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SHORT COMMUNICATION

Gene-Dose Effect on Carnitine Transport Activity in Embryonic Fibroblasts of JVS Mice as a Model of Human Carnitine Transporter Deficiency

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ABSTRACT. Recently, the marked decline in renal carnitine reabsorption has been thought to account for the systemic carnitine deficiency in juvenile visceral steatosis (JVS) mice. We have conducted a kinetic analysis using embryonic fibroblasts derived from normal, heterozygous, and homozygous *jvs* mice and found that the high-affinity carnitine transporter (K_m = 5.5 μM), which shows Na^+ and temperature dependency and stereospecificity, is defective in homozygous *jvs* mice. Moreover, a gene dose-dependent decrease of carnitine transport activity, which was due to a decrease in the number of the transporter molecules, was found in heterozygous *jvs* mice. Similar phenomena have been observed in human primary carnitine deficiency. Therefore, JVS mice may be useful for understanding this extremely rare human hereditary disorder. BIOCHEM PHARMACOL **55**;10:1729–1732, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. systemic carnitine deficiency; JVS mouse; membrane transporter; embryonic fibroblast; uptake kinetics

L-Carnitine is an essential cofactor in the transport of long-chain fatty acids across the inner membrane of mitochondria, and thus plays an important role in energy metabolism via β-oxidation. Moreover, recent analysis of a carnitine-deficient animal model has implied a further physiological role of carnitine in the secondary regulation of gene expression [1–3].

Human primary carnitine deficiency is a disease with autosomal recessive inheritance, and may be caused by a carnitine transporter defect [4, 5]; however, examination is difficult because the disease is so rare, and no relevant gene has been identified. JVS¶ mice show systemic carnitine deficiency that originates from the recessive mutant gene *jvs* on chromosome 11, and they exhibit symptoms remarkably similar to those of primary or secondary human carnitine deficiency [6–9]. Though the marked decline in renal carnitine reabsorption was thought to be the cause of the systemic carnitine deficiency in an early characterization of JVS mice [10], later investigations demonstrated a defect of carnitine transport activity in fibroblasts from the heart as well [11]. In addition, defects of carnitine transport activity have been found in kidney, skeletal muscle, and skin fibroblasts of human primary carnitine-deficiency patients [4, 12].

In this study, we established cultures of mouse embryonic fibroblasts having the *jvs* gene homo- and heterozygously and examined the uptake kinetics of carnitine in JVS and normal embryonic fibroblasts. Because the high-affinity transport activity was gene dose dependently decreased in JVS cells in a quantitative manner, these cells should be useful as a model for understanding human carnitine transporter deficiency, which is one type of primary systemic carnitine deficiency.

MATERIALS AND METHODS

Animals

JVS mice were originally found among mice of the C3H.OH strain in our laboratory (Institute for Experimental Animals, Faculty of Medicine, Kanazawa University). The autosomal recessive mutant gene *jvs* was then backcrossed into C57BL/6J mice (Clea Japan), and this congenic strain was called "B6-*jvs*." Animals were maintained under semi-barrier conditions at 24.0 ± 2.0° with a 12:12 hr light–dark cycle in compliance with the standards set forth in the "Guidelines for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University." Male and female littermates were housed separately on

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¶ *Abbreviations:* JVS, juvenile visceral steatosis; RAPD, random amplification polymorphic DNA; and HBSS, Hanks' balanced salt solution.

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wood shavings in metal cages and allowed access to laboratory chow and tap water *ad lib*.

Cell Experiments

The uptake of L-carnitine was investigated in primary cultured fibroblasts from homozygous *jvs*, heterozygous *jvs* and normal C57BL/6J embryos at 16–18 days of gestation, or in immortalized or cloned cells originated from the same embryos. The primary cultured fibroblasts from both homozygous *jvs* and normal embryos were serially passaged every 3 days. After 45–55 passages, the cells were used as "immortalized cells" and were cloned by a single colony formation. All mutant, *jvs*/*jvs* and *jvs*/+, and homozygously wild-type (+/+) embryos were produced by mating homozygous mutant female and male, homozygous mutant female and normal male, and normal female and male mice, respectively. The sex of the cells prepared from each embryo was identified using the Y chromosome-specific RAPD marker.

