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A Novel Mitochondrial Carnitine-acylcarnitine Translocase Induced by Partial Hepatectomy and Fasting*

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The carnitine-dependent transport of long-chain fatty acids is essential for fatty acid catabolism. In this system, the fatty acid moiety of acyl-CoA is transferred enzymatically to carnitine, and the resultant product, acylcarnitine, is imported into the mitochondrial matrix through a transporter named carnitine-acylcarnitine translocase (CACT). Here we report a novel mammalian protein homologous to CACT. The protein, designated as CACL (CACT-like), is localized to the mitochondria and has palmitoylcarnitine transporting activity. The tissue distribution of CACL is similar to that of CACT; both are expressed at a higher level in tissues using fatty acids as fuels, except in the brain, where only CACL is expressed. In addition, CACL is induced by partial hepatectomy or fasting. Thus, CACL may play an important role cooperatively with its homologue CACT in a stress-induced change of lipid metabolism, and may be specialized for the metabolism of a distinct class of fatty acids involved in brain function.

Long-chain polyunsaturated fatty acids, such as arachidonic acid and docosahexaenoic acid, are important nutritional components, serving as structural elements in mammalian cells. They confer fluidity, flexibility, and selective permeability to cellular membranes, and affect cellular and physiological processes (1). In addition, long-chain fatty acids are used as an energy source through the mitochondrial β-oxidation pathway, especially in tissues such as muscle, during periods of fasting and other metabolic stress (2).

The carnitine shuttle system in eukaryotic cells provides for the entry of long-chain fatty acids into the mitochondrial matrix, where β-oxidation takes place (3, 4). Acyl-CoA pools supply activated substrates for many key metabolic pathways, such as the tricarboxylic acid cycle and lipid synthesis. A wide range of activated acyl groups is transferred reversibly from acyl-CoA to carnitine through the actions of carnitine acyltransferases. The transfer from the limited pools of membrane-impermeable CoA to the abundant and mobile carnitine allows transport between compartments.

Acylcarnitines are imported into mitochondria through carnitine-acylcarnitine translocase (CACT)[1] (5, 6). This protein catalyzes a mole to mole exchange of carnitines and acylcarnitines, thereby permitting the fatty acid moieties to be translocated into the mitochondrial matrix. Several cases of CACT deficiency have been reported (7–9). Patients with these defects generally present in early infancy with acute, potentially life-threatening episodes of hypoketotic hypoglycemic coma, induced by fasting during intercurrent disease. The clinical features of these patients include hypoketotic hypoglycemia, mild hyperammonemia, variable dicarboxylic aciduria, hepatomegaly with abnormal liver functions, various cardiac symptoms, and skeletal muscle weakness.

In a search for genes that are up-regulated during liver regeneration after partial hepatectomy in rodents, we found a novel gene that encodes a protein homologous to CACT. Here we report the characterization of the gene product, CACL (for carnitine-acylcarnitine translocase-like). The protein exhibits a mitochondrial carnitine-acylcarnitine translocase activity, and its expression is induced by stresses such as hepatectomy and fasting.

EXPERIMENTAL PROCEDURES

Animals—Eight-week-old C57BL/6J male mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Animals were kept in a temperature-controlled animal room with a 12-h dark/light cycle and were maintained on a commercially available diet (CE-2, CLEA Japan Inc.) consisting (by energy) of 29.2% protein, 58.8% carbohydrates, and 12.0% fat. Mice were either fed *ad libitum* or fasted for 48 h, and had free access to water. A 70% partial hepatectomy was performed according to the method of Higgins and Anderson (10). The surgery was performed between 8 and 11 a.m. under ether anesthesia. Animals were sacrificed before partial hepatectomy and at 6, 12, 24, and 48 h after the operation. Hearts, livers, brains, and kidneys were excised, immediately frozen in liquid nitrogen, and stored at −80 °C. All experiments were conducted in accordance with the animal care guidelines of the National Institute for Longevity Sciences (Obu, Japan).

Cell Lines and Culture Conditions—NIH3T3 murine fibroblasts were maintained as monolayer cultures in Dulbecco's modified minimal essential medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (ICN Biomedicals Inc., Aurora, OH), penicillin (100 units/ml), and streptomycin (100 μg/ml; Invitrogen Corp.) at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. The GP2-293 retroviral packaging cell line was obtained from Clontech (Palo Alto, CA) and was maintained as monolayer cultures in Dulbecco's modified essential medium supplemented with 10% heat-inactivated fetal bovine serum. Puromycin (Sigma) was added at a final concentration of 3 μg/ml for the selection of pMXpuro-infected cells.

Construction of Plasmids—A retroviral vector derived from a murine leukemia virus, pMXpuro (11), was kindly provided by Prof. T. Kitamura (University of Tokyo, Tokyo, Japan). Open reading frame regions

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[1] The abbreviations used are: CACT, carnitine-acylcarnitine translocase; CACL, carnitine-acylcarnitine translocase-like; PBS, phosphate-buffered saline; PH, partial hepatectomy; MOPS, 3-morpholinopropanesulfonic acid.

of *mCACT* and *mCACL* were amplified by PCR using EST clones ME624844 and BE372112 as templates, respectively, and cloned into the *Bam*HI-*Bst*XI sites of pMXpuro. For epitope tagging of CACL, a myc-His_6 tag of the pEF4/mycHis vector (Invitrogen Corp.) was inserted immediately before the termination codon of *mCACL* cDNA, and the fusion gene was cloned into the pMXpuro vector.

For the functional expression of *mCACL* in yeast cells, the 1.2-kb *Eco*RI-*Eco*RV fragment of *mCACL* cDNA was inserted into the *Eco*RI-*Pvu*II sites of pKT10 (12). The resultant plasmid, pKT10-mCACL, expressed mCACL driven by promoter of *TDH3*, a gene for glyceraldehyde-3-phosphate dehydrogenase of the budding yeast. The p316CRC1 plasmid was constructed by inserting the yeast gene for CACT (*CRC1*) into the pRS316 vector (13).

For the expression of *mCACL* in *Escherichia coli* cells, the 0.9-kb *Bam*HI-*Xho*I fragment of *mCACL* was isolated from the pMXpuro/mCACL plasmid and inserted into the *BglII-SalI* sites of pBAD/gIIIA (Invitrogen Corp.). The resultant plasmid pBAD/CACL-His_6 encoded a CACL-His_6 fusion protein with the pIII signal sequence at the amino terminus, which allowed the recombinant protein to be secreted to plasma membranes. Expression of the fusion protein was induced by the addition of 0.2% arabinose to the LB medium.

Northern Blotting—Total RNA was isolated with TRIzol reagent (Invitrogen Corp.) according to the manufacturer's instructions. Poly(A)$^+$ RNA was isolated from total RNA with a Micro-FastTrack mRNA isolation kit (Invitrogen Corp.) and used for Northern blotting. Poly(A)$^+$ RNA (5 μg) was size-fractionated on a denaturing gel (1.2% agarose, 3.4% formaldehyde, 1× MOPS), transferred to a nylon membrane (Hybond-N, Amersham Biosciences) by capillary transfer, and fixed using standard techniques. A mouse multiple tissue Northern blot (number 7762-1) was purchased from Clontech. After prehybridization, the filters were probed with a 200-bp *CACL* or a 905-bp *CACT* cDNA fragment. An 857-bp β-actin fragment and a 400-bp β_2-microglobulin fragment of mouse were used as controls. The cDNA probes were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Biosciences) to a specific activity of >0.5 cpm/μg of DNA using the Megaprime DNA labeling system (Amersham Biosciences).

Antibodies—The following antibodies were purchased: anti-His_6 polyclonal antibody (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan), anti-myc monoclonal antibody (clone 9E10, CRP Inc., Denver, PA), anti-p53 monoclonal antibody (Ab-6, EMD Biosciences, Inc., Darmstadt, Germany), and anti-β-actin monoclonal antibody (clone AC-15, Sigma). Sheep polyclonal antibodies against CACT (14) were gifts from Dr. V. A. Zammit (Hannah Research Institute, Ayr, Scotland, United Kingdom). Anti-mouse CACL polyclonal antibodies were produced as follows. A cDNA fragment corresponding to the carboxyl-terminal 31 amino acids of mouse CACL was inserted into the pGEX-4T-2 vector (Amersham Biosciences). Glutathione *S*-transferase-mCACL fusion protein was produced in *E. coli* DH5α cells and was used as an antigen for immunizing rabbits. The antibodies were affinity purified against an MBP-mCACL fusion protein produced in *E. coli*.

Western Blot Analysis—NIH3T3 cells were washed twice with PBS and suspended in a lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% Na deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4. Frozen tissues were disrupted with a Multi-Beads Shocker (Yasui Kikai Co., Osaka, Japan) in 10 volumes of a buffer containing 0.25 M sucrose and 20 mM HEPES, and the homogenate was centrifuged at 600 × *g* for 10 min. The supernatant containing the postnuclear fraction was used as the protein lysate.

The protein lysates were incubated in 2× loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 12% β-mercaptoethanol) at 42 °C for 30 min, separated by SDS-PAGE in a 10% gel, and electroblotted onto polyvinylidene difluoride membranes. To verify the equal loading of proteins in each lane, the blotted membrane was stained with Ponceau-S solution (Sigma). The membranes were blocked in 5% nonfat dry milk in PBS containing 0.1% Tween 20. Immunoblotting was performed with the primary antibodies at dilutions of 1:100 for anti-mCACL, and 1:1000 for anti-CACT and anti-His_6. The incubation with rabbit anti-mCACL antibodies was performed at 4 °C overnight, whereas the incubation with the other antibodies was at room temperature for 1 h. After washing, the membranes were incubated for 60 min with a horseradish peroxidase-conjugated secondary antibody diluted in PBS containing 0.5% nonfat dry milk and 0.1% Tween 20. Blots were developed by enhanced chemiluminescence according to the manufacturer's instructions (ECL Western blotting detection system, Amersham Biosciences).

Immunocytochemistry—Cells were fixed in 4% paraformaldehyde/PBS at room temperature for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were then washed in PBS and incubated for 30 min with a blocking solution of 2% normal goat serum in PBS. Coverslips were then incubated for 1 h with the anti-CACL primary antibody diluted at 1:100 in PBS. After three 15-min washes in PBS, cells were incubated with secondary biotinylated anti-rabbit IgG antibody labeled with green fluorescent Alexa Fluor 488 (Molecular Probes Inc., Eugene, OR) at a 1:500 dilution in PBS for 1 h. Cells were washed again with PBS and mounted on glass slides with Vectashield (Vector Laboratories Inc., Burlingame, CA). To stain the mitochondria, 200 nM of a mitochondrion-specific dye (Mitotracker; Molecular Probes Inc.) was incubated with the cells for 30 min before fixation. Slides were examined on a confocal microscope (BX-FLA, Olympus, Tokyo, Japan) equipped for epifluorescence. Montages of images were prepared using PhotoShop 5.0 (Adobe Systems Inc., San Jose, CA).

Complementation of Yeast Mutation—*Saccharomyces cerevisiae* mutants defective in *CIT2* (for peroxisomal citrate synthase) or *CRC1* (for mitochondrial CACT) were obtained from Open Biosystems (Huntsville, AL). A *Δcit2::kanMX Δcrc1::kanMX* double mutant was constructed by a standard genetic cross. Yeast transformants were selected and grown on minimal medium containing 0.67% yeast nitrogen base without amino acids (YNB-WO, BD Diagnostic Systems, Sparks, MD) supplemented with 0.3% glucose and the appropriate amino acids. Minimal oleate medium contained YNB-WO with amino acids and 0.12% oleate, 0.2% Tween 40 as described previously (15).

