2DG uptake study

The glucose uptake of cultured neurons was measured by enzymatic fluorescence assay as described.[22] Incorporated 2DG is phosphorylated to form 2DG-6-phosphate, which is not further metabolized and can be used for quantifying glucose uptake. At day 14 after plating the hippocampal cells, neurons were washed twice with Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl, 3.6 mM NaHCO, 3.5 mM HEPES) without serum or glucose. Neurons were incubated in Locke's buffer for 1 h at 37°C in 5% CO_2 for insulin starvation with and without glucose, followed by exposure to insulin (1 μM) for 30 min. During exposure to insulin, $A\beta_{1-42}$ oligomers (1 μM) were added to culture media for 10 min, 20 min and 30 min (Fig. 2a).

After terminating the exposure to insulin and $A\beta_{1-42}$ oligomers, 2DG was added to cultures for 5 min at 1 μM. The incorporation of 2DG was terminated by three washes with ice-cold 0.1 mol PBS and the cells were digested with 0.1 N NaOH. To degrade NAD(P)H, $NAD(P)^+$ and any glycolytic enzymes derived from the digested neurons, the culture plate was incubated at 85°C for 60 min in a temperature-controlled bath. The components in the wells were then neutralized by the addition of 0.1 N HCl and then solubilized in 150 mM triethanolamine hydrochloride (TEA) buffer (pH 8.1). Then, incorporated 2DG was quantified by fluorescence assay of resorufin converted from resazurin.[22] A standard curve was generated by placing 2DG standard solutions in wells of a culture plate lacking cells but prepared similarly.

Measurement of ATP levels

At day 14 after plating the hippocampal cells, neurons were washed and incubated with $A\beta_{1-42}$ oligomers for 30 min and 60 min in the presence and absence of insulin (1 μM) and C-peptide (1 μM). They were immediately homogenized in 0.5 mM perchloric acid with 1 mM ethylenediamine-N,N,N′,N′-tetraacetic acid and centrifuged for 15 min at 300 *g*. The supernatant was neutralized with 2 mol $KHCO_3$, recentrifuged and stored at −30°C until assayed. ATP was quantified using a luciferin/luciferase luminescence assay kit (Invitrogen). The protein content of the cells was determined by the method of Lowry and Passonneau.[23]

Materials

2-Deoxy-d-glucose, recombinant G6P-DH, phloretin, C-peptide, sodium pyruvate and sodium lactate were obtained from Sigma-Aldrich (Tokyo, Japan), and all other chemicals were from Wako (Tokyo, Japan) or Nacalai Tesque (Kyoto, Japan).

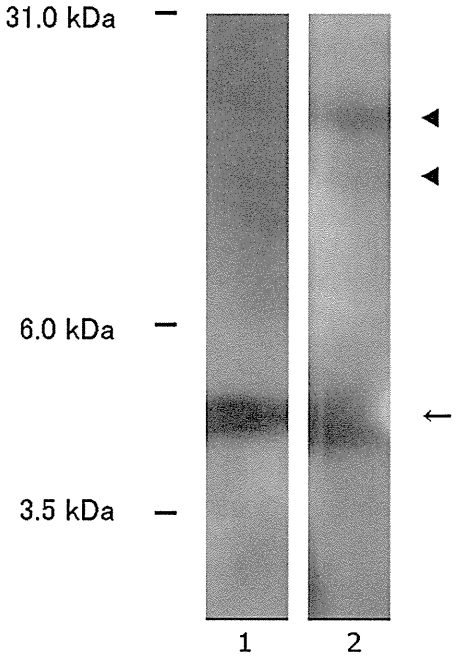


Figure 1 Immunoblotting of $A\beta_{1-42}$ monomer and oligomers. $A\beta_{1-42}$ oligomers were prepared as described.[19] The blots were probed with the 6E10 anti-Aβ antibody, followed by horseradish peroxidase-conjugated antimouse antibody and developed with ECL reagents. Representative blots of $A\beta_{1-42}$ monomers (arrow) and oligomers (arrowheads) are shown (lanes 1 and 2, respectively).

Statistical analysis

The data were analyzed using two-tailed Student's *t*-tests, and $P < 0.05$ was considered to be statistically significant.

Results

Immunoblotting of $A\beta_{1-42}$ monomer and oligomers

Figure 1 shows representative blots of $A\beta_{1-42}$ monomers and oligomers. The monomeric form of Aβ was observed at 5 kDa, whereas several oligomeric Aβ forms were at 10–20 kDa (lanes 1 and *2*). Furthermore, biological activity of the $A\beta_{1-42}$ oligomers was tested by electrophysiological experiments using hippocampal slice cultures.[24] We found that these oligomers effectively inhibited the induction of long-term potentiation (LTP) in the CA1 region of hippocampal slices after tetanic stimulation (100 Hz, 1 s), while application of $A\beta_{1-42}$ monomer at the identical concentration did not inhibit LTP (data not shown). These results confirmed that the 5–6 mers of $A\beta_{1-42}$ peptides used in this study were biologically active.

Specific uptake of 2DG in cultured hippocampal neurons

In these experiments, cultured neurons were exposed to glucose-free medium for more than 90 min before the 2DG uptake study (Fig. 2a), which could influence neuronal viability. Therefore, the rate of glucose incorporation was compared in neurons kept in glucose-free media and in medium containing 10 mM glucose. The 2DG uptake tended to increase in the absence of glucose, suggesting that cultured neurons were being maintained in relatively healthy conditions (data not shown). Based on this observation, glucose-free medium was used for following several sets of experimental conditions.

To confirm specific incorporation of 2DG in neurons, cultured hippocampal neurons were treated with phloretin, an inhibitor for glucose transporters, during 2DG uptake studies.[11] Administration of phloretin inhibited neuronal 2DG uptake significantly, indicating that 2DG was incorporated in a glucose transporter-mediated manner, but not by simple diffusion (data not shown).

Effects of insulin and $A\beta_{1-42}$ oligomers on 2DG uptake in cultured hippocampal neurons

To investigate the effects of insulin on neuronal 2DG uptake, hippocampal neurons were exposed to insulin (1 μM) for 30 min after insulin starvation (Fig. 2a). Because abundant distribution of insulin receptors and insulin-sensitive glucose transporter 4 has been identified in the hippocampus,[25] we expected an insulin-stimulated 2DG uptake in the cultured neurons. However, insulin-dependent increase of neuronal 2DG was not demonstrated after repeated experiments (Fig. 2b).

