DGAT2 are overexpressed in RH7777 cells, small lipid droplets around the cell periphery are observed in DGAT1-expressing cells, whereas numerous large cytosolic lipid droplets are observed in DGAT2-expressing cells (Fig. 1 from Ref. 10).

Judging from the abundance of DGAT2 mRNA, it was speculated that DGAT2 rather than DGAT1 is the major DGAT in the liver, although its protein levels and subcellular localization have not been determined (28, 29). In a previous study, DGAT1 and DGAT2 activities were distinguished by sensitivity to $MgCl_2$; DGAT2 but not DGAT1 activity is inhibited at higher concentrations of $MgCl_2$ (100 mM) (4). The recent observation that niacin inhibits both $MgCl_2$ (high concentration)-sensitive DGAT2 activity and overt DGAT activity in HepG2 cells also suggests that DGAT2 has potent overt DGAT activity (13). These observations confirm that DGATs in the liver have a distinct role; DGAT2 plays a major role in cytosolic lipid accumulation, whereas DGAT1 plays a role in VLDL assembly.

TABLE II
Body and tissue weights of Ad-GFP-, Ad-DGAT1-, and Ad-DGAT2-injected mice
Male mice were killed, and several tissues were weighed on day 12 after adenovirus injection. Data represent means ± S.E. (n = 9).

	Ad-GFP	Ad-DGAT1	Ad-DGAT2
Body weight (g)	25.2 ± 0.5	25.3 ± 0.4	24.5 ± 0.4
Liver weight (g)	1.59 ± 0.05	1.37 ± 0.02[a]	1.44 ± 0.08
Epididymal WAT weight (g)	0.30 ± 0.02	0.37 ± 0.02[b]	0.31 ± 0.02
Abdominal subcutaneous WAT weight (g)	0.24 ± 0.04	0.24 ± 0.04	0.21 ± 0.05
BAT weight (g)	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
Gastrocnemius weight (g)	0.33 ± 0.01	0.34 ± 0.01	0.31 ± 0.01
Quadriceps weight (g)	0.33 ± 0.02	0.30 ± 0.01	0.29 ± 0.01

[a] $p < 0.05$ *versus* Ad-GFP-injected mice.
[b] $p < 0.01$ *versus* Ad-GFP-injected mice.