Mitochondrial Preparations and Transport Assay—The transport of palmitoyl-[^{14}C]carnitine into mitochondria was measured as described previously (16). Cells grown to confluence on four dishes (15 cm in diameter) were washed twice with PBS and collected by centrifugation at 300 × *g* for 5 min. The cells were resuspended in 2 ml of a homogenization buffer containing 0.25 mM sucrose and 20 mM HEPES, pH 7.5, and were homogenized with 20 strokes in a Potter-Elvehjem (Teflon glass) homogenizer. The homogenate was centrifuged at 1,000 × *g* for 7 min. The resultant pellet was homogenized with 2 ml of the buffer and centrifuged again. The postnuclear supernatant was centrifuged at 2,000 × *g* for 30 min to obtain the mitochondrial fraction. The pellet, which contained ~0.2 mg of mitochondria, was resuspended in 250 μl of assay mixture containing 250 mM mannitol, 25 mM HEPES, pH 7.4, 50 μM EDTA, 3 mM ADP, 1 mM maleic acid, 5 mM potassium phosphate, pH 7.4, and palmitoyl-[^{14}C]carnitine (NEC-667, PerkinElmer Life Sciences)-bovine serum albumin complex (at a ratio of 1:3). Protein concentrations were determined using the BCA protein assay kit (Pierce).

Reconstitution of Carnitine-acylcarnitine Translocase in E. coli Cells—The plasmid pBAD/CACL-His_6 or the control vector pBAD/gIIIA was introduced into the *E. coli* strain Rosetta (F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm lacY1* pRARE(Cm^R); EMD Biosciences, Inc.). The transformants were grown in LB medium containing 0.2% L-arabinose for 18 h at 30 °C. The cultures were diluted with LB, and the transport reaction was initiated by the addition of palmitoyl-[^{14}C]carnitine (NEC-667, PerkinElmer Life Sciences) to the final concentration of 35 μM. After the reaction at 30 °C, cells were washed twice with PBS, and the incorporation of ^{14}C into the cells was quantified using an LSC-5100 liquid scintillation counter (Aloka, Tokyo, Japan).

Statistical Analysis—Data are expressed as mean ± S.D. The statistical significance of the differences between the control and the experimental group was determined by the unpaired Student's *t* test. Differences were considered significant at $p < 0.05$.

RESULTS

Identification of a Novel Protein Homologous to Carnitine-acylcarnitine Translocase—During a microarray analysis for genes induced after 70% partial hepatectomy (PH) in rats, we found a novel gene whose expression peaked at 6 h after the surgery (data not shown). A mouse clone homologous to the rat gene was obtained from the I.M.A.G.E. consortium, and the sequence analysis of the full-length cDNA identified an open reading frame of 918 nucleotides (Fig. 1*A*). The deduced protein, possessing six membrane-spanning regions (Fig. 1*B*), displayed homology with mitochondrial carrier family proteins. The protein showed the highest similarity (37% identity) with CACT, an inner mitochondrial membrane protein that is essential for the import of long-chain fatty acid moieties into the mitochondrial matrix (6). Thus, we designated the protein as CACL. A data base search using the BLAST program revealed that it is a conserved protein whose homologues are present in human, fly, and worm (Fig. 1*A*). The human orthologue

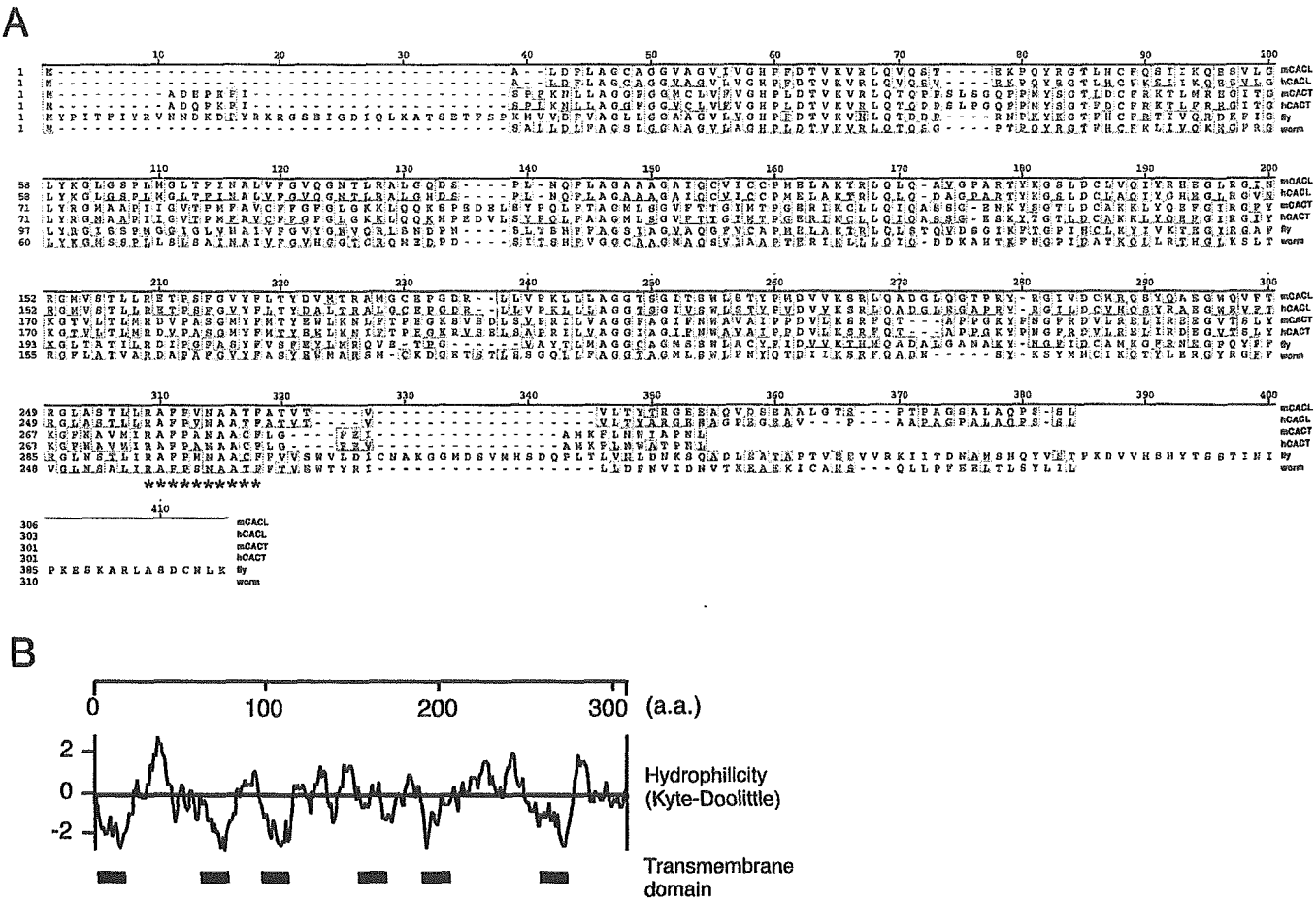


FIG. 1. **A novel CACL in mouse and human.** *A*, sequence alignment of CACLs with CACT. The deduced amino acid sequence of mouse CACL (*mCACL*) shows 37% identity with mouse and human CACT. The human homologue (*hCACL*) is highly conserved (97% identity to mCACL). A data base search identified homologues in *Caenorhabditis elegans* and *Drosophila melanogaster* (C54G10.4 and CG4995, respectively). Amino acid stretches characteristic of carnitine carriers (17) are marked with *asterisks*. *B*, hydrophilicity plot. Hydropathy scores were calculated by the method of Kyte and Doolittle (34). Putative transmembrane regions are shown with *lines*.

(hCACL) has 97% identity with mCACL at the amino acid level and is mapped on chromosome 14q32. A motif within the sixth hydrophobic domain, R(AS)(VF)PANAA(TC)F, has been shown to be conserved within the carnitine carrier subfamily (17). CACL and its homologous proteins all possess the motif, suggesting that they are involved in the transport of acylcarnitine across membranes.

Expression of CACL in Mouse Tissues—The expression pattern in tissues was surveyed using a mouse multiple tissue Northern blot filter. Mouse *CACL* mRNA, ~1.9 kb in length, was expressed in several tissues including heart, brain, liver, and kidney (Fig. 2). This pattern of expression was similar to that of its paralogue *mCACT*, except *CACT* mRNA was rare in brain tissue (Fig. 2*A*). The expression pattern was further confirmed by Western blot analysis using specific antibodies against CACL and CACT. The CACL protein was present at a comparable level in brain, liver, and kidney, whereas CACT expression was barely detectable in brain (Fig. 2*B*). These data suggest that CACL is involved in a biological process similar to that of CACT, but may play a specific role in certain tissues such as brain.

CACL Is Localized to Mitochondria—To determine the subcellular localization of the CACL protein, we constructed NIH3T3 mouse fibroblast cells that stably expressed a CACL fusion protein with a myc-His_6 tag in the carboxyl terminus. Using the anti-myc polyclonal antibody, CACL-myc-His_6 proteins in the fibroblasts were immunostained in a reticulated pattern, which coincided with the mitochondrial staining (Fig. 3, *A–C*). To exclude the possibility that the tag affected the subcellular localization of the protein, we further addressed the localization of CACL using an affinity purified antibody against mCACL. Although we could not detect specific signals of the endogenous protein (data not shown), overexpressed CACL protein without the tag was co-stained with the mitochondrial marker (Fig. 3, *D–F*). Based on these observations, we conclude that CACL, like its homologue CACT, is localized to mitochondria.

CACL Has Palmitoylcarnitine Transporting Activity—In yeast, the transport of acyl units to mitochondria is performed via two pathways, namely the glyoxylate cycle-mediated conversion of acetyl-CoA to succinate that occurs in peroxisomes and the carnitine-dependent acyl-CoA transport. The two pathways have been thought to act in parallel, because disruption of either the *CIT2* gene, which encodes the peroxisomal glyoxylate cycle enzyme citrate synthase, or one of the genes for the carnitine metabolism in mitochondria, did not affect the growth of yeast on oleate, whereas a mutant with both pathways disrupted failed to grow on the plate because of an inability to oxidize the fatty acid (18).

We constructed a double mutant defective in *CIT2* and *CRC1*, the mitochondrial CACT gene in yeast. As reported previously (15), the Δ*crc1* Δ*cit2* mutant could not form colonies on minimal medium containing oleate (Fig. 4*A*), and the defect was rescued by the introduction of the wild-type *CRC1* gene (Fig. 4*B*). Similarly, the heterologous expression of *mCACL* could relieve the growth impairment of the double mutant on

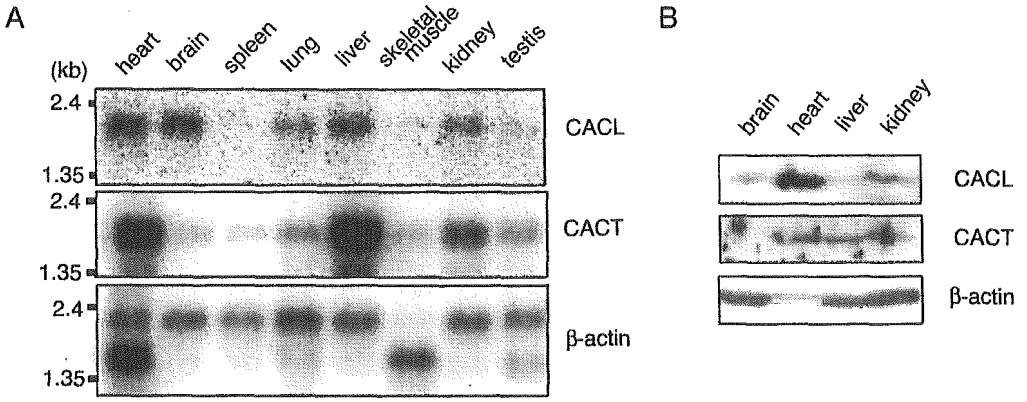


FIG. 2. **Expression of CACL in mouse tissues.** *A*, Northern blot analysis. A multiple tissue Northern blot filter (7762-1, Clontech) was hybridized with a probe corresponding to *mCACL* or *mCACT* cDNA. Each lane contains 2 μg of poly(A)$^+$ RNA. β-Actin cDNA (Clontech) was used as a control probe. Exposure time: 72 h for CACL and 24 h for CACT. *B*, Western blot analysis. An equal amount (100 μg/lane) of the protein lysates from various tissues was separated by SDS-PAGE, followed by Western blot analysis using antibodies against CACL, CACT, and β-actin.