The impact of $A\beta_{1-42}$ oligomers on 2DG uptake was tested next. Incubation with $A\beta_{1-42}$ oligomers for 10–30 min did not change neuronal 2DG uptake. In addition, no synergistic effects of insulin and $A\beta_{1-42}$ oligomers could be demonstrated. Prolonged treatment with $A\beta_{1-42}$ oligomers for more than 24 h showed no changes in 2DG uptake (data not shown). These results indicated that neither insulin nor $A\beta_{1-42}$ oligomers had any effects on neuronal glucose incorporation.

Changes in intracellular ATP levels by $A\beta_{1-42}$ oligomers and insulin/C-peptide

Adenosine triphosphate levels in the hippocampal neurons were determined for evaluating mitochondrial function. Because Wang *et al.* reported that Aβ toxicity depends on extracellular glucose concentrations,[24] cultured cells were incubated in media containing a variety of glucose concentrations (0–10 mM) with and without $A\beta_{1-42}$ oligomers. ATP concentrations in the cultured neurons were quantified by the luciferin/luciferase luminescence method. In the presence of 10 mM glucose, $A\beta_{1-42}$ oligomers gradually decreased the ATP levels, and a significant reduction in ATP concentration was observed after 60 min ($P < 0.05$), while $A\beta_{42-1}$ did not affect ATP contents (Fig. 3a). When the glucose concentration was less than 5 mM, $A\beta_{1-42}$ oligomers decreased ATP contents more rapidly but to similar final levels.

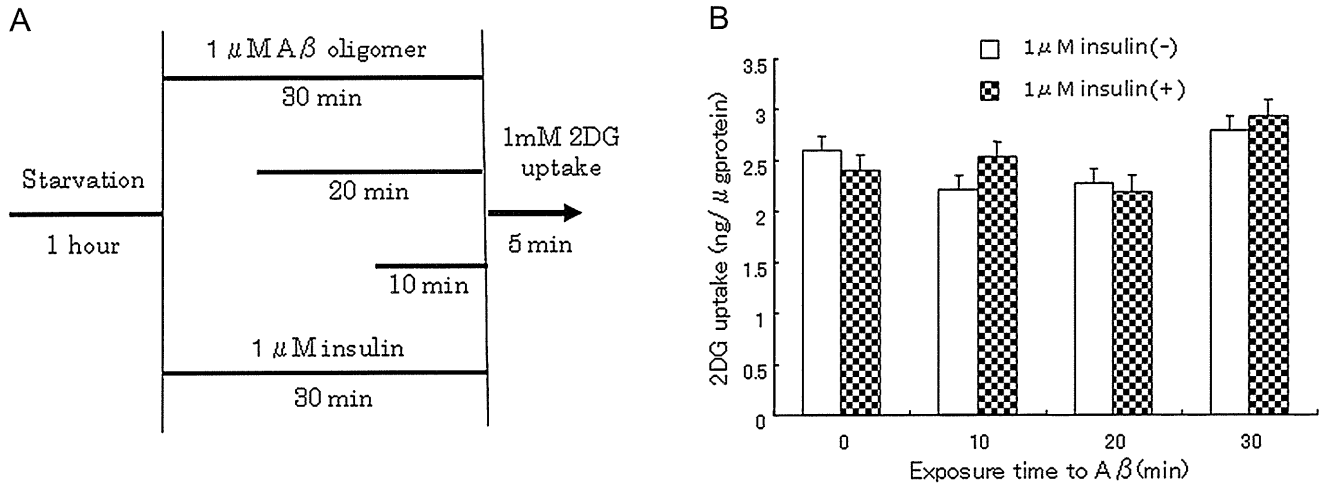


Figure 2 Effects of $A\beta_{1-42}$ oligomers and insulin on glucose incorporation in the hippocampal neurons. (a) Experimental protocols to investigate the effects of $A\beta_{1-42}$ oligomers and insulin on glucose uptake. After insulin starvation, hippocampal neurons were incubated with and without insulin (1 μM), and 2-deoxy-d-glucose (2DG) uptake was determined as described. (b) No insulin-dependent increase of neuronal 2DG could be demonstrated (Aβ exposure time 0). Incubation with $A\beta_{1-42}$ oligomers for 10–30 min did not change neuronal 2DG uptake. No synergistic effects of insulin and $A\beta_{1-42}$ oligomers were observed.