Briefly, 10^5 primary cultured cells or cloned cells were plated in a 2-cm^2 well embedded in a 14-mm diameter plastic disk and allowed to grow to a monolayer in Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum in humidified 5% CO_2 at 37° for 2 days. The medium was replaced with HBSS three times; then 0.5 mL of L-[*methyl*-^{3}H]carnitine (3.7×10^4 Bq/mL, 0.0117 μM) (Amersham) in HBSS was added without serum and with 0–40 mM of unlabeled L-carnitine. After incubation for 30 min at 37° in humidified 5% CO_2, the plastic disk was washed five times with ice-cold PBS and treated with 0.5 N of sodium hydroxide; then the radioactivity of the whole hydrolysate was measured. Data were normalized by protein contents of the cells in distinct wells. The kinetic parameters were estimated by nonlinear least-squares analysis from data of primary cultured heterozygous *jvs*, homozygous *jvs*, and normal cells.

Sodium-ion dependency and stereospecificity of carnitine uptake were also examined using immortalized cells from normal embryos by replacing sodium with potassium in the reaction buffer or using D-carnitine in place of unlabeled L-carnitine.

RESULTS

Carnitine Specific Transporter

Uptake of L-carnitine in immortalized normal mouse embryonic fibroblasts was temperature-dependent and linear for up to 4 hr and had a saturable, substrate concentration-dependent component at 37° (data not shown). This saturable uptake of L-carnitine showed both Na^+-dependence (Fig. 1) and stereospecificity (Fig. 2). These observations indicate that L-carnitine uptake occurs via a carnitine-specific transporter-mediated process in normal mouse embryonic fibroblasts.

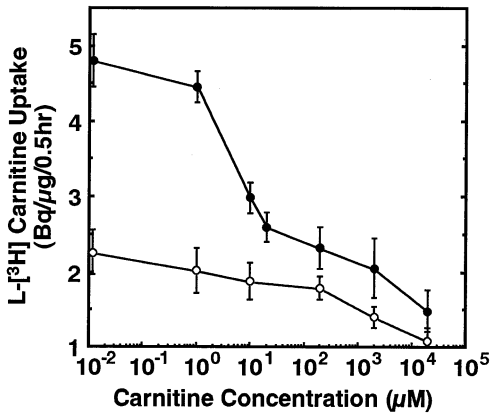

FIG. 1. Na^+-dependent uptake of L-[^{3}H]carnitine by immortalized normal mouse embryonic fibroblasts (passage 45). Cells were incubated with 0.0117 μM of L-[^{3}H]carnitine and with 0–40 mM of unlabeled L-carnitine for 30 min in HBSS with Na^+ (●) or without Na^+ (○) in 24-well plates. L-Carnitine uptake in each well was normalized by protein contents of cells and specific activities of each radiolabeled buffer. Values represent means ± SD (N = 3).

Kinetic Analysis of Concentration-Dependent Carnitine Transport

Carnitine uptake by primary cultured cells derived from normal, heterozygous, and homozygous *jvs* mice was kinetically analyzed by a nonlinear least-squares method. Panels A and B of Fig. 3 show Eadie–Hofstee plots of the carnitine uptake by the three kinds of cells at low concentrations (from tracer to 50 μM) and at high concentrations (from 50 μM to 40 mM), respectively. Cells from normal mice exhibited both high- and low-affinity transport activities, with a K_m for the high-affinity component of 5.5 μM