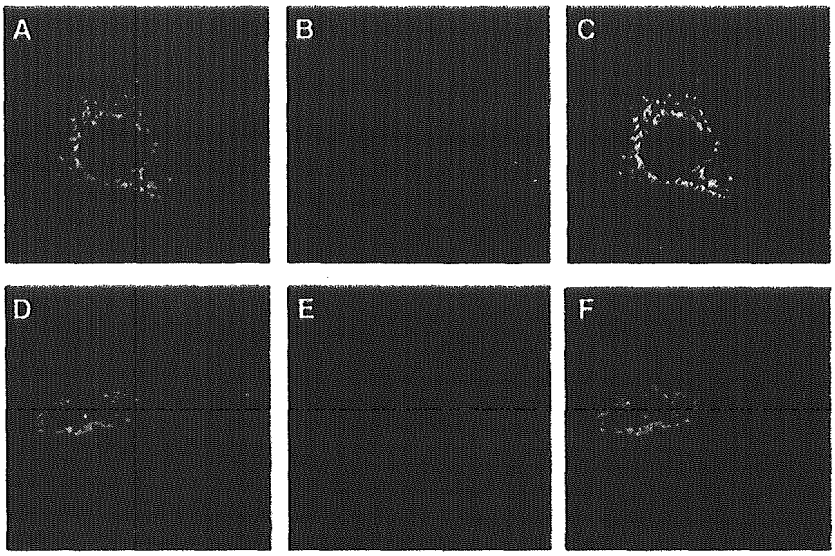


FIG. 3. **CACL is localized to mitochondria.** NIH3T3 cells overexpressing CACL with (*A–C*) or without (*D–F*) the myc-His$_6$ tag at the carboxyl terminus were fixed and subjected to immunostaining using the anti-His$_6$ or the anti-CACL antibody. Confocal images of anti-His$_6$ (*A*) and anti-CACL (*D*) staining (*green*), as well as images of mitochondria staining (*red*; *B* and *E*) with a fluorescent marker Mitotracker (Molecular Probes) are shown. Images were superimposed (*C* and *F*) using PhotoShop 5.0 (Adobe Systems Inc.).

the oleate plate (Fig. 4*C*), suggesting that the CACL protein possesses an enzymatic activity similar to Crc1p.

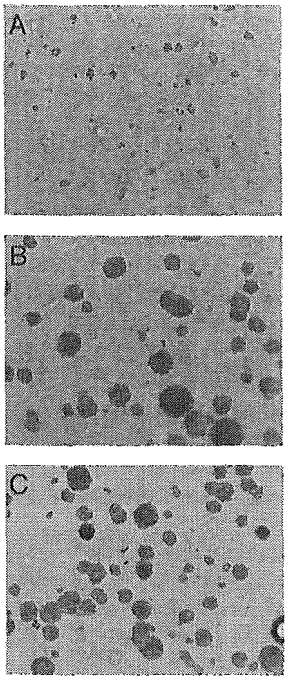
To examine more directly whether CACL possesses activity similar to that of CACT, we performed a biochemical assay for the acylcarnitine transporting activity of mitochondrial fractions using palmitoyl-[^{14}C]carnitine (16). To this end, we constructed NIH3T3 cells in which *CACL* or *CACT* was overexpressed under the control of the retroviral LTR promoter (Fig. 4*D*). As shown in Fig. 4*E*, liberation of [^{14}C]carnitine, which was produced enzymatically from palmitoyl-[^{14}C]carnitine in the mitochondrial matrix, was observed in the mitochondrial fractions harvested from cells infected with a control vector, and the activity was significantly elevated in cells overexpressing either *CACL* or *CACT*.

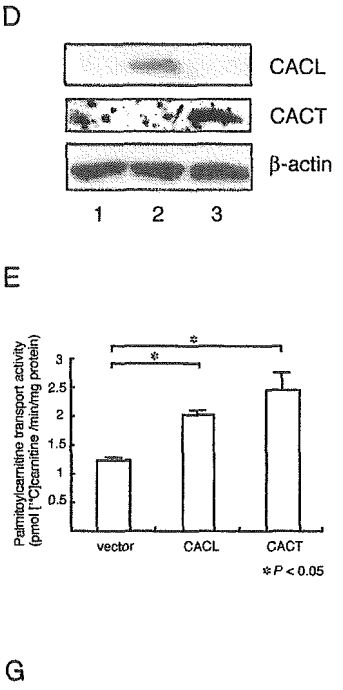
Moreover, we constructed a plasmid for the functional expression of the mCACL-His$_6$ fusion protein in *E. coli*. Cells harboring the plasmid expressed the recombinant protein in an arabinose-dependent manner (Fig. 4*F*), and the expression of mCACL conferred palmitoylcarnitine uptake activity to *E. coli* cells (Fig. 4*G*). Collectively, these data demonstrate that *CACL* indeed encodes a protein with acylcarnitine transporting activity in mitochondria.

Induction of CACL after Partial Hepatectomy and Fasting—As described above, *CACL* was found as a gene whose expression was up-regulated after PH in rats. We addressed whether *CACL* expression was altered in mouse livers after PH.

As shown in Fig. 5*A*, the expression level of the *CACL* transcript in the liver before the operation was low, and was increased at 6–12 h after PH (Fig. 5*A*, *left*). The *CACL* transcript level was slightly increased in sham-operated mice at 6 h (Fig. 5*A*, *right*). Consistently, the amount of the CACL protein was increased at 12 h after PH, whereas the increase was slight after the sham operation (Fig. 5, *B* and *C*). In contrast, a modest increase in *CACT* expression was observed, but its protein level was not increased significantly (Fig. 5, *A–C*). The hepatic surgeries did not affect the protein levels of CACL and CACT in other tissues such as heart (Fig. 5*D*).

Fasting is a stress that is known to cause a metabolic shift to preferentially use free fatty acids. We found that the transcript corresponding to *CACL* was induced markedly in liver after 12 h of fasting (Fig. 6*A*). *CACT* mRNA was also increased under the same condition (Fig. 6*A*). We further examined the amount of CACL protein in several tissues by Western blot analysis using the anti-CACL antibody. As shown in Fig. 6, *B–D*, the protein levels of CACL after fasting were markedly elevated in liver, and increased modestly in heart. In contrast, up-regulation was slight in kidney. We also observed an increase in the amount of CACT in livers and hearts after fasting. These data indicate that the expression of *CACL* and *CACT* is regulated by fasting in a tissue-specific manner and suggest that the induction of these carnitine carriers may contribute to a metabolic change in specific tissues such as the liver.





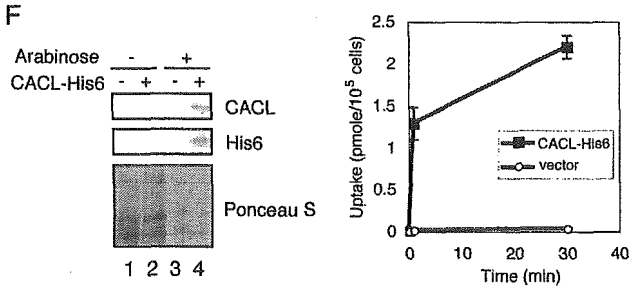
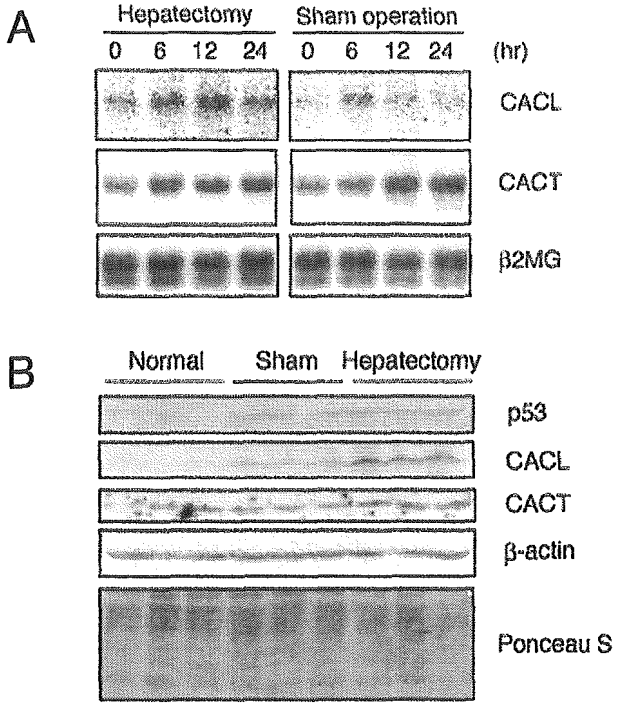


FIG. 4. **CACL possesses acylcarnitine importing activity.** *A–C*, rescue of the growth impairment of the yeast Δ*cit2* Δ*crc1* mutant on oleate by the expression of Crc1p or mCACL. The Δ*cit2* Δ*crc1* cells were transformed with control vector pRS316 (*A*), p316CRC1 (*B*), or pKT10-mCACL (*C*), and the transformants were grown in minimal medium containing 0.3% glucose for 24 h. The cultures were diluted with H_2O and spread onto agar plates of minimal oleate medium. Photographs were taken after incubation for 5 days at 30 °C. *D*, establishment of cells overexpressing *CACL* or *CACT*. NIH3T3 cells were infected with pMXpuro/mCACL (*lane 2*), pMXpuro/mCACT (*lane 3*), or a control vector pMXpuro (*lane 1*), and stable infectants were selected. Cell lysates (100 μg/lane) were analyzed by Western blot analysis using antibodies against mCACL, CACT, and β-actin. *E*, acylcarnitine transport activity in the mitochondrial fraction. Mitochondrial fractions were prepared and used for the acylcarnitine transport assay with palmitoyl-[^{14}C]carnitine (NEC-667, PerkinElmer Life Sciences) as described under "Experimental Procedures." The data presented are average ± S.D. of three independent experiments. *F–G*, functional expression of mCACL in *E. coli*. *E. coli* cells possessing pBAD/CACL-His_6 (*lanes 2* and *4*) or a control vector pBAD/gIIIA (*lanes 1* and *3*) were grown in the presence (*lanes 3* and *4*) or absence (*lanes 1* and *2*) of 0.2% arabinose for 18 h at 30 °C. Lysates were subjected to Western blot analysis using anti-CACL or anti-His_6 antibodies (*F*). In *G*, *E. coli* cells grown in LB + 0.2% arabinose were incubated with palmitoyl-[^{14}C]carnitine, and the incorporation of ^{14}C into cells at 30 °C was measured as described under "Experimental Procedures." The data presented are average ± S.D. of three independent experiments.