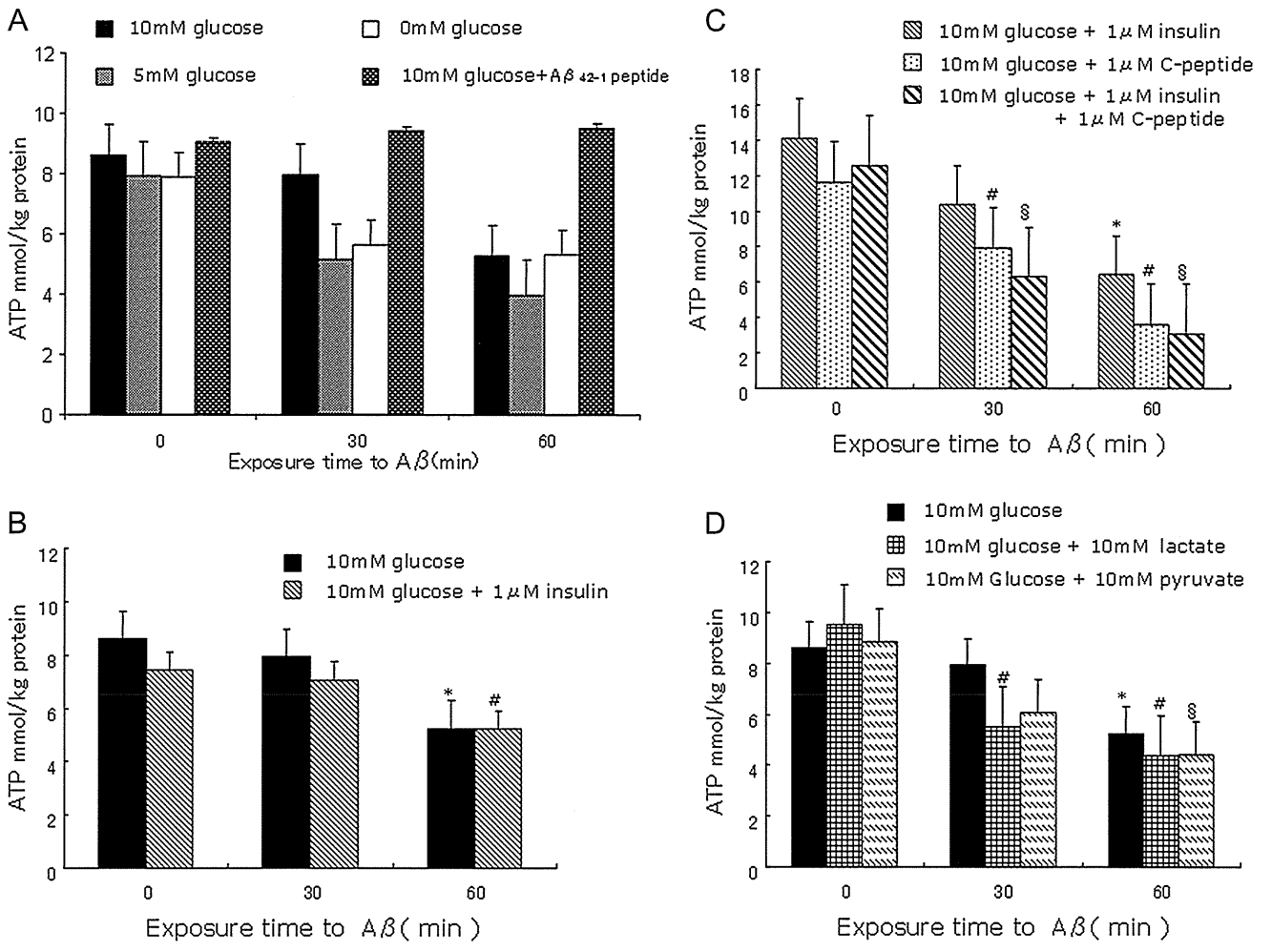


Figure 3 Intracellular adenosine triphosphate (ATP) levels after application of $A\beta_{1-42}$ oligomers, insulin/C-peptide and monocarboxylates. At day 14 post-plating, hippocampal neurons were incubated with $A\beta_{1-42}$ oligomers for 30–60 min in the presence and absence of insulin, C-peptide and lactate/pyruvate. ATP concentration was quantified using a luciferin/luciferase luminescence assay. (a) Cultured cells were incubated in media containing a variety of glucose concentrations (0–10 mM) with and without $A\beta_{1-42}$ oligomers. Each bar represents the mean ± standard error of the mean ($n = 4$). In the presence of 10 mM glucose, $A\beta_{1-42}$ oligomers gradually decreased ATP levels, and significant reduction of ATP was observed after 60 min ($^{\S}P < 0.05$ compared with time 0). $A\beta_{42-1}$ peptide did not decrease neuronal ATP levels. When glucose concentration was less than 5 mM, $A\beta_{1-42}$ oligomer decreased ATP contents more rapidly: $^{\#}P < 0.05$ (5 mM glucose) and $^{*}P < 0.05$ (0 mM glucose) compared with time 0. (b) Insulin did not change ATP contents in the cultured hippocampal neurons over 60 min of incubation. Concomitant application of $A\beta_{1-42}$ oligomers significantly reduced ATP concentration. Each bar represents the mean ± SEM ($n = 4$). (c) In the presence of insulin and/or C-peptide, addition of $A\beta_{1-42}$ oligomers similarly reduced intracellular ATP levels. Each bar represents the mean ± SEM ($n = 4$). $^{*}P < 0.05$ (10 mM glucose + 1 μM insulin), $^{\#}P < 0.05$ (10 mM glucose + 1 μM C-peptide) and $^{\S}P < 0.05$ (10 mM glucose + 1 μM insulin + 1 μM C-peptide) compared with time 0. (d) $A\beta_{1-42}$ oligomers decreased intracellular ATP concentrations in hippocampal neurons in the presence of lactate and pyruvate plus glucose. Each bar represents the mean ± SEM ($n = 4$). $^{*}P < 0.05$ (10 mM glucose), $^{\#}P < 0.05$ (10 mM glucose + 10 mM lactate) and $^{\S}P < 0.05$ (10 mM glucose + 10 mM pyruvate), compared with time 0.

The effect of insulin on ATP production was examined next (Fig. 3b). Insulin did not change ATP contents in the cultured hippocampal neurons during 60 min of incubation, and concomitant application of $A\beta_{1-42}$ oligomers significantly reduced the concentration. Because C-peptide, another derivative of proinsulin, has been shown to have neuroprotective effects,[26] we also examined the effects of C-peptide on the intracellular ATP concentrations. The addition of $A\beta_{1-42}$ oligomers also reduced intracellular ATP levels in the presence of insulin and/or C-peptide ($P < 0.05$, Fig. 3c).

Finally, the impacts of $A\beta_{1-42}$ oligomers on ATP concentrations in hippocampal neurons were determined in the presence of lactate and pyruvate plus 10 mM glucose, because these monocarboxylates are incorporated into neuronal cells via monocarboxylate transporters and

are metabolized in mitochondria to produce ATP directly without using anaerobic glycolytic pathways.[27] It has been shown that pyruvate serves as a scavenger of reactive oxygen species (ROS) induced by $A\beta_{1-42}$ oligomers.[28] However, incubation of hippocampal slices in Locke's buffer containing glucose plus lactate or pyruvate did not influence the reduction of neuronal ATP levels by $A\beta_{1-42}$ oligomers ($P < 0.05$, Fig. 3d).