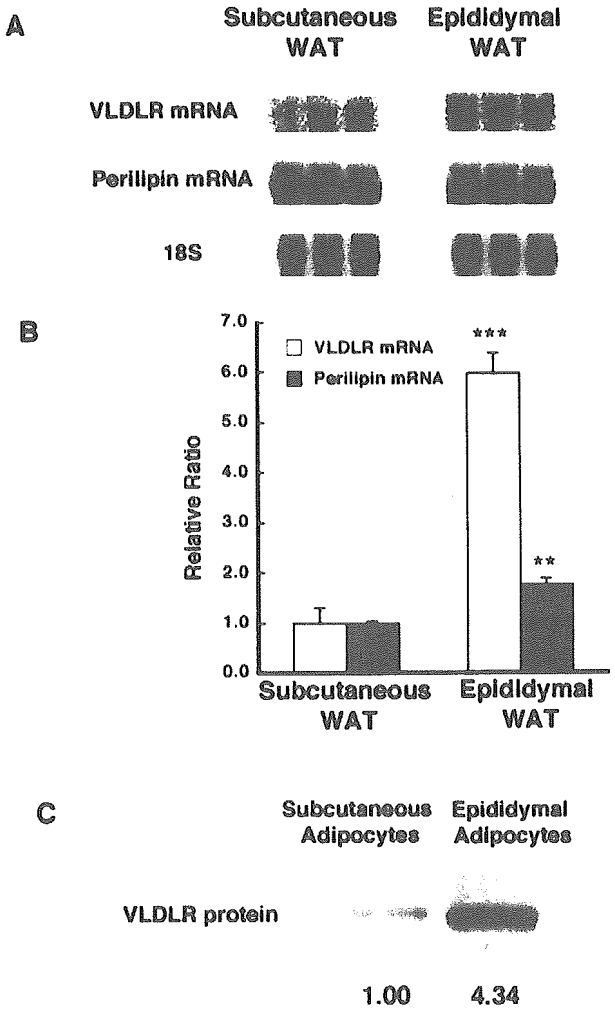

FIG. 7. **Increased VLDLR expression in epididymal WAT.** *A* and *B*, total RNA was isolated from abdominal subcutaneous WAT and epididymal WAT, and 20 μg of RNA was subjected to electrophoresis, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled VLDLR and perilipin cDNA probes. Perilipin mRNA was measured on the same membrane sheet as a control. Each lane represents a sample from an individual mouse. The molecular sizes of VLDLR and perilipin mRNAs are 3.4 and 2.7 kb, respectively (*A*). Quantification of each mRNA is expressed relative to subcutaneous WAT (*B*). Data represent means ± S.E. (n = 3). **, $p < 0.01$ (significantly different); ***, $p < 0.001$ (significantly different). *C*, VLDLR proteins in pooled membrane fractions of isolated adipocytes from either abdominal subcutaneous WAT or epididymal WAT from three mice were measured by immunoblotting. The molecular size of the VLDLR protein is 135 kDa.

However, contrary to this hypothesis, DGAT1-null mice fail to decrease 4-h fasted serum TG concentrations (5). We speculated that a 4-h fasting might not be long enough to eliminate the contribution of ingested dietary fat to serum TG levels, and the actual VLDL TG levels might be decreased in a 12-h fasted state. It is also conceivable that ablation of DGAT1 in the whole body might up-regulate adaptive mechanism(s) that enhance VLDL secretion. Regulation of VLDL assembly and secretion is a complex process that requires a coordinated function of many enzymes such as acyl-CoA:cholesterol acyltransferases and MTP and those related to apolipoprotein B synthesis and transport of acylcarnitine from the cytosol to the ER, including DGATs (30–32). Thus, these other players might be involved in the adaptive mechanism(s).

Plasma TG levels are reduced by 70–90% in DGAT2-null mice at birth relative to littermate controls (10). This result can be explained as follows. Plasma free fatty acids are markedly reduced by 70–90% in DGAT2-null mice, with a marked reduction in fatty acid supply from fat mass (10). Because it is well known that plasma free fatty acids promote VLDL secretion (33), a decrease in plasma free fatty acids might lead to a decrease in fasting plasma TG levels in DGAT2-null mice.

The idea that a similar enzyme but in a different orientation of the ER plays a different role is not new. Acyl-CoA:cholesterol acyltransferases catalyze the formation of cholesteryl ester from cholesterol and fatty acyl-CoA (34), whereas DGATs catalyze the formation of TG from diacylglycerol and fatty acyl-CoA. Like DGATs, two genes that encode ACAT1 and ACAT2 have been identified with distinct roles (35–37). Although the membrane topology of ACAT2 is still controversial (38, 39), it secretes more VLDL cholesterol compared with ACAT1 when overexpressed in RH7777 cells (27). These data suggest that DGAT1 and ACAT2 might coordinately participate in VLDL formation within the ER lumen.

The other important finding of this study is that VLDL increased gonadal fat mass weight. The common causes of increased VLDL synthesis include increased free fatty acid flux into hepatocytes, as seen in obesity, and increased hepatic *de novo* lipogenesis, as seen in excessive intake of alcohol or carbohydrate (40). VLDL transports fatty acids from the liver to adipose and other peripheral tissues. Because adipose tissues receive fat from VLDL and chylomicrons, an increase in blood VLDL concentrations might be one of the causes of obesity. However, it has not been elucidated whether the observed increased VLDL secretion is a cause or a result of obesity. Our results that gonadal adipose tissues expressed a large amount of VLDLR (Fig. 7) and that liver DGAT1-overexpressing mice showed enhanced VLDL secretion and increased gonadal adipose tissue weight, whereas DGAT2-overexpressing mice did not show enhanced VLDL secretion or increased fat mass weights (Table II), support the hypothesis that VLDL causes some types of obesity, *viz.* abdominal obesity. Ablation of the *Srebp-1* (sterol regulatory element-binding protein-1) gene results in marked reductions in hepatic lipogenesis, but does not decrease VLDL secretion or the amount of WAT in hybrids between C57BL/6J and 129Sv/Ev mouse strains (41) and in

ob/ob mice (42). Mice lacking the VLDLR exhibit normal lipid levels and a modest decrease in epididymal adipose tissue weights and are protected from diet-induced obesity (43, 44). These knockout mouse studies and our DGAT study support the hypothesis that an increased plasma VLDL concentration is an important determinant of intra-abdominal obesity, possibly mediated by the VLDLR.