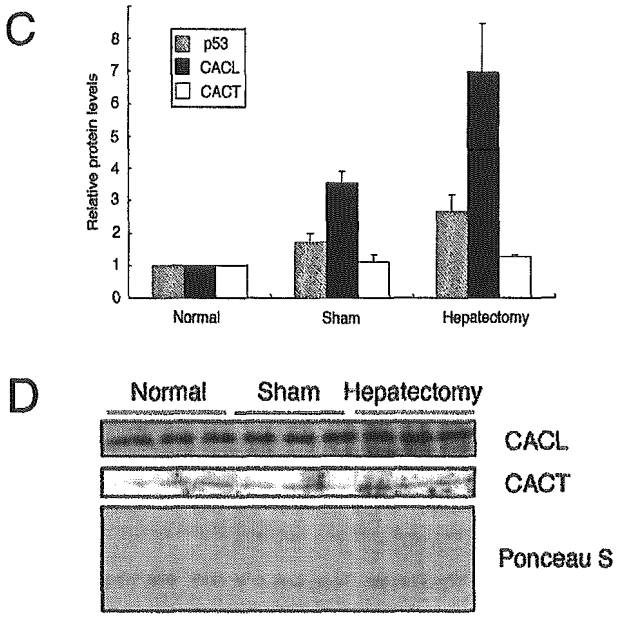


FIG. 5. **Induction of CACL after partial hepatectomy.** *A*, Northern blot analysis. A 70% hepatectomy was performed at time 0, and the remnant regenerating livers were collected at the indicated times after surgery. Livers were also collected from sham-operated C57BL/6 mice. Poly(A)$^+$ RNAs (5 μg/lane) were subjected to Northern blot analysis with *mCACL*, *mCACT*, and β_2-microglobulin cDNA probes. *B* and *C*, Western blot analysis of liver lysates. Livers were collected from three mice without operations and from mice 12 h after the hepatectomy or a sham operation. Postnuclear lysates were subjected to Western blot analysis using antibodies against p53, mCACL, CACT, and β-actin. In *C*, protein levels were quantitatively measured, and normalized -fold induction after the operations was calculated. Values shown are average ± S.D. for three different mice. Note that the protein levels of p53 increased after the partial hepatectomy, as reported previously (35). *D*, Western blot analysis of heart lysates. Hearts were collected at the same time points as in *B*, and postnuclear lysates were subjected to Western blot analysis.

DISCUSSION

In the present study we identified CACL, a novel mammalian protein that is localized to mitochondria and exhibits acylcarnitine transporting activity. The *CACL* transcript was found in tissues such as heart and liver, where its homologue *CACT* was expressed at a high level. In humans, patients with a CACT deficiency exhibited various cardiac symptoms and abnormal liver functions (7–9). Thus, CACL may not be able to compensate for CACT function in fatty acid metabolism of these tissues.

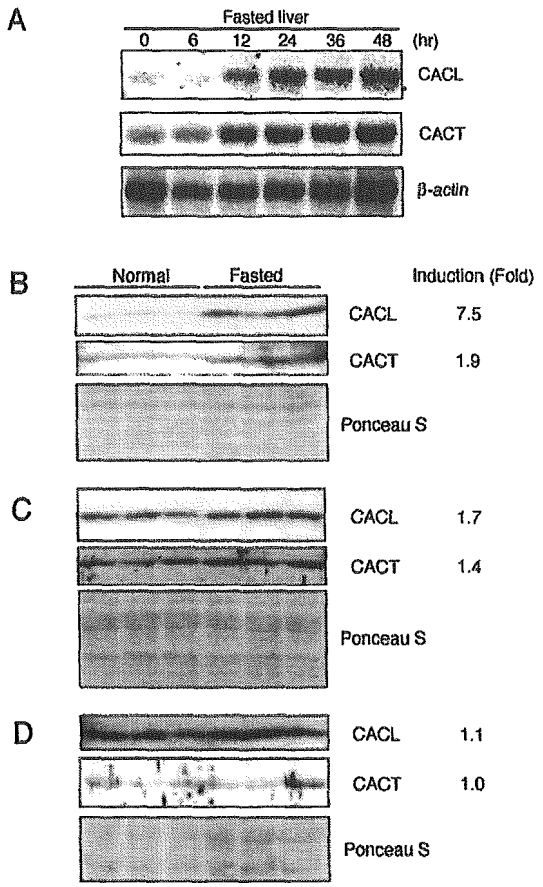


FIG. 6. **Induction of CACL after fasting.** *A*, Northern blot analysis. Mice that fasted for the indicated times were sacrificed, and poly(A)$^+$ RNAs were collected from the livers. Northern blot analysis was performed with probes for *mCACL* mRNA, *mCACT* mRNA, and β-actin mRNA. Each lane contained 10 μg of poly(A)$^+$ RNA. *B–D*, Western blot analysis. Protein lysates were prepared from the tissues of three mice before or after a 48-h fast. Western blot analysis was performed using antibodies against CACL and CACT. Each lane contained 100 μg of protein. Protein levels were quantitatively measured, and normalized -fold induction after fasting was calculated. Values shown are average for three different mice. Tissues examined were as follows: *B*, liver; *C*, heart; and *D*, kidney.

In contrast, the brain is a unique organ in which *CACL* is expressed at higher levels than is *CACT*. Although peroxisomal β-oxidation enzymes are expressed in brain (19), there have been no reports to show that the mitochondrial β-oxidation pathway is operating in the brain. The presence of the *CACL* transcript suggests that acylcarnitine might be used in this organ. The brain contains relatively high amounts of long-chain polyunsaturated fatty acids, such as docosahexaenoic acid, which are critical for its functions (20). It was recently reported that a novel carnitine palmitoyltransferase was expressed specifically in brain and testis (21) and that carnitine transporters on the plasma membrane, which are involved in carnitine uptake, were expressed in brain (22). Together with the carnitine-handling enzymes expressed in brain, CACL might have specialized roles in the metabolism of a distinct class of fatty acids that are involved in brain function.

The liver is a unique organ with a regenerative capacity. After 70% of the mass is surgically removed, the residual hepatic lobes enlarge to restore the original mass within 7 days, and vascularization is completed within the subsequent 7 days (23). A variety of genes are involved in the whole process of liver regeneration, although the molecular mechanisms underlying the process remain unknown. Recently, Su *et al.* (24) reported a microarray-based study of the gene expression profile during the priming phase of liver regeneration in mice. They reported up-regulation of phosphoenolpyruvate carboxykinase and glucose 6-phosphatase, which are involved in maintaining glucose levels after an acute loss of liver mass. Thus, a subtle regulation that switches energy metabolism appears to occur in the regenerating liver.

Carnitine has been thought to be one of the key factors in the regulation of liver regeneration, because the carnitine content in the liver increases after PH (25) and liver regeneration is accelerated by the administration of carnitine to hepatectomized rats (26). In addition, increases in the mRNA levels of carnitine palmitoyltransferase I and II have been observed in regenerating livers (27). Taken together, these results suggest that the carnitine-dependent pathway is important for energy supply when the liver, a major organ critical to maintaining metabolic and biosynthetic homeostasis, is partially removed. In agreement with this notion, we found that the level of CACL protein was elevated after PH. This acylcarnitine carrier may be involved in one of the key steps that regulate cellular metabolism during liver regeneration.

A change in the energy source from glucose to free fatty acids has been widely observed as an adaptive response to fasting (28). In the fasting heart, intracellular droplet accumulation was observed (29), and the content of glycerides and glycogen was increased through the inhibition of the glycolytic pathway and the enhancement of the β-oxidation pathway (30). A recent study using oligonucleotide microarrays revealed that the expression of a wide range of cardiac genes was affected by fasting, including the up-regulation of genes for fatty acid oxidation and gluconeogenesis and the down-regulation of genes for glycolysis (31). We found that *CACL* and *CACT* were up-regulated at the mRNA level in liver, and furthermore, fasting increased the amount of the CACL and CACT proteins in heart and liver. These two organs are prominent in the use of fatty acids upon starvation; fasted cardiac muscles directly use fatty acids as an energy source, whereas hepatic metabolism of fatty acids is mostly directed toward the synthesis of ketone bodies for use as energy sources in tissues such as brain (32). Thus, the up-regulation of CACL and CACT may contribute to the adaptation of the whole body to fasting.

Systemic energy metabolism has been shown to be tightly regulated by the action of hormones, and a disruption of this coordinated regulation causes disorders such as obesity and diabetes (33). Further studies of the coordinating mechanisms of glucose and lipid metabolism in the responsible organs will provide insights for the development of novel approaches to therapy or prevention of these disorders.

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● プラクティカル　フレボロジー

向血栓性素因のスクリーニング検査

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●要　約：名古屋大医学部学附属病院では，術後深部静脈血栓・肺塞栓の予防を目的に，大手術や経大腿動静脈的な血管内操作を受ける患者の向血栓性素因を検査している．測定が容易で臨床的に向血栓性素因とされている，プロテインC，プロテインS，アンチトロンビン，β2グリコプロテインI依存性カルジオライピン抗体に関して概説した．

●索引用語：向血栓性素因，プロテインC，プロテインS，アンチトロンビン，β2グリコプロテインI依存性カルジオライピン抗体

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下肢深部静脈血栓症は，エコノミークラス症候群という名前で頻回にマスコミを賑わすようになって一般の人にもよく知られるようになった．従来，術後下肢深部静脈血栓症による肺塞栓症は術後突然死の一因であった．しかし，欧米に比較して致死的肺塞栓症の頻度が低いとされてきた日本では，末梢血管疾患に興味をもつ者以外には静脈血栓症の予防・治療方法などが知られていなかった．

名古屋大学医学部附属病院では医療安全管理の一環として，術後下肢深部静脈血栓症・肺塞栓症の予防に向けて統一したガイドラインを作成している．本稿では，このガイドラインで採用した向血栓素因のスクリーニング検査の一部を概説する．

遺伝子多型と向血栓性素因

向血栓性素因とは血栓症発症の傾向を高める多くの血液凝固異常を意味する因子である．この凝固異常には先天性(遺伝性)と後天性の場合があり，TransAtrantic Inter-Society Consensus による下肢閉塞性動脈硬化症の診断・治療指針では，1)完全に解明されている遺伝性凝固亢進状態，2)十分に説明されている後天性凝固亢進状態，3)推定上の遺伝性凝固亢進状態に分類されている(**Table**)．さらに先天性血栓性素因は，凝固因子の機能亢進，凝固制御因子の機能不全，線溶系の機能不全に分類される．凝固因子機能亢進は，Factor V Leiden がよく知られているが，これは活性化したプロテインCによる活性化第V因子の分解に抵抗を示して凝固亢進になる状態で，白人の7％ほどにみられる．また，プロトロンビンG20210A多型は，血中のプロトロンビン量が増加し凝固亢進状態となるもので，白人の1～2％に見られる．これらの遺伝子多型は，欧米在住の人種に多く見られるため，日本での血栓性疾患の発生率を欧米と比較すると低い理由であると考えられる．最近，食生活の西欧化により深部静脈血栓症・肺塞栓症が増加してきた，と疾患の増加要因を閉塞性動脈硬化症と同じようにとらえたイントロダクションも見かけるが，魚肉由来の蛋白・油の摂取から獣肉由来のものに変わってきた食生活の変化が一因と考えられる閉塞

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Table　Type of hereditary and acquired hypercoagulable states

Well-proven hereditary hypercoagulable states
Factor V Leiden mutation (APC resistance)
Protein C deficiency
Protein S deficiency
Antithrombin deficiency
Dysfibrinogenemia
Prothrombin mutation
Well-described acquired hypercoagulable states
Antiphospholipid syndrome
Cancer-related (Trousseau's) thrombosis
Thrombosis associated with inflammatory bowel disease
Antithrombin deficiency due to low-protein states
Hyperhomocysteinemia
Presumed inheritable hypercoagulable states
Plasminogen deficiency
Anbormal plasminogen
Plasminogen activeter-1 deficiency
Factor XII deficiency
Abnormal platelet reactivity

性動脈硬化症と，深部静脈血栓症の原因を混同してはならない．ちなみに，上記のどちらの遺伝子多型も日本人に見つかったという報告は，今のところない．

一方，日本人に多く報告されているのは様々なプラスミノーゲン異常症である．これによる向血栓性素因は比較的弱いと考えられているが，この異常は日本人の３～４％に見られる．