Discussion

This study has provided evidence for the impacts of $A\beta_{1-42}$ oligomers and insulin on glucose metabolism and energy homeostasis *in vitro*. Although an inhibitory effect of high concentrations of Aβ peptides on glucose uptake has been suggested,[10,11] here we clearly demonstrate that Aβ oligomers did not suppress the 2DG uptake reflecting the activities of glucose transport and/or hexokinase but disrupted ATP contents in the presence and absence of monocarboxylates (lactate/pyruvate). These results imply that the primary target of $A\beta_{1-42}$ oligomers could be mitochondria but not anaerobic glycolysis. This could result in the reduced cerebral glucose metabolism observed in patients with AD. On the other hand, insulin and C-peptide were not directly linked to glucose uptake or ATP production, although insulin receptors and GLUT 4, an insulin-sensitive glucose transporter, are known to be distributed abundantly in the hippocampal neurons.[25]

In the present study, we have demonstrated that Aβ oligomers can cause reduction in neuronal ATP levels. In this connection, Saraiva *et al.* have recently reported decreased ATP levels and impaired mitochondrial enzymes in mature cortical neurons by exposure to Aβ oligomers.[29] Aβ oligomers have now been identified in mitochondrial membranes in the neurons of postmortem brain specimens from patients with AD, in brain neurons of mice with AD and in neuronal cells expressing mutant amyloid precursor protein (APP).[30] In mitochondria, Aβ oligomers induce elevated levels of ROS, deterioration of mitochondrial enzymes and failure of Ca^{2+} homeostasis *in vivo*.[30] However, the mechanism by which Aβ is transported to the mitochondrial membrane is not fully understood. Besides the plasma membrane, APP generated in neurons localizes to the Golgi apparatus, to the endoplasmic reticulum and to the endosomal, lysosomal and mitochondrial membranes. Aβ is produced by the sequential cleavage of APP by β-secretase and γ-secretase. In addition, previously secreted Aβ can be also taken up by cells and internalized into intracellular Aβ pools through various receptors and transporters.[30]

Furthermore, extracellular Aβ oligomers activate nerve growth factor receptors to induce apoptotic cell death and N-methyl-d-aspartate (NMDA)-type glutamate receptors to cause abnormal calcium homeostasis, leading to increased oxidative stress and synapse loss.[31] Aβ oligomers cause a rapid and substantial loss of neuronal surface insulin receptors specifically on dendrites, with increased receptor immunoreactivity in the cell body, indicating the redistribution of insulin receptors. Aβ oligomers reduce autophosphorylation of the insulin receptor mediated by the NMDA receptor in both hippocampal and cortical neurons, and impair LTP-associated kinase activity.[18,32] Involvement of the Frizzled (Fz) receptor, an acceptor of the Wnt protein, has been proposed.[33] Wnt signaling promotes progenitor cell proliferation and directs cells into a neuronal phenotype during brain development. This inactivates glycogen synthase kinase-3β (GSK-3β) and increases β-catenin levels. Inhibition of Wnt signaling by Aβ oligomers through the Fz receptor could cause tau phosphorylation and neurofibrillary tangles, which supports the idea of a Wnt/β-catenin toxicity pathway.[33] Aβ oligomers can impair presynaptic P/Q-type calcium currents, which are related to neurotransmission and synaptic plasticity.[34] Direct or indirect metabolic signals originating from Aβ binding sites on the neuronal surface could lead to alterations in mitochondrial structure and function.

It should be noted that Aβ was used at 1 μM in this study. Most estimates for its concentration in the human brain have been in the low nanomolar range.[35] To span this large concentration gap, several potential mechanisms have been proposed. Thus, Hu *et al.* reported that low, physiologically-relevant concentrations of extracellular Aβ can be taken up by neurons and then concentrated into endosomes/lysosomes (>2.5 μM).[36] Our experimental conditions might have accelerated this process by using higher concentrations of Aβ, leading to increased Aβ neurotoxicity. Detailed studies are needed to reveal how Aβ oligomers induce mitochondrial insufficiency.

Interactions of insulin signaling and Aβ oligomers have been proposed.[37] De Felice *et al.* have reported that Aβ oligomers cause major downregulation of plasma membrane insulin receptors in hippocampal cortical cultures.[37] Aβ oligomer-induced oxidative stress and synaptic spine deterioration could be completely prevented by insulin at 1 μM. Therefore, we used 1 μM insulin in the present experiment.

It has been postulated that insulin resistance plays a critical role in the development and progression of AD.[15] The beneficial effects of insulin in the central nervous system include improved glucose metabolism and energy homeostasis. Increased synaptic plasticity and modulation of Aβ and tau metabolism have also been proposed. Acute administration of insulin with sustained blood glucose concentrations facilitates memory in healthy elderly people as well as in patients with AD. This effect occurs at lower insulin doses for the healthy elderly group than for patients with AD and is suppressed by insulin-induced elevations of Aβ in

cerebrospinal fluid.[38] However, our results here clearly indicate that insulin signaling was not directly linked to glucose metabolism or energy homeostasis in hippocampal cultured neurons with and without Aβ oligomers. This suggests that insufficient insulin action might contribute to progression of the AD pathology by accelerating impaired synaptic activity and metabolic changes in the Aβ cascade. Because insulin could not ameliorate the disruption of energetic homeostasis in neurons induced by Aβ oligomers, it seems plausible that therapeutic strategies to prevent or correct insulin resistance should be conducted in the earlier stages of AD when synaptic abnormalities are irreversible. Further intensive studies are needed to develop new drugs for improving mitochondrial impairment in patients developing AD.

Acknowledgments

Financial support was provided by grants from the National Center of Geriatrics and Gerontology (22–5), from the Japan Foundation for Aging and Health (H21-chojuippann-005) and from the Ministry of Education, Culture, Sports, Science and Technology (22590654), to T. S.