In conclusion, DGAT1 but not DGAT2 has a role in VLDL synthesis, and increased blood VLDL concentrations may promote obesity, whereas increased DGAT2 activity has a role in steatosis. In addition, the finding that gonadal fat expresses a large amount of VLDLR highlights the importance of the VLDLR in the etiology of intra-abdominal obesity. Further studies using liver-specific DGAT knockout mice are needed to examine whether a decrease in liver DGAT activity is safe and effective in preventing fatty liver and VLDL oversecretion.

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Lack of potential of low dose *N*-nitrosodimethylamine to induce preneoplastic lesions, glutathione *S*-transferase placental form-positive foci, in rat liver

Shoji Fukushima[a,*], Hideki Wanibuchi[a], Keiichirou Morimura[a], Dai Nakae[b], Hiroyuki Tsuda[c,1], Katsumi Imaida[d], Tomoyuki Shirai[e], Masae Tatematsu[f], Tetsuya Tsukamoto[f], Masao Hirose[g], Fumio Furukawa[g]

[a]*Department of Pathology, Osaka City University Medical School, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan*
[b]*Department of Pathology, Sasaki Institute, Sasaki Foundation, 2-2 Kanda-Surugadai, Chiyoda, Tokyo 101-0062, Japan*
[c]*Experimental Pathology and Chemotherapy Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[d]*Onco-Pathology, Department of Pathology and Host-Defence, Faculty of Medicine, Kagawa University, 1750 Ikenobe, Miki-cho, Kagawa 761-0793, Japan*
[e]*Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan*
[f]*Division of Oncological Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan*
[g]*Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*

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Abstract

Induction of liver lesions in male F344 rats by the genotoxic and carcinogenic *N*-nitrosodimethylamine (NDMA) was studied at a wide range of dose levels, i.e. from 0.001 to 10 ppm, in drinking water for 16 weeks. Dose related and statistically significant increase of glutathione *S*-transferase placental form-positive foci, endpoint markers for hepatocarcinogenesis in rats, at 1 and 10 ppm dose groups was obtained, but no increment in foci could be detected with the lower doses (0.001, 0.01, and 0.1 ppm). This finding of a no-observed effect level supports our hypothesis that a threshold, at least in practical terms, exists in carcinogenesis proposed on the basis of extensive wide range dose-dependence studies of other genotoxic carcinogens.

Keywords: *N*-nitrosodimethylamine; Risk assessment; Carcinogenicity dose threshold

* Corresponding author. Tel.: +81 6 6645 3735; fax: +81 6 6646 3093.
E-mail address: fukuchan@med.osaka-cu.ac.jp (S. Fukushima).
[1] Present address: Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Science, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan.

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1. Introduction

Chemical carcinogens are generally classified into two categories, genotoxic and non-genotoxic. Concerning cancer risk assessment, it is considered that genotoxic carcinogens exert carcinogenic potential regardless of the animal species, so that chemicals which are carcinogenic to rodents are carcinogenic to humans as well. Because genotoxic carcinogens are mutagenic and seem to act through interaction with DNA to produce irreversible genetic changes in target organ cells, it has been generally concluded that they have no dose threshold in their carcinogenic potential [1,2]. Therefore, there is widespread acceptance of a linear curve extending to zero at very low doses for chemicals found to be carcinogenic with in vivo carcinogenicity tests. However, while there are data supporting the non-threshold theory [3–5], and we recently provided evidence of thresholds for the hepatocarcinogenicity of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and *N*-nitrosodiethylamine (NDEA) in rats [6,7]. Williams et al. [8,9] also earlier reported the existence of similar thresholds for NDEA and 2-acetylaminofluorene hepatocarcinogenicity.