向血栓性素因のスクリーニング検査として遺伝子多型を解析することは効率的ではないので，我々は問診等により向血栓性素因が疑われる患者や下肢深部静脈血栓症発生のおそれが高い処置を受ける患者には，プロテインC活性，プロテインS活性，アンチトロンビン活性，および抗カルジオライピン-β2 糖蛋白抗体を測定することとした．つまり，凝固制御に関して重要な役割を果たしているプロテインC/プロテインS系とトロンビン/アンチトロンビン系の，また後天性凝固亢進状態をもたらす抗リン脂質抗体症候群の(一部の)スクリーニングとなる．プロトロンビン時間や活性化部分トロンボプラスチン時間は一般的血液凝固試験として施行している．

プロテインC/プロテインS系による凝固制御

凝固系が活性化されることでプロトロンビンから切り出されたトロンビンは，凝固カスケードの下流でフィブリノーゲンをフィブリンに変換し血液凝固を起こすとともに，カスケード上流の第Ｖ因子，第VIII因子，第XIII因子を活性化することで，さらに凝固を促進させる．しかし，このトロンビンが血管内皮細胞表面のトロンボモジュリンと結合すると，このトロンビンは血小板を活性化したり，フィブリノーゲンをフィブリンに変換する機能を失い，選択的にプロテインCを活性化させ，活性化プロテインCに変換する．この活性化プロテインCは，血小板や血管内皮のリン脂質膜表面で進行する活性化第V因子，活性化第VIII因子を分解し失活させる．遊離型のプロテインSはこのときに補酵素としてプロテインCによる活性化凝固因子の分解・失活を促進させる．また，プロテインSには単独で活性化第Ｖ因子，活性化第X因子に結合して，活性型第V因子/活性型第X因子/リン脂質/カルシウム複合体の形成を阻害し，凝固を抑制する機能もある．

なお，プロテインCは肝，プロテインSは肝，血管内皮，巨核球などで合成されるビタミンK依存性蛋白である．

プロテインC欠損症

先天性プロテインC欠損症は常染色体性優性の遺伝形式を示す．病型はプロテインCの抗原量，活性値ともに低下しているものをType I，抗原量は正常で活性値のみ低下しているものをType IIと呼ぶ．ホモ接合体のプロテインC欠損症では，新生児期に全身の出血性梗塞を生じ(電撃性紫斑病)予後不良とされている．ヘテロ接合体は0.06～0.3％との報告がある．ヘテロ接合体では健常人に比し，深部静脈血栓症発症の危険は７倍とするものもいる．また，深部静脈血栓症患者の5.4％にプロテインC欠損症を認めたとの報告もある．

プロテインS欠損症

先天性プロテインS欠損症は常染色体性優性の遺伝形式を示す．プロテインSの60％は補体系制御蛋白のC4b結合蛋白と結びついていて，残りの40％がプロテインCの補酵素として機能する．先天性プロテインS欠損症の多くは，総プロテインS(抗原量)の大部分がC4b結合蛋白と結びついて機能しないと考えられている．そこで，抗原量はほぼ正常だが遊離型プロテインSが

著明に低下しているものをType I，抗原量は正常で活性値のみ低下しているものをType IIと呼ぶ．ホモ接合体のプロテインS欠損症も，プロテインC欠損症と同様に新生児期に血栓症により死亡するとされている．ヘテロ接合体の頻度はよく知られていないが，プロテインC欠損症より多いとされている．ヘテロ接合体では健常人に比し，深部静脈血栓症発症の危険は6倍とするものもいる．また，深部静脈血栓症患者の12.7%にプロテインS欠損症を認めたとの報告もある．

アンチトロンビンによる凝固制御

アンチトロンビン(AT)は，トロンビン(活性化第II因子)だけではなく活性化第X因子，活性化第IX因子，活性化第XII因子，活性化第XI因子，活性化第VII因子－組織因子複合体などと安定した複合体を形成することで，凝固反応の進行を阻害する．よく知られているように，AT分子のヘパリン結合部位にヘパリンが結合すると，この複合体形成反応は速やかに進行し，凝固反応の進行が即時に阻害される．なお，ATも肝で合成されるビタミンK依存性蛋白である．

AT欠損症

先天性AT欠損症は常染色体性優性の遺伝形式を示す．AT欠損症では若年(35歳以前)での血栓症発症が多く報告され，80～90%の患者で60歳までに血栓症を経験すると言われる．病型はATの抗原量，活性値ともに低下しているものをType I，抗原量は正常で活性値のみ低下しているものをType IIと呼ぶ．ホモ接合体のAT欠損症は致死的で，出産に至らない．ヘテロ接合体は0.02～1.1%との報告がある．ヘテロ接合体では健常人に比し，深部静脈血栓症発症の危険は5倍とするものもいる．また，深部静脈血栓症患者の1.8%にAT欠損症を認めたとの報告もある．

β2グリコプロテインI依存性カルジオライピン抗体

抗リン脂質抗体症候群は，現在はSapporo Criteriaによって一定の診断基準が示されているが，様々な疾患名が混在した経緯がある．梅毒患者では抗カルジオライピン抗体が出現するが，これは，他の感染症や自己免疫疾患でも出現し，その場合には生物学的偽陽性と呼ばれる．生物学的偽陽性が診断基準の一つであるSLE患者血漿中の凝固阻害物質としてLupus (like) anticoagulantが発見され，リン脂質に対する抗体であることが明らかにされた．Lupus anticoagulantは定量的検査が困難であったが，抗カルジオライピン抗体はRIAやELISAで直接測定可能になった．抗カルジオライピン抗体が高値の患者では，血栓症，流産を生じ，Lupus anticoagulant陽性者が多いことから抗カルジオライピン症候群の概念が作られた．その後，この抗体は陰性荷電をもつ他のリン脂質と反応することが明らかになり，抗リン脂質抗体症候群と呼ばれるようになった．

現在の抗リン脂質抗体症候群の診断基準は，検査上β2グリコプロテインI依存性カルジオライピン抗体またはLupus anticoagulantのいずれかを有し，臨床症状として動静脈血栓症または流産・不育症をきたすものである．

抗リン脂質抗体症候群での血栓発生機序は，血小板の活性化，内皮細胞機能の障害，単球を介する組織因子活性，抗β2グリコプロテインI抗体による作用，血漿蛋白に対する作用など，様々な機序が報告されているが，今後の研究結果が待たれる．

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Abstract

Clinical Screening Test for Thrombophilic Predispositions

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Key words : Thrombophilia,, Protein C, Protein S, Antithrombin, Anti cardiolipin- β2- Glycoprotein

Prevention of deep vein thrombosis (DVT) is a key to avoid pulmonary embolism (PE), that is a postoperative fatal complication. Number of patient with postoperative DVT and PE is increasing in Japan with a wide recognition of its pathophysiology.

Screening test is important to find out patients with a thrombophilic predisposition; Protein C deficiency, Protein S deficiency, Anti-thrombin deficiency and positive anti cardiolipin- β2- Glycoprotein 1 antibody. These were examined routinely before major surgery and femoral catheter procedure in Nagoya University Hospital. This paper is a brief summary of these thrombophilic predispositions.

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4. 高齢者食道癌手術の術中・術後管理

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はじめに

高齢者においては一般に加齢に伴う臓器機能の低下による生理的予備能の低下が認められる．検査所見上は正常値を示す場合でも，食道癌根治術のような大きな侵襲が加わると臓器障害が顕在化することも少なくない．また，高齢であればあるほど臓器機能には個人差が大きく，暦年齢だけで判断せず，綿密な術前評価が必要である．近年，集学的治療の導入が進んでいる食道癌治療においては，手術療法のみに固執することなく病期，全身臓器機能，既往症，社会的背景などを考慮した柔軟な適応決定を行うことも重要である．また，手術療法を選択した場合にも少しでも低侵襲な術式を模索し，根治性とのバランスを考慮しながら術式を工夫するなど柔軟に対応し，予想される合併症に対する予防措置，合併症発症の早期発見，迅速な対処が重要である．

1 高齢者の定義

何歳以上をもって高齢者とするかの確定した定義はない．一般には75歳をもって一定の目安とする施設が多いようである．しかし，高齢であればあるほど，微小血管の老化，臓器障害，既往症などにおいて個人差が大きく，あくまで個々の症例において術前機能評価を行うべきである．75歳以上の症例ではデータ上リスクがなくとも潜在的な臓器予備能の低下が潜んでいる可能性を考えるべきである[1]．

[表1] 高齢者食道癌手術術前評価項目

- performance status
- 精神障害の有無（老人性痴呆など）
- 喫煙歴，慢性呼吸器疾患の既往
- 呼吸機能（1秒量 1.5l 以上，PaO_2 60mmHg 以上，肺内シャント率 15%以下，respiratory index 2 以下）
- 心機能（ECG，負荷 ECG，ホルター ECG，心エコー）
- 腎機能（1日尿量 600ml 以上，尿比重 1.010～1.030，血清 BUN 25mg/dl 以下，血清クレアチニン 2.0mg/ml 以下）
- 肝機能（ICG 15 分値）

1. 術前評価と手術適応

根治術の適応を判断するうえでperformance status（PS）の評価，老人性の痴呆など精神障害の有無をチェックする必要がある．総合的に評価して，大きな手術侵襲を加えたのちにこれに見合うPSの改善が望めない，あるいはむしろ低下する危険があるようであれば手術以外の治療法を考慮すべきである．

食道癌術後において手術死亡の原因となる可能性の高い合併症である肺合併症に直結する呼吸機能の評価は最重要である．フローボリュームカーブをチェックし，特に1秒量低下例（1.5l以下）では術後の喀痰排出能が不良であり，無気肺，術後肺炎の発症の危険が高い．加齢，慢性呼吸器疾患による低酸素血症，肺内シャント率の増加もみられる．PaO_2 60mmHg以上，肺内シャント率15%以下，respiratory index ($RI = AaDO_2/PaO_2$) 2以下が目安となる[2]．

循環器系では術前心電図，負荷心電図，ホルタ

一心電図，心エコーにより虚血性心疾患，不整脈の存在をチェックする．

加齢により腎血流は低下し，尿細管機能も低下する．1日尿量600m*l*以上，尿比重1.010～1.030，血清BUN 25mg/d*l*以下，クレアチニン2.0mg/m*l*以下が安全限界である．加齢による肝機能障害は他の重要臓器に比して少ない[2]．

1 合併症予防策

術直前に施行可能な予防策の中心は呼吸器系合併症予防のため呼吸訓練である．十分な禁煙期間をとり，去痰剤，気管支拡張剤を使用する．

また，術前からの精神機能の把握は重要である．高齢者は術後せん妄をきたしやすく，食道癌手術ではその頻度も高い．術前から治療内容や術後集中治療室での状況などについて理解を得ておく必要がある．しかし，術後せん妄の出現は高頻度であり，術前から精神神経科専門医の診断を仰ぎ，術後もその管理下とすることが望ましい．

2 術式選択の工夫

従来，耐術能不良の高齢者，特に低肺機能例では開胸に伴う侵襲を軽減する目的で非開胸食道抜去術を選択することもあった．しかし，この術式は，リンパ節郭清を施行しないために姑息手術となる反面，胸部食道を切除する術侵襲は無視できず化学放射線療法や放射線療法を選択する施設も増えその適応は限られてきている．近年，胸腔鏡，腹腔鏡補助下の食道癌根治術が普及してきている．器材，技術の改良によりリンパ節郭清の質も向上し，術時間も短縮してきている．体壁破壊が軽減されるため術後の疼痛は軽減され，呼吸機能に関しては通常の開胸開腹術に比して有利であると考えられる．今後高齢者を対象とした手術にも応用されていくことが予想される．