References

1 Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* 2001; **81**: 741–766.
2 Mattson MP. Pathways towards and away from Alzheimer's disease. *Nature* 2004; **430**: 631–639.
3 LaFerla FM, Green KN, Oddo S. Intracellular amyloid-beta in Alzheimer's disease. *Nat Rev Neurosci* 2007; **8**: 499–509.
4 Reddy PH, Beal MF. Amyloid beta, mitochondrial dysfunction and synaptic damage: implications for cognitive decline in aging and Alzheimer's disease. *Trends Mol Med* 2008; **14**: 45–53.
5 Small GW, Ercoli LM, Silverman DH *et al.* Cerebral metabolic and cognitive decline in persons at genetic risk for Alzheimer's disease. *Proc Natl Acad Sci U S A* 2000; **97**: 6037–6042.
6 DeLeon MJ, Convit A, Wolf OT *et al.* Prediction of cognitive decline in normal elderly subjects with 2-[(18)F]fluoro-2-deoxy-D-glucose/poitron-emission tomography (FDG/PET). *Proc Natl Acad Sci U S A* 2001; **98**: 10966–10971.
7 Reiman EM, Caselli RJ, Chen K, Alexander GE, Bandy D, Frost J. Declining brain activity in cognitively normal apolipoprotein E epsilon 4 heterozygotes: a foundation for using positron emission tomography to efficiently test treatments to prevent Alzheimer's disease. *Proc Natl Acad Sci U S A* 2001; **98**: 3334–3339.
8 Mosconi L, DeSanti S, Li J *et al.* Hippocampal hypometabolism predicts cognitive decline from normal aging. *Neurobiol Aging* 2008; **29**: 676–692.
9 Bigl M, Brückner MK, Arendt T, Bigl V, Eschrich K. Activities of key glycolytic enzymes in the brains of patients with Alzheimer's disease. *J Neural Transm* 1999; **106**: 499–511.
10 Uemura E, Greenlee HW. Amyloid β-peptide inhibits neuronal glucose uptake by preventing exocytosis. *Exp Neurol* 2001; **170**: 270–276.
11 Prapong T, Buss J, Hsu WH, Heine P, Greenlee HW, Uemura E. Amyloid β-peptide decreases neuronal glucose uptake despite causing increase in GLUT3 mRNA transcription and GLUT3 translocation to the plasma membrane. *Exp Neurol* 2002; **174**: 253–258.
12 Parihar MS, Brewer GJ. Mitoenergetic failure in Alzheimer disease. *Am J Physiol Cell Phisiol* 2007; **292**: C8–C23.
13 Walsh DM, Klyubin I, Fadeeva JV *et al.* Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 2002; **418**: 535–539.
14 Dahlgren KN, Manelli AM, Stine WB Jr, Baker LK, Krafft GA, LaDu MJ. Oligomeric and fibrillar species of amyloid-β peptides differentially affect neuronal viability. *J Biol Chem* 2002; **277**: 32046–32053.
15 Biessels GJ, Staekenborg S, Brunner E, Brayne C, Scheltens P. Risk of dementia in diabetes mellitus: a systematic review. *Lancet Neurol* 2006; **5**: 64–74.
16 Vanhanen M, Koivisto K, Moilanen L *et al.* Association of metabolic syndrome with Alzheimer disease: a population-based study. *Neurology* 2006; **67**: 843–847.
17 Whitmer RA. The epidemiology of adiposity and dementia. *Curr Alzheimer Res* 2007; **4**: 117–122.
18 Zhao WQ, DeFelice FG, Fernandez S *et al.* Amyloid beta oligomers induce impairment of neuronal insulin receptors. *FASEB J* 2008; **22**: 246–260.
19 Roselli F, Tirard M, Lu J *et al.* Soluble β-Amyloid$_{1-40}$ induces NMDA-dependent degradation of postsynaptic density-95 at glutamatergic synapses. *J Neurosci* 2005; **25**: 11061–11070.
20 Fusheng Y, Giselle PL, Aynun NB *et al.* Curcumin inhibits formation of amyloid β oligomers and fibrils, binds plaques, and reduces amyloid *in vivo*. *J Biol Chem* 2005; **280**: 5892–5901.
21 Brewer GJ, Torricelli JR, Evege EK, Price PJ. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum free medium combination. *J Neurosci Res* 1993; **35**: 567–576.
22 Yamamoto N, Sato T, Kawasaki K, Murosaki S, Yamamoto Y. A nonradioisotope, enzymatic assay for 2-deoxyglucose uptake in L6 skeletal muscle cells cultured in a 96-well microplate. *Anal Biochem* 2006; **351**: 139–145.
23 Sakurai T, Yang B, Takata T, Yokono K. Synaptic adaptation to repeated hypoglycemia depends on the utilization of monocarboxylates in Guinea pig hippocampal slices. *Diabetes* 2002; **51**: 430–438.
24 Wang X, Song X, Takata T, Miichi Y, Yokono K, Sakurai T. Amyloid-β neurotoxicity restricts glucose window for neuronal survival in rat hippocampal slice cultures. *Exp Geront* 2010; **45**: 904–908.
25 McEwena BS, Reagan LP. Glucose transporter expression in the central nervous system: relationship to synaptic function. *Eur J Pharmacol* 2004; **490**: 13– 24.
26 Zhang W, Yorek M, Pierson CR, Murakawa Y, Breidenbach A, Sima AAF. Human C-peptide dose dependently prevents early neuropathy in the BB/Wor-rat. *Int J Exp Diabetes Res* 2001; **2**: 187–193.
27 Bergersen LH. Is lactate food for neurons? Comparison of monocarboxylater substrate in brain and muscle. *Neuroscience* 2007; **145**: 11–19.
28 Wang XN, Takata T, Sakurai T, Yokono K. Different effects of monocarboxylates on neuronal survival and β-amyloid toxicity. *Eur J Neurosci* 2007; **26**: 2142–2150.
29 Saraiva LM, da Silva GS, Galina A *et al.* Amyloid-β Triggers the Release of Neuronal Hexokinase 1 from Mitochondria. *PLoS ONE* 2010; **5**: e15230.

30 Reddy PH. Amyloid beta, mitochondrial structural and functional dynamics in Alzheimer's disease. *Exp Neurol* 2009; **218**: 286–292.
31 Sakono M, Zako T. Amyloid oligomers: formation and toxicity of Abeta oligomers. *FEBS J* 2010; **277**: 1348–1358.
32 Townsend M, Mehta T, Selkoe DJ. Soluble Abeta inhibits specific signal transduction cascades common to the insulin receptor pathway. *J Biol Chem* 2007; **282**: 33305–33312.
33 Magdesian MH, Carvalho MM, Mendes FA *et al.* Amyloid-beta binds to the extracellular cysteine-rich domain of Frizzled and inhibits Wnt / beta-catenin signaling. *J Biol Chem* 2008; **283**: 9359–9368.
34 Nimmrich V, Grimm C, Draguhn A *et al.* Amyloid beta oligomers (A beta(1-42) globulomer) suppress spontaneous synaptic activity by inhibition of P / Q-type calcium currents. *J Neurosci* 2008; **28**: 788–797.
35 Brody D, Magnoni S, Schwetye K *et al.* Amyloid-beta dynamics correlate with neurological status in the injured human brain. *Science* 2008; **321**: 1221–1224.
36 Hu X, Crick SL, Bu G, Frieden C, Pappu RV, Lee JM. Amyloid seeds formed by cellular uptake, concentration, and aggregation of the amyloid-beta peptide. *Proc Natl Acad Sci U S A* 2009; **106**: 20324–20329.
37 De Felice FG, Vieira MNN, Bomfim TR *et al.* Protection of synapses against Alzheimer's-linked toxins: insulin signaling prevents the pathogenic binding of Aβ oligomers. *Proc Natl Acad Sci U S A* 2009; **106**: 1971–1976.
38 Watson GS, Craft S. Modulation of memory by insulin and glucose: neuropsychological observations in Alzheimer's disease. *Eur J Pharmacol* 2004; **490**: 97–113.