N-nitrosodimethylamine (NDMA), an *N*-nitroso compound, is well established as a hepatocarcinogen in rodents. Humans are exposed to NDMA from occupational and environmental sources and through in vivo formation of ingested precursor amines and nitrosating agents [10]. In particular its endogenous formation from ingested precursors has been indicated to be a major source of exposure. Previously Peto et al. [5] reported non-threshold of NDMN hepatocarcinogenicity based on statistical analysis of results from a long-term carcinogenicity test at low doses in 4080 rats.

Recently in vivo medium-term bioassays for carcinogens have become accepted as alternatives to long-term carcinogenicity tests. Particularly, the liver medium-term bioassay has been developed as useful for detecting hepatocarcinogenicity of chemicals [11]. Recently we found a 21-day-old rat, a medium-term model to be very useful for assessment of low dose carcinogenicity of hepatocarcinogens such as MeIQx and NDEA because of high sensitivity [6]. In this medium-term bioassay, the animal treatment duration was 16 weeks and glutathione *S*-transferase placental form (GST-P)-positive foci, established preneoplastic lesions in the livers of rats [11,12], were taken as end-point markers of carcinogenicity.

In the present study, we examined low dose carcinogenicity of NDMA in the rat liver from viewpoint of 'weight of evidence' for clarification of human risk assessment of genotoxic carcinogens. For this purpose we employed the same experimental protocol with which MeIQx and NDEA were earlier examined for low dose carcinogenicity [6].

2. Materials and methods

2.1. Animals and chemicals

A total of 540 male 20-day-old F344 rats were obtained from Charles River Japan, Inc. (Atsugi, Kanagawa, Japan) and housed in rooms maintained on a 12 h light/dark cycle, at constant temperature and humidity, and observed daily. Numbers of the rats employed in the present study were decided on the basis results of previous, low dose carcinogenicity studies [6,7]. NDMA (purity >99%) was purchased from Sakai Research Laboratory (Fukui, Japan).

2.2. Experimental procedures

The experiment was started when the animals were aged 21 days. They received NDMA at doses of 0 (group 1, a control, 90 rats), 0.001 (group 2, 89 rats), 0.01 (group 3, 89 rats), 0.1 (group 4, 90 rats), 1 (group 5, 91 rats), or 10 ppm (group 6, 91 rats), in drinking water for 16 weeks. The lowest level, 0.001 ppm, was established with reference to daily exposure of humans to this carcinogen [10]. The animals had free access to Oriental MF diet (Oriental Yeast Co., Tokyo, Japan) throughout the experiment and were killed at the end of week 16 under ether anesthesia for examination of lesion development.

Ten percent phosphate-buffered formalin-fixed liver tissues (a total of 9 slices per animal, 3 each from the left lateral, medial, and right lateral lobes) were embedded in paraffin wax for immunohistochemical examination of GST-P-positive foci in the liver, as described previously [6]. Hepatocellular foci comprising of two and more cells were counted under a light microscope. They were categorized as

Table 1
Final average body, absolute and relative liver weights, and average total NDMA intakes

Groups	NDMA doses (ppm)	No. of rats	Final body weights (g)	Liver		Total NDMA intake (mg/rat)
				Absolute (g)	Relative (%)	
1	0	90	327±15[a]	9.6±0.9	3.0±0.2	0
2	0.001	89	325±17	9.6±0.7	2.9±0.2	0.00151
3	0.01	89	325±16	9.5±0.7	2.9±0.2	0.0145
4	0.1	90	327±18	9.5±0.8	2.9±0.1	0.1505
5	1	91	326±19	9.9±1.0	3.0±0.2	1.5117
6	10	91	315±19	9.1±0.9	2.9±0.2	15.0680

[a] Values are mean±SD.

having a total of 11 and more cells. Total areas of livers were measured using a color image processor (IPAP, Sumica Technos, Osaka, Japan) and the numbers of foci per cm^2 of liver tissue were calculated.

2.3. Statistical analysis

Statistical analysis of the data was performed using the StatView-J 5.0 program (Abacus Concepts, Inc., Berkeley, CA). Differences from control values were evaluated for significance with the Dunnett two-tailed post hoc test.