2. 高齢者手術の麻酔，術中管理

全身麻酔と硬膜外麻酔に代表される局所麻酔の利点を生かし両者を併用する[3]．吸入麻酔薬は一般に強い循環抑制を起こすため使用は最低限にとどめる．術中は大幅な血圧変動を極力避け，術前血圧の80%程度とする．術中輸液は尿量1m*l*/kg/hrを目安とするが，輸血量とともに過剰とならない注意が必要である．輸血は出血実測値の60%程度を目安にする．血液粘度の上昇による末梢循環障害を考慮してヘマトクリット値は35%以上とならないよう留意する．高齢者の場合筋弛緩薬による遅発性呼吸抑制の恐れもあり，術後低酸素血症を予防する目的でオーバーナイトで人工呼吸管理を行うことが多い．

また，高齢者では，体温調節機能の低下により低体温，これによるアシドーシスを呈することがあり十分な加温が必要である．

3. 術後管理

1 呼吸管理

高齢者に限ったことではないが，上縦隔郭清により気管周囲のリンパ流，血流が途絶し，気道内分泌物の排出能は低下し，微小無気肺が生じやすい．手術侵襲により体液は3rd spaceに移動し，肺間質水分量も増加する．術前肺機能低下傾向にある高齢者では術直後より低酸素血症を呈しやすい．これを防止し，かつ十分な循環血液量を確保するための安全な輸液管理を行うためには，陽圧換気を併用した人工呼吸器による呼吸管理を行う．翌日以降，血液ガスデータ，喀痰の量・性状，胸部X線所見を参考に，鎮静剤の投与を漸減し呼吸器よりの離脱を図る．高齢者では呼吸筋の疲弊や，鎮静剤効果の遷延の恐れもあり，抜管後も自発呼吸，喀痰排出能を厳重に監視する．気管穿刺による喀痰除去は有用である．術直後挿管中より頻回に気管支鏡を施行し喀痰の性状，量をチェックし，肺炎発生時に備えて喀痰の監視培養を施行する．

2 輸液管理

術後体液変動，輸液管理の基本方針は高齢者といえども通常と同様である．しかし，高齢者では体内総水分量，細胞内液が減少しており，術侵襲

Knack & Pitfalls

◎高齢者の臓器機能には個人差が大きく，暦年齢だけで判断せず綿密な術前評価が必要である．

◎術後合併症として術後肺炎，せん妄などの頻度が高いが特に前者は予後にかかわる重要な因子となりうる．

◎各種臓器予備能，体液変動に対する緩衝能は低下しており，リスクに応じて手術侵襲自体を軽減する工夫が必要である．

に伴う細胞外液量，浸透圧の変動，電解質の変化に対する緩衝作用は低下している．したがって循環動態の変化を的確かつきめ細かくチェックする必要がある．すなわち輸液量が不適正であった場合，心肺にかかる負荷が大きい．第3～4病日のreilling stateにおいては肺水腫となりやすい．一方，極端に輸液量を減らすと腎血流の低下から腎機能低下を招いたり，血液粘度の上昇に伴う血栓性の病変を合併することもある．このように術後管理上の許容範囲が狭いのが高齢者における特徴である．

4. 高齢者において留意すべき術後合併症

1 術後せん妄

術後一過性の精神障害，特に術後せん妄は高齢者に多くみられる．麻酔時間の長い手術，代謝変化の大きな手術でその発症頻度は高く，食道癌手術はその代表的なものの一つである．鎮静剤，睡眠剤などの薬剤療法を施行するが通常数日以内で軽快し，後遺症を残すことはない．脳血管障害などの器質的な変化による精神症状との鑑別を要する．

この時期，ドレーン類などの自己抜去による事故に注意する．

2 誤嚥性肺炎

食道癌手術では，上縦隔郭清により反回神経麻痺を生ずる危険もあり，また明らかな反回神経麻痺を合併していない症例でも頸部操作により嚥下機能が一過性に低下している場合がある．高齢者ではこの傾向が強く摂食開始後の誤嚥性肺炎には十分留意する．

おわりに

高齢化社会を迎え，自ずと高齢者食道癌症例を取り扱う機会も増えよう．個々の症例の耐術能を考慮して，手術療法以外の選択肢も視野に入れた柔軟な対応が求められる．また，手術症例においても少しでも手術侵襲を軽減する工夫が重要である．また術中，術後管理においては臓器予備能が少ないことによる許容範囲の狭さを認識し，厳重かつ繊細な観察，対処が肝要である．

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The function of GADD34 is a recovery from a shutoff of protein synthesis induced by ER stress: elucidation by GADD34-deficient mice[1]

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SPECIFIC AIMS

GADD34 is a member of the protein family whose expression is up-regulated by growth arrest and DNA damage, but its function has not yet been elucidated in vivo. We tried to find the function of GADD34 by constructing GADD34-deficient mice.

PRINCIPAL FINDINGS

1. GADD34 is highly expressed at the fetal stage and in adult mouse organs

At the fetal stage of C57BL/6CR mice, GADD34 mRNA expression was detected on embryonic day 8.5 (E8.5), E9.5, E12.5 (especially strong), E14.5, and E18.5. Almost no expression was detected on E10.5, E11.5, and E13.5. The distribution of GADD34 was ubiquitously expressed on E12.5; it is expressed at high levels in the brain, spinal cord, tongue, lung, and genital tubercle. In adult mice, GADD34 expression was strong in the spleen and lung, moderate in the thymus and muscle, weak in the brain, and almost undetectable in the other organs examined (liver, small intestine, testis, and ovary).

2. GADD34 knockout mice show normal development and normal fertility

To elucidate the role of GADD34 in vivo, we generated GADD34$^{-/-}$ mice. We replaced a portion of GADD34 including a translation initiation codon with the neomycin resistance gene from pMC1-neo (Stratagene, San Diego, CA, USA). Matings between GADD34 heterozygous mice yielded the expected frequency of wild-type, nullizygous (GADD34$^{-/-}$), and heterozygous (GADD34$^{+/-}$) offspring (n=176; 44, 46, and 86, respectively). These results demonstrate that GADD34 deficiency has no severe effects on normal mouse development. GADD34$^{-/-}$ mice did not show abnormal phenotypes or signs of disease during the first 12 months of life.

3. GADD34 is strongly induced in response to endoplasmic reticulum (ER) stress

Previous studies demonstrated that rodent GADD34 was inducible by treatment of cell lines with various DNA-damaging agents such as alkylating agent and that it was mediated by some of the agents causing unfolded protein response (UPR). Tunicamycin (Tm) inhibits asparagines (N) -linked glycosylation and dithiothreitol (DTT) disrupts disulfide bond formation. Thapsigargin (Tg) is an irreversible inhibitor of the Ca^{2+} ATPase transporter known to rapidly activate the ER stress response. We investigated the levels of GADD34 expression by these ER stress-inducible agents. Tm induced GADD34 in a dosage-dependent manner, whereas Tg and DTT induced GADD34 at >0.05 μM and 5 mM, respectively, with levels remaining unchanged. All three ER stress-inducible agents elevated the level of GADD34 in a time-dependent manner.

4. GADD34 is required for eukaryotic translation initiation factor 2α (eIF2α) dephosphorylation and recovery from a shutoff of total protein synthesis in response to ER stress

The COOH terminus of GADD34 is homologous to the corresponding domain of the herpes simplex virus-encoded protein $\gamma_1$34.5. Both $\gamma_1$34.5 and GADD34 interact with PP1 and dephosphorylate eIF2α. To determine whether the expression of GADD34 affects eIF2α phosphorylation levels, we compared those levels of GADD34$^{+/+}$ MEFs treated with ER stress-inducing

[1]To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.02-1184fje; doi: 10.1096/fj.02-1184fje

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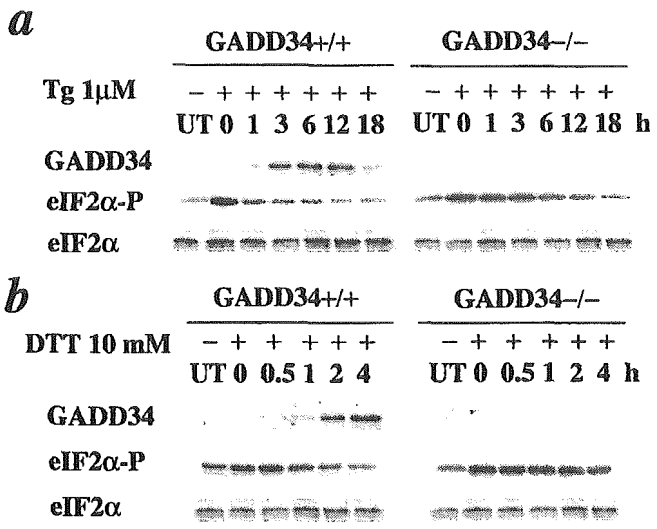

Figure 1. Phosphorylation of eIF2α induced by Tg or DTT is reversed by GADD34. GADD34$^{+/+}$ MEFs or GADD34$^{-/-}$ MEFs were treated with 1 μM of Tg (*a*) or 10 mM of DTT (*b*), washed off, and changed to fresh medium at 0.5 h. Total cell extracts were taken from these cells at different times after treatment. Shown are immunoblots with a rabbit polyclonal antibody against GADD34 and eIF2α phosphorylated on Ser51 and a goat polyclonal antibody that recognizes phosphorylated and unphosphorylated eIF2α.

agent with those of GADD34$^{-/-}$ MEFs. In wild-type MEFs, the eIF2α phosphorylation level was temporarily increased just after treatment with Tg or DTT and promptly decreased, whereas in GADD34-deficient MEFs eIF2α is strongly phosphorylated and sustained a high level of phosphorylation (**Fig. 1*a*, *b***). By contrast, after treatment with tunicamycin, the eIF2α phosphorylation level did not change in either GADD34$^{+/+}$ or GADD34$^{-/-}$ cells. Phosphorylation of eIF2α reduces its functional level and limits initiation translation on all cellular mRNAs within the cells. To clarify whether strongly phosphorylated eIF2α in Tg- or DTT-treated GADD34$^{-/-}$ MEFs affects translation, we examined the new protein synthesis rates in the treated MEFs. In both genotypes of MEFs, protein synthesis was severely reduced just after Tg treatment. Protein synthesis gradually recovered in GADD34$^{+/+}$ MEFs but remained at lower levels even after 12 h of Tg treatment in GADD34$^{-/-}$ MEFs (**Fig. 2*a***). By treatment with another ER stress agent, DTT, GADD34$^{-/-}$ cells also kept protein synthesis at a low level until 2 h after treatment (Fig. 2*b*). These results indicate that highly phosphorylated eIF2α in GADD34$^{-/-}$ MEFs leads to a reduction of newly synthesized protein.