Biochemical and Biophysical Research Communications 408 (2011) 306–311

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Acceleration of autoimmune diabetes in Rheb-congenic NOD mice with β-cell-specific mTORC1 activation

Hirotomo Sasaki [a], Hisafumi Yasuda [a,b,*], Hiroaki Moriyama [a], Akira Nakamura [a], Mami Shimizu [a], Takashi Arai [a], Masao Nagata [a], Kenta Hara [a], Koichi Yokono [a]

[a] Department of Internal and Geriatric Medicine, Kobe University Graduate School of Medicine, Kobe, Japan
[b] Division of Integrated Medical Education, Department of Social/Community Medicine and Health Science, Kobe University Graduate School of Medicine, Kobe, Japan

ARTICLE INFO

Article history:
Received 5 April 2011
Available online 13 April 2011

Keywords:
Type 1 diabetes
NOD
Rheb
mTOR
CFA
GLP-1

ABSTRACT

The protein Ras homolog enriched in brain (Rheb) is a Ras-like small GTPase that activates the mechanistic target of rapamycin complex 1 (mTORC1), which promotes cell growth. We previously generated transgenic C57BL/6 mice overexpressing Rheb in β-cells ($B6^{Rheb}$), which exhibited increased β-cell size and improved glucose tolerance with higher insulin secretion than wild type C57BL/6 mice. The mice also showed resistance to obesity-induced hyperglycemia, a model of type 2 diabetes, and to multiple low-dose-streptozotocin (MLDS)-induced hyperglycemia, a model of type 1 diabetes (T1D). To investigate whether the effects of mTORC1 activation by Rheb in $B6^{Rheb}$ mice would also be evident in NOD mice, a spontaneous autoimmune T1D model, we created two NOD mouse lines overexpressing Rheb in their β-cells (NOD^{Rheb}; R3 and R20). We verified Rheb overexpression in β-cells, the relative activation of mTORC1 and β-cell enlargement. By 35 weeks of age, diabetes incidence was significantly greater in the R3 line and tended to be greater in the R20 line than in NOD mice. Histological analysis demonstrated that insulitis was significantly accelerated in 12-week-old R3 NOD^{Rheb} mice compared with NOD mice. Furthermore, serum insulin autoantibody (IAA) expression was significantly higher than that of NOD mice. We also examined whether complete Freund's adjuvant (CFA) treatment alone or with glucagon-like peptide-1 (GLP-1) analog would reverse the hyperglycemia of NOD^{Rheb} mice; unexpectedly, almost none achieved normoglycemia. In summary, diabetes progression was significantly accelerated rather than prevented in NOD^{Rheb} mice. Our results suggest that the β-cell enlargement might merely enhance the autoimmunity of pathogenic T-cells against islets, leading to acceleration of autoimmune diabetes. We conclude that not only enlargement but also regeneration of β-cells in addition to the prevention of β-cell destruction will be required for the ideal therapy of autoimmune T1D.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by T-cell mediated pancreatic β-cell destruction [1]: β-cells secrete insulin, the only hormone that downregulates serum glucose levels. In treatment of T1D, sufficient insulin replacement is essential to achieve near-normal glucose control. Insulin is, however, considered one of the targets of autoimmunity in T1D [2]. In nonobese diabetic (NOD) mice, a well-established animal model for T1D, administration of insulin or insulin peptide through various routes prevents diabetes [3–6]. These studies have raised the possibility that the clinical application of prophylactic insulin in humans could delay or prevent the onset of T1D, and have led to human trials such as the Diabetes Prevention Trial-Type 1 (DPT-1). In DPT-1, low-dose subcutaneous and intravenous insulin was administered to nondiabetic relatives of patients with T1D, but had no preventive effect in individuals with autoimmune prediabetes [7]. Previous reports also suggested that islet regeneration can occur even in T1D, but that the imbalance between regeneration and autoimmune destruction of β-cells would eventually lead to hyperglycemia and onset of T1D [8,9]. Our ultimate goal is to achieve normoglycemia in T1D patients by establishing a therapy that protects pancreatic β-cells from ongoing autoimmune attack and increases the β-cell mass.

Growing evidence has indicated that insulin and insulin-like growth factor-1 (IGF-1) receptor-mediated signaling pathways are involved in the regulation of β-cell number and size. Activation of insulin or IGF-1 receptors induces tyrosine phosphorylation of insulin receptor substrates 1 (IRS-1) and 2 (IRS-2), followed by activation of phosphoinositide-3 kinase (PI3-K), resulting in activation of the proline-rich protein kinase B (PKB)/Akt signaling pathway. The mechanistic target of rapamycin (mTOR) is a

* Corresponding author at: 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Fax: +81 78 382 6597.
E-mail address: yasuda@med.kobe-u.ac.jp (H. Yasuda).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.04.026

serine-threonine protein kinase that forms two multiprotein complexes called mTOR complex 1 (mTORC1) and 2 (mTORC2) [10]. The mTORC1 is composed of mTOR, raptor, mammalian G-protein β-subunit-like protein/lethal with Sec thirteen 8 (GβL/LST8) and proline-rich protein kinase B (PKB)/Akt substrate 40 kDa (PRAS40). The mTORC1 promotes cell growth and proliferation via phosphorylation of translation regulator targets such as eukaryotic initiation factor 4E binding protein 1 (4EBP1) and p70 S6 kinase (p70S6K) [10–13].