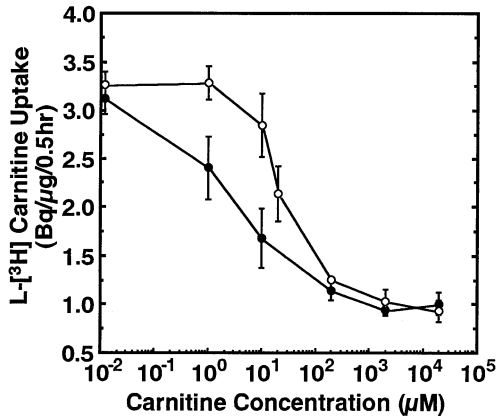

FIG. 2. Stereospecific uptake of L-[^{3}H]carnitine by immortalized normal mouse embryonic fibroblasts (passage 54). Cells were incubated with 0.0117 μM of L-[^{3}H]carnitine and with 0–40 mM of unlabeled L-carnitine (●) or D-carnitine (○) for 30 min in HBSS. L-Carnitine uptake in each well was normalized by protein contents of cells and specific activities of each radiolabeled buffer. Values represent means ± SD (N = 3).

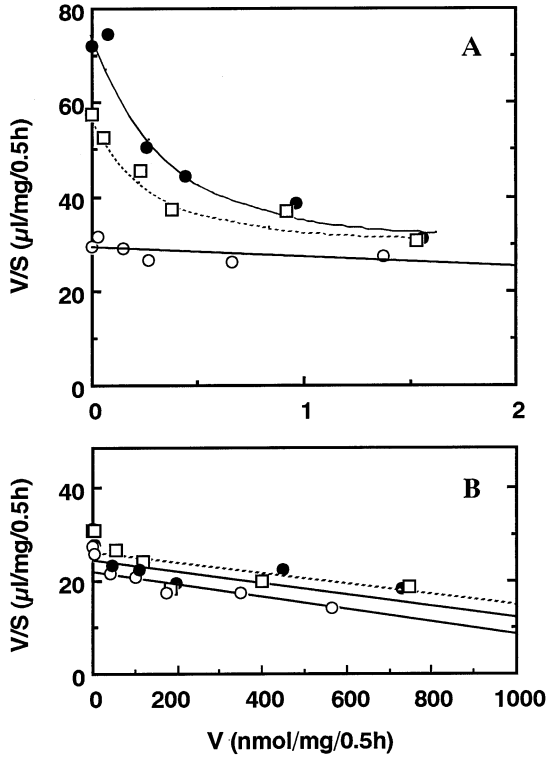

FIG. 3. Eadie–Hofstee plots of concentration-dependent uptake of L-[^{3}H]carnitine by primary cultured embryonic fibroblasts from normal (●), heterozygous *jvs* (□), and homozygous *jvs* (○) mice. Cells were incubated with 0.0117 μM of L-[^{3}H]carnitine and with 0–40 mM of unlabeled L-carnitine for 30 min in HBSS in 24-well plates (N = 4). L-Carnitine uptake in each well was normalized by protein contents of cells and specific activities of each radiolabeled buffer. (A) Plots at low L-carnitine concentrations, from 0.0117 μM (tracer only) to 50 μM. (B) Plots at high L-carnitine concentrations, from 50 μM to 40 mM.