3. Results

3.1. General findings

All the rats survived in good condition until the scheduled sacrifice. No macroscopic lesions were apparent in any organ including the liver. No adverse effects on average body weight gain were observed in rats treated with NDMA at any of the doses (Table 1). Absolute liver weights were not increased in the groups given NDMA and relative liver weights did not differ among the groups. Average total NDMA intake in each group was dose-dependent.

3.2. Induction of GST-P-positive foci in the liver

In livers of rats treated with NDMA, total numbers of GST-P-positive foci per unit area in the groups receiving 0.001 to 0.1 ppm of the carcinogen did not differ from the control value (non-treatment group, Table 2 and Fig. 1), in contrast to the significant increase observed in rats treated with the 1 and 10 ppm doses. In fact, total values in groups treated with NDMA at a dose of 0.01 ppm showed a slight decrease as compared to the control value. Moreover, numbers of GST-P-positive foci comprising ≥11 cells in the groups given 0.001–0.1 ppm NDMA were not different from the control values, while these values in rats treated with 1 ppm NDMA, and particularly 10 ppm NDMA, were significantly increased.

Table 2
The development of GST-P-positive foci in the livers of rats treated with NDMA at various doses

Groups	NDMA doses (ppm)	No. of rats	Size distribution of GST-P-positive foci (no./cm^2)	
			Total	≥11 cells
1	0	90	0.375±0.545[a]	0.012±0.066
2	0.001	89	0.366±0.586	0.018±0.077
3	0.01	89	0.276±0.582	0.011±0.056
4	0.1	90	0.377±0.519	0.025±0.074
5	1	91	1.905±2.399*	0.117±0.200*
6	10	91	24.875±13.267*	11.063±6.986*

*$P<0.01$ (vs. group 1).
[a] Values are mean±SD.

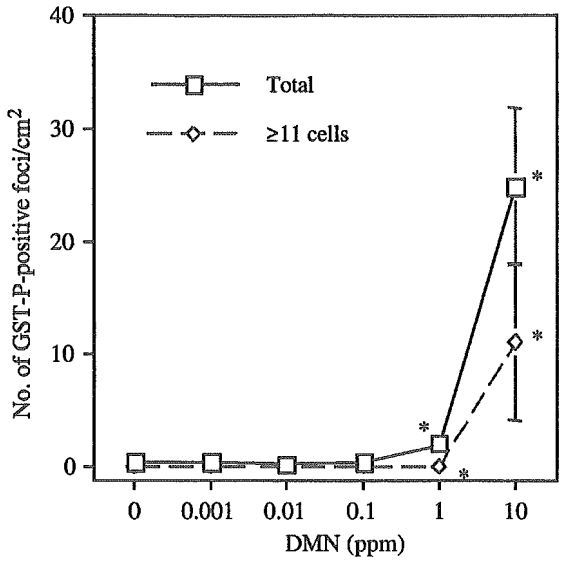

Fig. 1. Induction of GST-P-positive foci in the liver of rats treated with NDMA. *$P < 0.01$ (vs. group 1). Numbers of rats are shown in Table 1. Bars, SD.

4. Discussion

Previously Peto et al. [5] examined the carcinogenicity of NDMA or NDEA at low doses (the so-called ED01 study) and found no indication of any threshold for liver tumor induction of rats. They speculated that NDMA or NDEA at 0.1 ppm in drinking water cause about 2.5% of animals to develop liver tumors and therefore a dose of 0.01 ppm would yield a 0.25% incidence. However, the issue of true and practical thresholds for carcinogenicity has attracted increasing interest [13] and recently Waddell [14] reanalyzed data of the rat carcinogenicity study using NDEA at low doses. His speculation pointed to the existence of a threshold for NDEA carcinogenicity in the liver and esophagus. Recently Williams et al. [9] also suggested the existence of threshold for NDEA hepatocarcinogenicity in rats. In the present study, a dose related and statistically significant increase of GST-P-positive foci in the liver, established endpoint markers for hepatocarcinogenesis in rats [11,12], was obtained with the 1 and 10 ppm doses of NDMN, but the lower doses (0.001, 0.01, and 0.1 ppm) did not cause significant increment in the foci, in line with thresholds found for MeIQx and NDEA previously [6].