The protein Ras homolog enriched in brain (Rheb) is a Ras-like small GTPase. Rheb binds GTP after stimulation by nutrients such as amino acids and glucose, and insulin also enhances Rheb GTP charging via inhibition of the function of tuberous sclerosis complex 1/2 (TSC1/2) heterodimer. GTP-charged Rheb binds directly to the mTOR catalytic domain and activates the mTORC1 cascade, leading to promotion of cell growth [14].

We previously generated transgenic mice that overexpress Rheb in β-cells on a C57BL/6 genetic background, called $B6^{Rheb}$ mice [15]. Compared with C57BL/6 mice, the $B6^{Rheb}$ mice exhibited improved glucose tolerance with higher insulin secretion resulting from increased β-cell size, and resistance to obesity- and multiple low-dose-streptozotocin (MLDS)-induced hyperglycemia. To investigate whether the beneficial effects that Rheb displayed in $B6^{Rheb}$ mice would also be evident in NOD mice, a spontaneous autoimmune T1D model, we generated NOD^{Rheb} mice that overexpressed Rheb in β-cells on a NOD genetic background.

2. Materials and methods

2.1. Mice

To generate congenic NOD mice that expressed the Rheb transgene in β-cells (NOD^{Rheb}), $B6^{Rheb}$ mice were backcrossed to NOD mice by speed congenics [16]. To confirm the presence of the Rheb transgene, polymerase chain reaction (PCR) amplification was performed with genomic DNA isolated from mouse tails. The primers are shown in Table 1. All experiments were done using female mice. All mice were fed in pathogen-free facilities and handled under the guidelines for Animal Experimentation of Kobe University School of Medicine.

2.2. Metabolic studies

Blood glucose was monitored weekly. The mice were considered diabetic when two consecutive blood glucose measurements exceeded 200 mg/dL. The first day of hyperglycemia was considered as diabetes onset.

2.3. Immunoblotting

Pancreata obtained from the mice were homogenized in lysis buffer, and the supernatants were collected and analyzed by immunoprecipitation and immunoblotting with anti-FLAG (M2) antibody (Sigma–Aldrich, St. Louis, MO) as previously described [15].

Table 1
Primer sequences for PCR.

Rheb forward:	Sense	5′-TTT CCT CAG ACA TAC TCC ATA G-3′
Rheb transgene forward:	Sense	5′-TAC AAG GAC GAC GAT GAC AAG-3′
Rheb and transgene reverse:	Antisense	5′-TGA TTT TCT TTA GCA GAA GAT TCC-3′

2.4. Histological analysis

Pancreata obtained from the mice were fixed in 10% formalin, embedded in paraffin and sectioned at a thickness of 5 μm. The sections were stained with hematoxylin and eosin. Islets were sorted into four categories based on the relative degree of leukocyte infiltration: 0 = no insulitis; 1 = peri-insulitis (mononuclear cell infiltration in <25% of the islet); 2 = invasive insulitis (25–50% infiltration); and 3 = severe insulitis (>50% infiltration or small, retracted islet). Immunostaining of FLAG and phosphorylated S6 was performed with the anti-FLAG antibody (DYKDDDDK Tag antibody) and anti-phospho-S6 ribosomal protein (Ser235/236), which were purchased from Cell Signaling Technologies (Beverly, MA). Immunostaining of insulin and glucagon was performed with guinea pig anti-insulin antibody and rabbit anti-glucagon antibody (Dako Japan, Kyoto, Japan). For quantification of β-cells, images of β-cells were obtained using a digital camera (BZ-8000, Keyence Co., Osaka, Japan) and analyzed using WinROOF software (Mitani Corp., Fukui, Japan).

2.5. Insulin autoantibody (IAA) assay

Serum IAA expression of the mice was evaluated at 12 weeks of age. IAA was measured with a 96-well filtration plate micro-IAA assay as previously described [17]. Briefly, serum of the mice preincubated with ^{125}I-insulin (GE Healthcare, Little Chalfont, England) was immunoprecipitated with protein A and protein G in a 96-well filtration plate and the radioactivity was counted with a TopCount scintillation counter (96-well plate β-counter; Packard). The result was expressed as an index where the index = (sample Δ_{cpm} − negative control Δ_{cpm})/(positive control Δ_{cpm} − negative control Δ_{cpm}). A value of 0.01 or greater was considered positive.

2.6. Complete Freund's adjuvant (CFA) treatment with or without human glucagon-like peptide 1 (GLP-1) analog

A single injection of CFA (Sigma–Aldrich, St. Louis, MO) combined with an equal volume (50 μl) of saline was administered subcutaneously into newly-diabetic NOD and NOD^{Rheb} mice, with or without the addition intraperitoneal injections of a long-acting human GLP-1 analog, Liraglutide (Novo Nordisk, Bagsvard, Denmark) at 9 μg for five consecutive days (Monday to Friday) in two consecutive weeks (10 injections total).

2.7. Statistical analysis

Diabetes incidence was analyzed by the log-rank test and Kaplan–Meier method. To analyze the insulitis score and IAA, the Mann–Whitney *U* test was used. For these analyses, GraphPad Prism 4 for Windows (GraphPad Software, San Diego, CA) was used.

3. Results

3.1. Rheb expression induces activation of the mTORC1 pathway in β-cells of NOD^{Rheb} mice

NOD^{Rheb} mice were created by intercrossing NOD mice with two independent $B6^{Rheb}$ lines (R3 and R20) that expressed different amounts of Rheb, and were verified as a congenic NOD strain by analysis of insulin-dependent diabetes susceptibility (Idd) genes 1–26 (data not shown). PCR amplification detected endogenous Rheb in both NOD and NOD^{Rheb} mice, but FLAG-Rheb was detected only in NOD^{Rheb} mice (Fig. 1A). To confirm transgenic Rheb expression in the pancreata of NOD^{Rheb} mice, FLAG-Rheb was

immunoprecipitated from tissue lysates of isolated pancreata with the anti-Flag antibody and analyzed by immunoblotting with the same antibody. The transgene product was detected in the pancreata of NOD^{Rheb} mice but not of NOD mice (Fig. 1B). Immunostaining of the pancreas sections with the anti-FLAG antibody also confirmed the expression of FLAG-Rheb in β-cells of NOD^{Rheb} mice, but not in NOD mice (Fig. 1C). In addition, to confirm the effect of FLAG-Rheb expression on mTORC1 in β-cells, we examined phosphorylation of ribosomal protein S6 as a readout of the mTORC1 pathway. Immunostaining with the anti-phospho-S6 antibody showed higher immunoreactivity in islets from NOD^{Rheb} mice than in those from NOD mice (Fig. 1D), indicating that the mTORC1 pathway is more highly activated in NOD^{Rheb} mice compared with NOD mice.