(Table 1). Although the low-affinity component appeared to have a K_m value higher than 40 mM, this could not be evaluated, since the highest concentration examined was 40 mM, which was considered high enough from a physiological point of view. High-affinity transport activity was completely absent in the cells derived from homozygous *jvs* mice at low concentrations of carnitine (Fig. 3A), whereas low-affinity transport activity was comparable with that of the cells from normal animals (Fig. 3B). All of these experiments were performed using cells from male homozygous *jvs* mice, but no sex difference in the lack of saturable transport activity at low concentration was observed (data not shown). In addition, in the process of immortalization and cloning of the cultured cells, the feature of high-affinity transport deficiency was maintained (data not shown). The uptake by the cells from heterozygous animals showed intermediate activity between those of normal and homozygous *jvs* mice at low concentrations, while the low-affinity transport activity was comparable to those of normal and homozygous mice. The kinetic analysis demonstrated that the intermediate high-affinity transport activity can be explained by a decrease of maximum transport activity to 151 pmol/mg/30 min from 256 pmol/mg/30 min in the normal mice without alteration of K_m (5.3 and 5.5 μM in heterozygous *jvs* and normal mice, respectively). These results demonstrate that, although the low-affinity transport component is maintained in both normal and JVS mice, the high-affinity transport activity was gene-dose-dependently decreased in heterozygous and homozygous *jvs* mice through a decrease in the number of transporter molecules (V_{max}), but not the affinity (K_m).

TABLE 1. Carnitine uptake kinetic parameters for primary cultured embryo cells from JVS mice*

Genotype	V_{max} (pmol/mg/30 min)	K_m (μM)	k_d (μL/mg/30 min)
+/+	256 ± 81	5.48 ± 1.66	27.8 ± 2.7
jvs/+	151 ± 64	5.30 ± 2.09	28.5 ± 2.3
jvs/*jvs*	0	—	27.0 ± 0.7

*The kinetic parameters were estimated by nonlinear least-squares analysis and are expressed as means ± SD, $N = 4$.

DISCUSSION

Recently, a prenatal diagnosis of human carnitine transporter defect was attempted in a fetus at high risk of having this disorder [13]. In this and some other case studies of primary carnitine-deficiency patients, the defect of carnitine transport activities showed autosomal recessive inheritance (i.e. the parents or nonaffected brother of affected patients showed transport activities intermediate between patients and control) [14, 15].

The JVS mouse was found as a mutant mouse with a hereditary disorder originating from a single autosomal recessive mutant gene, *jvs*, on chromosome 11; it develops a severe fatty liver a few days after birth, and infantile death occurs before weaning [6]. The JVS mouse was then characterized as a model of human primary and secondary systemic carnitine deficiency, showing symptoms of hyperammonemia, hypoglycemia, and cardiac hypertrophy [7]. Kuwajima *et al.* [11] reported that fibroblasts from the heart of the JVS mouse show a defect of high-affinity carnitine transport. We have confirmed this defect of carnitine transport activity in the present study using embryonic fibroblasts from homozygous *jvs* mice, though the low-affinity transport remained intact.

In this study, primary cultured fibroblasts from heterozygous *jvs* embryos showed a high-affinity carnitine transport intermediate between those of normal and homozygous *jvs* cells. Further, the serum level of free carnitine in heterozygous *jvs* mice (26.4 ± 8.0 μM) was lower than that in normal mice (37.5 ± 13.3 μM) even though heterozygous *jvs* mice showed no symptoms at all ($P < 0.005$). These results support the validity of the JVS mouse as an animal model for human primary carnitine deficiency, especially from the viewpoint of the carnitine transporter defect. It is not clear whether a functionally abnormal molecule is produced by the *jvs* gene, because carnitine itself has a role in the regulation of gene expression [2], and this may be the

cause of the reduced transport activity in heterozygous *jvs*. However, normalizing transcription through carnitine administration, which corrects the suppressed expression of urea cycle enzymes sufficiently, did not correct all the symptoms caused by the carnitine defect in JVS mice [1].

In conclusion, the high-affinity carnitine transport activity (K_m = 5.5 μM) in normal mouse embryonic fibroblasts is Na^+-dependent and stereospecific, and the gene-dose-dependent decrease of the transport activity in JVS mice is due to a decrease in the number of the transporter molecules. The results indicate that the JVS mouse will be a useful animal model not only for studying human primary carnitine deficiency, especially carnitine transporter defect with recessive inheritance, but also for the elucidation of the pathological and physiological roles of carnitine.

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