Recently we documented that MeIQx and NDEA do not induce GST-P-positive foci in rat liver at very low doses [6]. Moreover, formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG), the most abundant species of adduct associated with oxidative stress, also demonstrated a no-observed effect level. We also reported that the curve for induction of aberrant crypt foci, preneoplastic markers in the colon of rats by 2-amino-1-methyl-6-phenolimidazo[4,5-*b*]pyridine (PhIP) is not linear down to zero [15]. Similarly, no-response levels were evident for both PhIP–DNA adducts and 8-OHdG formation. The present study also clearly indicates that the curve for induction of GST-P-positive foci in the liver is not linear down to zero. Taking all the evidence together, we conclude that genotoxic carcinogens have a threshold, at least in practical terms, for their carcinogenicity.

The question of whether there is a threshold for chemical carcinogenesis, particularly with genotoxic agents is clearly controversial in risk assessment and the non-threshold theory continues to hold sway in the regulatory area for carcinogenic toxicology. However, the findings for a threshold in the genotoxicity of MeIQx [16,17] and the evidence of practical thresholds for genotoxic carcinogenicity from recent in vivo studies including the present result [6–9,15] indicates that this area requires more attention and careful consideration.

In conclusion, the present finding of no-observed effect level on induction of GST-P-positive foci supports our hypothesis that a threshold, at least in practical terms, exists with regard carcinogenesis due to genotoxic agents, from our extensive wide range dose-dependence studies of a variety of carcinogens.

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Review

Current and emerging challenges in toxicopathology: Carcinogenic threshold of phenobarbital and proof of arsenic carcinogenicity using rat medium-term bioassays for carcinogens

Shoji Fukushima*, Keiichirou Morimura, Hideki Wanibuchi, Anna Kinoshita, Elsayed I. Salim

First Department of Pathology, Osaka City University Medical School, Abeno-ku, Asahi-machi 1-4-3, Osaka 545-8585, Japan

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Abstract

For the last 25 years, Prof. Nobuyuki Ito and his laboratory have focused on the development of liver medium-term bioassay system for detection of carcinogens in F344 rats utilizing glutathione *S*-transferase placental form (GST-P)-positive foci as an end point marker. In this presentation, the outline and samples of medium-term bioassay systems were described. Furthermore, our data demonstrated the presence of a threshold for the non-genotoxic carcinogen, phenobarbital (PB), and the lack of linearity in the low-dose area of the dose–response curve, providing evidence for hormesis. In addition, the establishment and applications of multiorgan carcinogenicity bioassay (DMBDD model), used for the examination of the carcinogenicity of genotoxic and non-genotoxic chemicals, are discussed. Dimethylarsinic acid, one of organic arsenics, was found to be carcinogenic in rat bladder using DMBDD model and carcinogenicity test.

Keywords: Medium-term liver bioassay; Hormesis; Multiorgan carcinogenicity test; Arsenics carcinogenicity

Contents

Introduction

Chemicals with carcinogenic potential cannot be completely eliminated from our environment and it is difficult to decrease exposure to safe levels in actual practice; therefore, it is of prime importance to reduce its carcinogenic risk to humans. Thus, carcinogenicity tests in rodents are required for this purpose. However, these tests, particularly in long-term, are very expensive in terms of financial and human resources and are time-consuming. In addition, good animal facilities with experts in toxicology and toxicologic pathology are essential for performing such tests. Accordingly, alternative medium-term screening assays which are very rapid and economical were desired, though their validity is still limited.

* Corresponding author. Fax: +81 6 6646 3093.
E-mail address: fukuchan@med.osaka-cu.ac.jp (S. Fukushima).

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The rat liver medium-term bioassay for chemical carcinogens