5. GADD34 affects expression of BiP/GRP78 (binding Ig protein/glucose regulated protein of molecular weight 78 kDa) and CHOP (C/EBP homologous protein)/GADD153 in response to ER stress

Bip/GRP78 and CHOP/GADD153 have been shown to be downstream target proteins in ER stress responses. Bip, a classical marker for UPR activation, acts as a chaperone in the UPR. CHOP has been reported as an ER stress-associated apoptosis factor. In the case of ER stress, eIF2α are phosphorylated and subsequently ATF4 (activating transcription factor 4) induced by phosphorylated eIF2α. Transcription of Bip and CHOP are both activated by ATF4 through binding their amino acid response element. We analyzed ATF4, Bip, and CHOP expressions in GADD34-deficient MEF un-

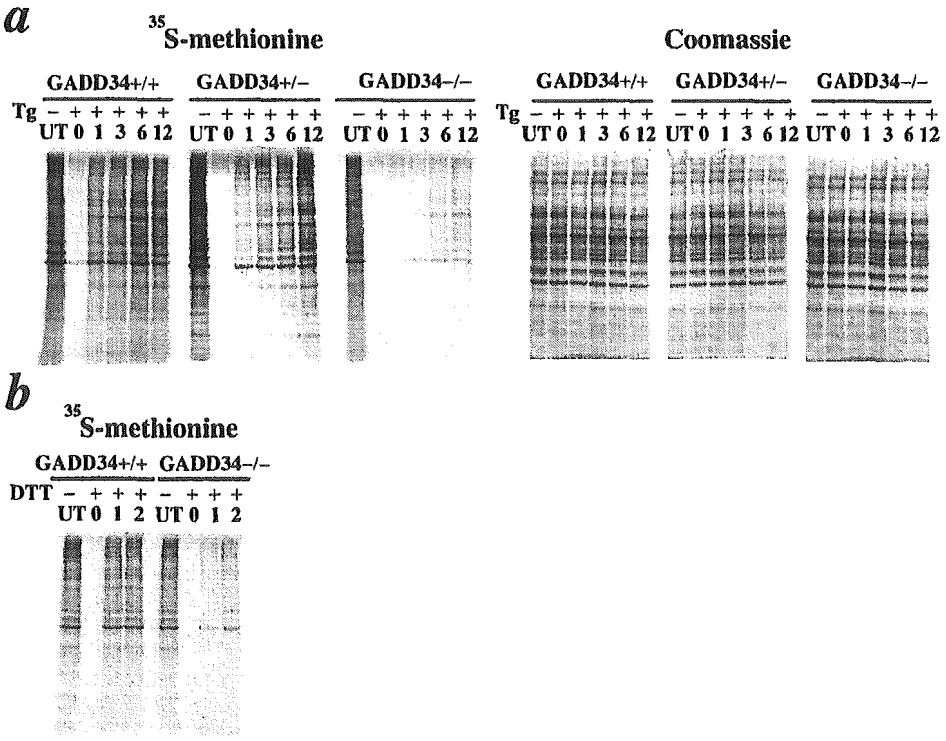

Figure 2. Protein synthesis after Tg treatment in GADD34$^{+/+}$, GADD34$^{+/-}$, and GADD34$^{-/-}$ MEFs. MEFs were exposed to Tg (1 μM) for 0.5 h, followed by different recovery times. After pulse-labeling with [^{35}S] methionine, whole cell lysates (10 μg) were resolved by 12% SDS-PAGE. *a)* The nascent protein synthesis after Tg treatment was determined by autoradiography (left panel). The right panel shows a Coomassie blue stain of the same gel to confirm the equal amount of total proteins in each lane. Results are representative of the 4 separate experiments using different pairs of MEFs. *b)* The nascent protein synthesis after DTT treatment was determined by autoradiography.

der ER stress. Although ATF4, Bip, and CHOP are strongly induced by Tg treatment in wild-type MEF, they are weakly induced by Tg treatment in GADD34-deficient MEF. This indicates that GADD34 exists upstream of ATF4, BiP, and CHOP and regulates their expressions in the UPR.

CONCLUSIONS

We have shown here that GADD34 was expressed in normal tissues in an unstimulated state and that its expression in the fetus was unexpected. We showed that at the fetal stage, GADD34 expression was especially strong on E12.5. Almost no expression was detected on E10.5, E11.5, and E13.5. It is of interest to know why GADD34 expression was different in the fetal stages. Despite the stage-specific expression of GADD34, mice deficient in it had no abnormalities under normal breeding conditions. Other proteins with functions similar to GADD34 may compensate for the GADD34 functions.

Although GADD34$^{-/-}$ mice show a normal phenotype, ER stress induces dramatic differences in MEF between a wild-type and a GADD34 deficiency. We show that GADD34 reverts the phosphorylation of eIF2α induced by Tg or DTT. These treatments, which induce an ER stress response, shut off the protein synthesis of both GADD34-deficient and wild-type MEF. However, in wild-type MEF an early recovery from the shutoff is observed that correlates with GADD34 expression. Despite the agent that disrupts protein folding in the ER, in the present study Tm induced neither phosphorylation of eIF2α nor shutoff of the protein synthesis. This result may be caused by the difference of its mechanism in the UPR. It has been shown that cells expressing GADD34 by in vitro transfection experiments diminished the phosphorylation of eIF2α levels in response to ER stress. GADD34 formed a complex with the catalytic subunit of protein phosphatase 1 (PP1α) that specifically promoted the dephosphorylation of eIF2α These results correlate with the function of $\gamma_1$34.5 of herpes simplex virus (HSV), which is found to combine with PP1α and dephosphorylates eIF2α, thereby precluding the shutoff of protein synthesis. It has been reported that transcriptional activation of CHOP and BiP upon activation of the UPR requires eIF2α phosphorylation. We have shown here that transcriptional and translational expression of CHOP and BiP are both strongly induced by ER stresses in wild-type MEF, presumably through ATF4. On the other hand, this induction is weak in GADD34-deficient MEF despite sustaining a high level of phosphorylated eIF2α. These results suggest that a GADD34-related pathway of CHOP and BiP expression in response to ER stress exists regardless of the eIF2α phosphorylation level (**Fig. 3**).

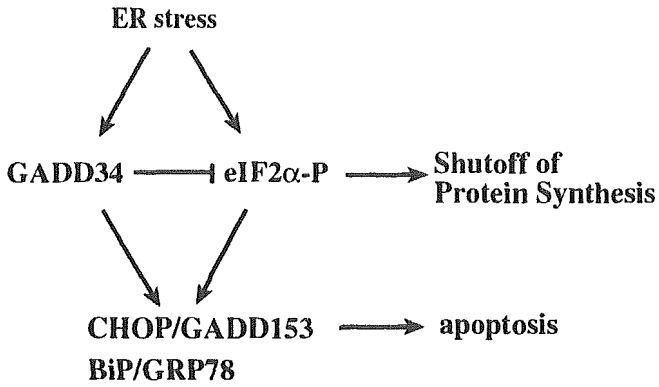

Figure 3. Schematic diagram of the function of GADD34 in response to ER stress. ER stress induces GADD34 expression. Under the condition of highly phosphorylated eIF2α by ER stress, GADD34 positively dephosphorylates eIF2α and induces a recovery from shutoff of protein synthesis. GADD34 also induces CHOP and Bip expression via ATF4.

Although translational attenuation caused by ER stress has been discussed in great detail, a recovery from shutoff of protein synthesis has not been extensively studied. What is the biological significance of recovery from translational attenuation in response to ER stress? GADD34, which functions as a recovery from a shutoff of protein synthesis, may be important in maintaining homeostasis of cells in the UPR. Recent work provided us with possible roles for the recovery from a shutoff of protein synthesis caused by GADD34 in vivo. It was reported recently that GADD34 is expressed in the peri-infarct zone after focal cerebral ischemia in rats. It has also been reported that mammalian eIF2α kinase-dependent autophagy is antagonized by the HSV-encoded neurovirulence gene product ICP34.5. Further analysis of the GADD34 engaged molecular pathway of the recovery from a shutoff of protein synthesis will open a new field in the pathophysiology of the mammalian system.

Dramatic increase of telomerase activity during dendritic cell differentiation and maturation

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Abstract: Telomerase, the reverse transcriptase that maintains telomere DNA, is usually undetectable in most adult tissues but is positive in embryonic tissues and in cancers. In addition, freshly islolated or in vitro-activated lymphocytes were shown to express high levels of telomerase activity, although its expression in myeloid cells including dendritic cells (DCs) is largely unknown. Here, we investigated telomerase activity during the differentiation and maturation process of DCs. In vitro culture of bone marrow (BM) cells with granulocyte macrophage-colony stimulating factor and interleukin-4 induced a dramatic increase of telomerase activity accompanied with their differentiation into DCs. Furthermore, stimulation with microbial components such as lipopolysaccharide (LPS), which triggers maturation of DCs, augmented the activity. In vivo responses of telomerase activity were also observed in splenic DCs by injection of LPS intraperitoneally. It is interesting that in old mice, telomerase activity of splenic DCs was significantly higher than young mice but rather decreased after LPS stimulation. By measuring expression of cell-surface activation markers, splenic DCs of old mice responded poorly to LPS stimulation. Such poor responses to LPS were also observed in BM-derived DCs. These different features of DCs between young and old mice may contribute to a pathogenesis to microbial infections. *J. Leukoc. Biol.* 74: 270–276; 2003.

Key Words: *DC · aging · telomere*

INTRODUCTION

Dendritic cells (DCs) are professional antigen presenting cells (APCs) and play a critical role in primary immune responses [1, 2]. DCs reside in an immature state in nonlymphoid tissues, where they efficiently capture and process antigens. Upon activation, they initiate a complex maturation process, which results in decreased antigen-processing capacities, enhanced expression of major histocompatibility complex (MHC) and costimulatory molecules, and migration into secondary lymphoid organs to prime acquired immune responses [3–6]. The maturation process is central to the function of DCs and enables them to perform different, highly specialized actions sequentially. There are many stimuli that can initiate this maturation process in vivo and in vitro. In fact, DCs are sensitive to many different indicators of infection, reflecting the key role to recognize a variety of pathogens. Inflammatory products and microbial components such as lipopolysaccharide (LPS), bacterial DNA [CpG-oligodeoxynucleotide (ODN)], and a synthetic dsRNA (poly I:C), which is often used as a model of viral infection, are all able to stimulate DCs to become activated and matured, professional APCs [7–10].

The ends of linear eukaryotic chromosomes are capped by specialized DNA–protein structures, called telomeres, which are composed of hexanucleotide repeats (TTAGGG)n [11, 12]. Telomeric DNA is lost every time somatic cells divide, and such shortening may act as a mitotic clock, regulating the number of cell divisions. When telomeres are shortened to such a critical point that they may no longer stabilize chromosome ends, most of the cells exit from the cell cycle and die [13–15]. Telomerase is a ribonucleoprotein enzyme that is able to add telomeric repeats to chromosome ends. In the presence of telomerase, telomere lengths are extended or maintained, and replicative senescence is avoided [16, 17]. In initial analysis, telomerase activity was only detectable in the early, immature cells, such as hematopoietic precursor cells in the bone marrow (BM) at infantile ages, but recent studies have demonstrated that it can be detected in normal, somatic cells. It has been shown that telomerase activity is up-regulated during T cell activation [18–21] and B cell differentiation [22], and high levels of telomerase activity were observed in thymocytes and germinal center B cells. However, a limited number of studies have examined the expression of telomerase activity in mouse tissues and reported low levels of activity in a variety of mouse tissues.

Here, we analyzed telomerase activity of DCs at various differentiation stages. A significant increase of telomerase activity was observed during the differentiation and maturation process of DCs, which was led in vitro and in vivo. We also measured telomerase activity of DCs with or without stimulation by microbial components and made a comparison between young and old mice.

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MATERIALS AND METHODS

Mice

C57BL/6J male mice were purchased from Chubu-Kagaku Laboratory (Aichi, Japan) and were kept under specific, pathogen-free conditions at our animal facilities. They were used between 6 and 10 weeks of age (young mice). C57BL/6J male mice aged 2 years (old mice) and 1 year (adult mice) were obtained from our animal center.

Reagents

LPS was purchased from Sigma Chemical Co. (St. Louis, MO) and was used at 200 ng/ml for stimulation of cultured cell. CpG-ODN, the oligonucleotide 1668 containing a "CpG-motif" marked with bold letters (5′-TCC-AT**G-ACG-TT**C-CTG-ATG-CT), was phosphorothioate-stabilized and synthesized by Hokkaido System Science Co. (Hokkaido, Japan) and was used at 1 μM in culture. Poly I:C was purchased from Sigma Chemical Co. and was used at 25 μg/ml for stimulation. Mouse granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4 were purchased from eBioscience (San Diego, CA) and were used at 20 ng/ml and 10 ng/ml, respectively, in DC culture.

Generation of DC from BM cells and stimulation in vitro

BM-derived DCs (BM DCs) were generated by a modification of the protocol as described previously [23]. Briefly, BM cells were collected from tibias and femurs of C57BL/6J mice and passed through a nylon mesh to remove small pieces. After an incubation period of one-half day, nonadherent cells were removed by washing, and the plastic-adherent cells were cultured in RPMI 1640 (Sigma Chemical Co.) containing GM-CSF and IL-4 with 10% fetal calf serum. Half of the medium was exchanged with fresh medium every 2 days. At day 6, loosely adherent cells [immature DCs (imDCs)] were harvested by gentle pipetting, plated at 5×10^5 cells/ml, and cultured with LPS, CpG-ODN, or poly I:C for an additional 2 days to generate mature DCs (mDCs).

Measurement of telomerase activity

Harvested cells were washed twice with ice-cold phosphate-buffered saline (PBS). The pellets were resuspended and incubated with lysis buffer of the telomerase polymerase chain reaction (PCR)-enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Mannheim, Germany) on ice for 30 min and then centrifuged for 20 min at 10,000 *g* at 4°C, and the supernatant was collected as an extract of telomerase. Then, telomerase activity of extracts was measured by the telomerase PCR-ELISA, according to the manufacturer's instructions or a modified version of the standard telomerase repeat amplification protocol (TRAP). In brief, the cell extract (representing 1×10^3 cells) was incubated for 30 min at 25°C in a mixture containing the reaction mixture of the telomerase PCR-ELISA kit and was heated at 94°C for 5 min and then subjected to 40 cycles of PCR amplification (94°C for 30 s, 58°C for 30 s, and 72°C for 90 s). PCR products were detected by the photometric enzyme immunoassay, and the Microtiter plate reader (Bio-Rad, Hercules, CA) measured the absorbance of samples at 450 nm. All assays were performed in triplicate. In the telomerase PCR-ELISA, the level of telomerase activity in positive-control cell extract supplied in the kit was set to 100%, and the relative specific telomerase activity (RTA) of each extract was expressed as a percentage of the positive-control standard (mean±SD). In the TRAP assay, the cell extracts were subjected to PCR with 2 μCi α-^{32}P-dCTP, and after the reaction, direct visualization of the TRAP ladder is possible on a 12.5% nondenaturing polyacrylamide gel electrophoresis, followed by autoradiography.

Flow cytometry

Expression of cell-surface molecules was analyzed by flow cytometry with standard procedures. Anti-CD11c (HL3), anti-CD40 (3/23), anti-CD86 (GL1), anti-I-A/I-E (M5/114.15.2), and an isotype-control antibody (Ab) were purchased from BD PharMingen (Los Angeles, CA). Dead cells were excluded by staining with propidium iodide, and data were analyzed by Cell Quest software.

LPS stimulation in vivo and isolation of splenic cells and peritoneal macrophages

LPS (10 μg) in PBS or only PBS was injected into mice intraperitoneally (i.p.). Every group has five mice, and three independent experiments were performed. Twenty-four hours later, spleens were dissected, and single-cell suspensions were prepared by passing through nylon mesh. Cells were separated by leukocyte separation medium (ICN Biomedicals, Aurora, OH). The low-density fraction was collected and incubated on ice with anti-CD11c microbeads (N418), anti-CD11b microbeads (M1/70.15.11.5), anti-CD90 microbeads (30-H12), or anti-CD19 microbeads (1D3; all microbeads were obtained from Miltenyi Biotec, Gladbach, Germany). An automated magnetic cell sorter (Miltenyi Biotec) sorted the positive cells, according to the manufacturer's instruction. Peritoneal macrophages were collected by recovering attached cells after 3 h incubation of peritoneal exudates from i.p.-injected PBS. For in vitro stimulation, splenic DCs were cultured with 200 ng/ml LPS in the presence of GM-CSF and IL-4 for 48 h. Purified cells were assessed by flow cytometry and were subjected to telomerase activity measurement.

RESULTS

Telomerase activity is up-regulated during in vitro differentiation of DCs

To address whether telomerase activity has changed during differentiation of DCs, in the first place, we took the well-performed and standardized method to induce DCs in vitro. ImDCs were generated from BM cells of 6- to 8-week-old mice by culturing with GM-CSF and IL-4, and telomerase activity and surface phenotype were checked every 2 days (**Fig. 1A**). Telomerase activity of cells started to increase immediately after they were put in culture and then showed a sharp increase and reached the maximum level at day 8. Simultaneously, the ratio of CD11c-positive cells indicating differentiation to DCs was also increased in a similar manner (Fig. 1A).

Exposure to microbial components such as LPS can induce mDC, characterized by up-regulation of costimulatory molecules. To assess the effect of LPS, we measured the telomerase activity of cells at several time points of culture with or without LPS stimulation. Although we could not see a LPS effect on telomerase activity at day 2 when BM cells were not well differentiated to DCs yet, telomerase activity of cells at day 8 stimulated with LPS (mDCs) was significantly higher than unstimulated ones (imDCs), expressing a middle level of telomerase activity (Fig. 1B). The same results were observed in the TRAP assay (Fig. 1C). To confirm the correlation between telomerase activity and DC maturation, we used other microbial origin inflammatory agents to activate imDCs. Recent studies have shown that LPS, CpG, and dsRNA are capable of triggering maturation of DCs via Toll-like receptor (TLR) signaling [10, 24]. Our experiments showed CpG and poly I:C were also able to increase telomerase activity of imDCs comparably with LPS (Fig. 1D). These results proved that telomerase activity of imDCs was indeed up-regulated during the maturation process by infectious agents. We obtained similar results in six independent experiments.

LPS can enhance telomerase activity of splenic DCs and peritoneal macrophages in vivo

LPS has also been shown to lead the maturation of DCs in vivo, and injection of mice with LPS can induce up-regulation of

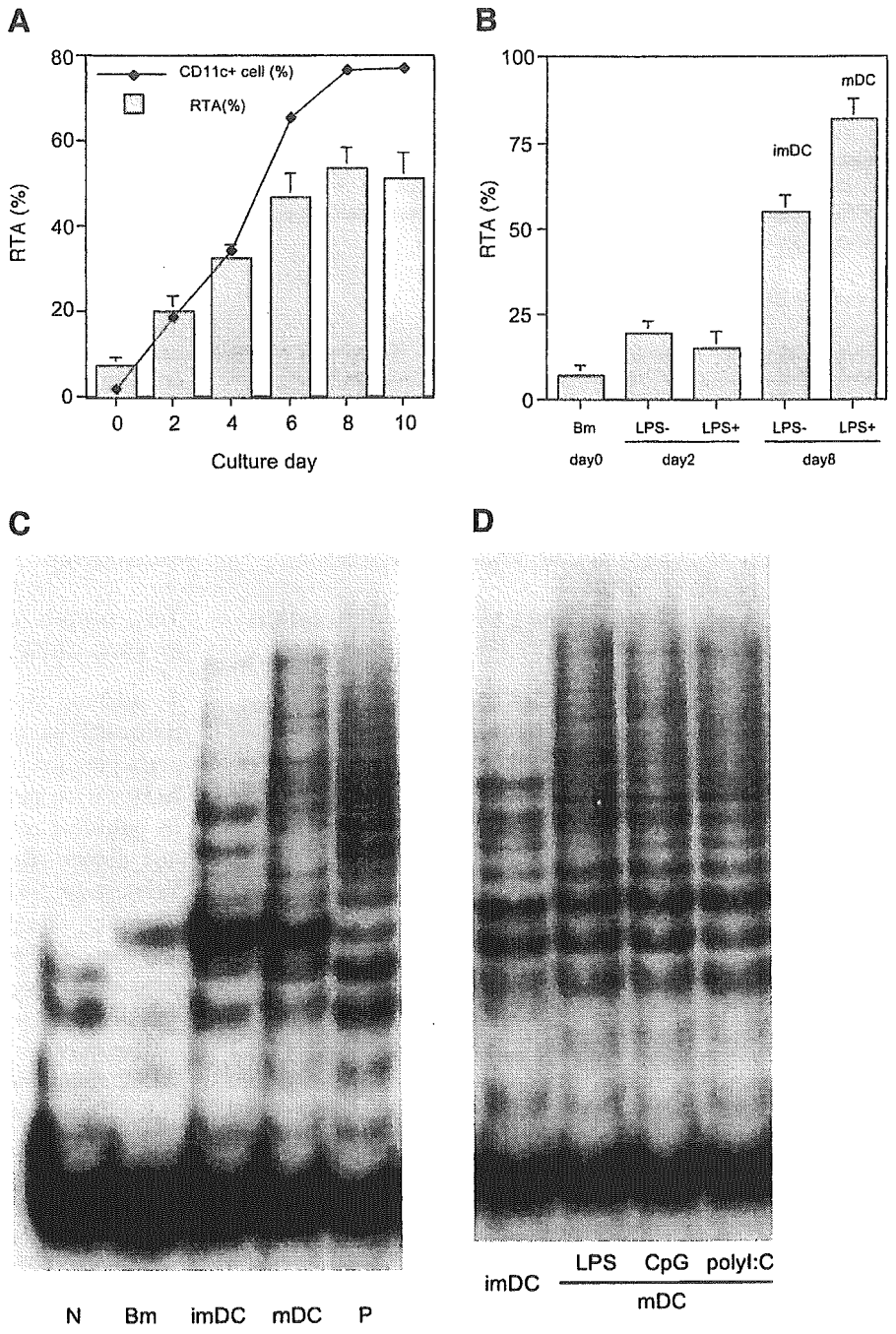

Fig. 1. Induction of telomerase activity during DC differentiation and maturation. (A) BM cells were isolated and cultured in medium containing GM-CSF and IL-4. The cells were collected every 2 days from days 0 to 10, and ELISA measured telomerase activity. The same cells were stained with anti-CD11c Ab and measured in flow cytometry. Subtracted by an isotype-control Ab staining, the percentage of the CD11c$^+$ cell was calculated from whole events. (B) At days 0 and 6, nonadherent cells were collected and cultured for an additional 2 days with or without LPS and measured telomerase activity. (C) Telomerase activity was visualized by TRAP. The PCR products from BM (day 0), imDCs and mDCs (day 8) were subjected to electrophoretic gel. (D) At day 6 of BM culture, cells were harvested and incubated with or without CpG-ODN, poly I:C, or LPS for another 2 days. Telomerase activity of imDCs without stimulation and mDCs with stimulation was compared with TRAP. P, positive control (telomerase activity in positive-control cell extract supplied); N, negative control (telomerase activity measured without cell extract).

costimulatory molecule expression on splenic DCs [25]. To further explore the correlation between regulation of telomerase activity and stimulation of DCs, we investigated effects of LPS in vivo. Splenic cells from 10-week-old mice injected with LPS or only PBS were sorted by antibody-coated microbeads: CD11c beads for DCs, CD11b for monocytes (macrophages), CD90 for T cells, and CD19 for B cells. After sorting, cells were checked by flow cytometry and were shown to have over 90% purity (data not shown). Analysis of telomerase activity in splenic cells injected with only PBS revealed that myeloid lineage cells such as DCs and monocytes have middle or low levels of telomerase activity, and lymphoid cells (B cells and T cells) have relatively high levels of activity. However, by injection of LPS, the telomerase activity of DCs was enhanced dramatically, whereas an increase in other hematopoietic cells was only modest (**Fig. 2A**). Together, these findings clearly indicate that LPS can also induce up-regulation of telomerase activity on DCs in vivo, and DCs are the potent responding cells to LPS stimulation in the spleen. Among CD11b$^+$ cells sorted, ~25% cells also expressed CD11c that represented DCs. To investigate responses of other myeloid cells to LPS, we also measured telomerase activity of peritoneal macrophages (Fig. 2B). Similar to DCs, peritoneal macrophages expressed the middle level of telomerase activity (~50% RTA) without stimulation and rose up to ~70% RTA after LPS stimulation in vivo.

Different features of DCs between young and old mice

To investigate the effect of age on changes of telomerase activity, we compared telomerase activity of purified splenic