Various approaches using liver, skin, mammary gland and urinary bladder as test organs in mice and rats has been introduced for early detection of chemical carcinogens. Among these, several medium-term bioassays appear to offer particular promise. About 60% of known chemical carcinogens were found to exert carcinogenic potential to the liver of rodents. So, the rat liver medium-term bioassay for carcinogens has much advantage among different bioassays. Based on the two-step initiation and promotion concept of liver carcinogenesis, the screening assays have the important advantage of easy detection of the preneoplastic enzyme-altered lesions which are widely accepted as early indicators of neoplastic development. A liver medium-term bioassay system (Ito test) in male F344 rats utilizing glutathione *S*-transferase placental form (GST-P)-positive foci as an end-point marker has been introduced recently (Ito et al., 1991, 2003) (Fig. 1). The system consists of initiation with a single i.p. injection of diethylnitrosamine (DEN) (200 mg/kg b.w.) followed by administration of test compound for 6 weeks from week 2 to week 8 in conjunction with 2/3 partial hepatectomy performed at week 3 to accelerate liver cellular proliferation and subsequently reduce the time needed for the desired effects. Test chemicals are usually given in the diet or drinking water. The numbers and sizes of the GST-P-positive foci which are analyzed using image-analyzer are expressed as values per unit liver section (1 cm^2). When the yield of GST-P-positive foci is significantly enhanced over the control value, a chemical is judged to possess carcinogenenic or promoting potential for the liver. So far, 313 chemicals have been tested (Table 1). Of the liver carcinogens, 97% of the mutagenic chemicals gave positive results, and 88% of the non-mutagenic examples demonstrated positivity. It is particularly important that the system has a very low false-positive rate. Since the two-step liver assay model is based on the induction of preneoplastic hepatocyte lesions, it primarily provides information of as to whether a test compound is carcinogenic for the liver. Moreover, the results obtained in the assays have good correlation with data of 2-year carcinogenicity tests.

Table 1
Positive rates for 313 chemicals of different categories (%)

Category of chemicals	Ames test (%)			Total
	Positive	Negative	Unknown	
Hepatocarcinogen	30/31 (97)	29/33 (88)	1/1 (100)	60/65[a] (92)
Non-hepatocarcinogen	7/26 (27)	2/15 (13)	1/2 (50)	10/43 (23)
Non-carcinogen	0/6 (0)	1/40[b] (3)	0/2 (0)	1/48[b] (2)
Unknown	4/14 (29)	30/86 (36)	14/57 (24)	48/157 (31)
Total	41/77 (53)	62/174 (36)	16/62 (25)	119/313 (38)

[a] Negative; DDPM and 4 peroxisome proliferators, such as clofibrate, and DEHP etc.
[b] Positive.

Phenobarbital carcinogenicity and hormesis

Risk of human cancer is associated with environmental, occupational, and recreational exposure to carcinogens. These carcinogens are usually classified as genotoxic or non-genotoxic, according to their ability to bind to DNA and the form of adducts. However, important cellular mechanisms for defense against genotoxic and non-genotoxic stress are complex and involve many factors that form

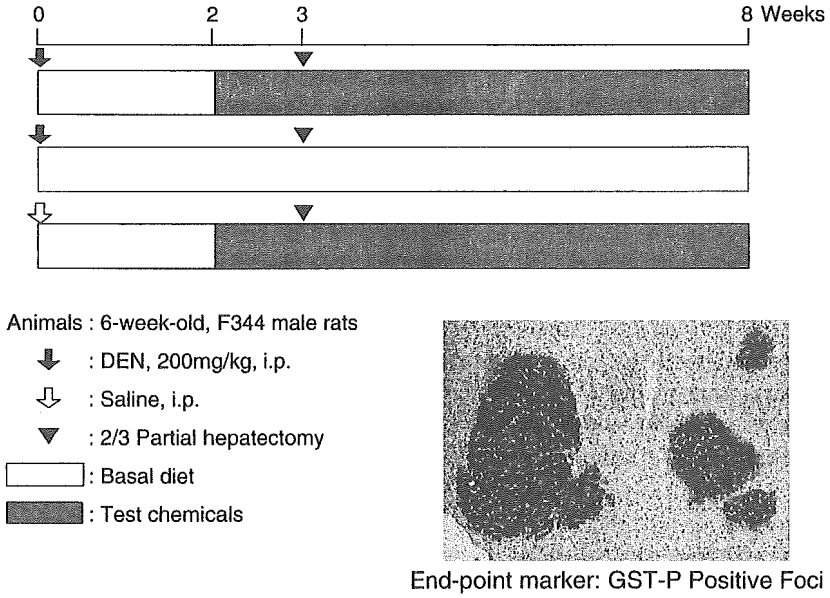

Fig. 1. Rat medium-term liver bioassay for carcinogens (Ito test).