3.2. Diabetes progression in NOD^{Rheb} mice

In the R3 line, 84% (21/25) of the NOD^{Rheb} mice and 55% (11/20) of the NOD mice developed diabetes by 35 weeks of age. The median age of diabetes onset was 24 weeks for NOD^{Rheb} mice and 34 weeks for NOD mice, respectively. Diabetes progression was significantly accelerated in NOD^{Rheb} mice compared with NOD mice ($P = 0.0342$, log-rank test) (Fig. 2A). In the R20 line, 79% (19/24) of the NOD^{Rheb} mice and 68% (13/19) of the NOD mice developed diabetes by 35 weeks of age. The median age of diabetes onset was 22 weeks for NOD^{Rheb} mice and 25 weeks for NOD mice, respectively. Although the rate of diabetes progression was not significantly different between NOD^{Rheb} and NOD mice ($P = 0.2366$, log-rank test), it showed a tendency to acceleration (Fig. 2B).

3.3. NOD^{Rheb} islets

Immunostaining with anti-insulin and anti-glucagon antibodies was performed on pancreas from 4-week-old NOD^{Rheb} and NOD mice, before insulitis developed. NOD^{Rheb} mice showed β-cell enlargement compared with NOD mice (Fig. 2C). The area of β-cells relative to the area of the whole pancreas was much greater in NOD^{Rheb} mice than in NOD mice. To investigate whether Rheb overexpression in β-cells influences insulitis in NOD mice, the insulitis score was calculated based on the degree of leukocyte infiltration. In 5-week-old R3 mice, there was little insulitis and no difference between NOD^{Rheb} and NOD mice. At 12 weeks of age, insulitis increased in both NOD^{Rheb} and NOD mice, but NOD^{Rheb} mice showed significantly more severe insulitis than NOD mice ($P = 0.0411$, Mann–Whitney U test) (Fig. 2D). These results showed that Rheb overexpression promoted β-cell enlargement before insulitis and leukocyte infiltration into islets in NOD^{Rheb} mice, which is consistent with the accelerated diabetes progression.

3.4. The induction of IAA in NOD^{Rheb} mice

On histological analysis, NOD^{Rheb} mice showed more severe infiltration of immune cells into pancreatic islets than NOD mice. To investigate the difference in the autoimmune process in the presence or absence of transgenic Rheb, we examined serum IAA expression in NOD^{Rheb} of the R3 line and NOD mice at 12 weeks of age. Serum IAA expression in NOD^{Rheb} mice at 12 weeks of age was significantly higher than that in NOD mice ($P = 0.0418$, Mann–Whitney U test) (Fig. 3). This result suggests that the rapid induction of IAA indicates the severity of autoimmunity and accelerated disease progression in NOD^{Rheb} mice.

3.5. Reversal of hyperglycemia in newly-diabetic NOD and NOD^{Rheb} mice receiving CFA treatment with or without human GLP-1 analog

In the R3 line, one of three NOD mice and one of five NOD^{Rheb} mice achieved normoglycemia (Fig. 4A). In the R20 line, CFA treatment with or without human GLP-1 analog was attempted. Four of eight NOD mice achieved normoglycemia, whereas zero of nine NOD^{Rheb} mice, including two mice that received combination therapy, achieved normoglycemia (Fig. 4B). These results indicate that CFA treatment with or without human GLP-1 analog could not reverse hyperglycemia, suggesting that the β-cell enlargement because of Rheb overexpression might enhance the autoimmunity of pathogenic T-cells against islets and lead to acceleration of autoimmune diabetes.

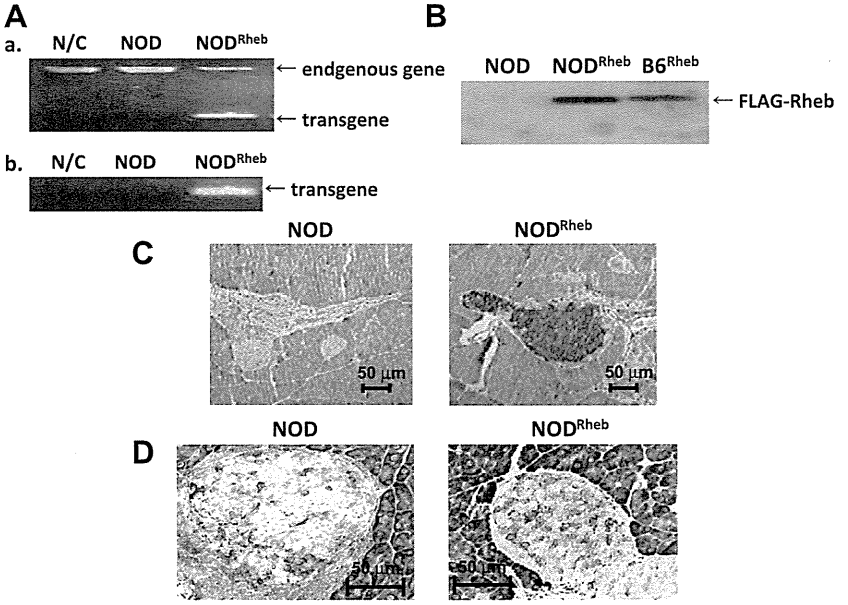


Fig. 1. Rheb overexpression in NOD^{Rheb} mice. (A) Rheb gene expression demonstrated by PCR amplification in NOD^{Rheb} mice (NOD^{Rheb}), wild type littermate NOD mice (NOD) and wild type NOD mice (N/C). (a) Endogenous and transgenic Rheb expression and (b) transgenic Rheb expression. (B) Immunoprecipitation and immunoblotting with anti-FLAG antibody performed using lysates of the pancreata isolated from NOD^{Rheb}, wild type littermate NOD and B6^{Rheb} mice as a positive control. (C) Immunostaining with the anti-FLAG antibody and (D) anti-phospho-S6 antibody of representative pancreas sections from 8-week-old R3 NOD^{Rheb} mice and wild type littermate NOD mice. (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

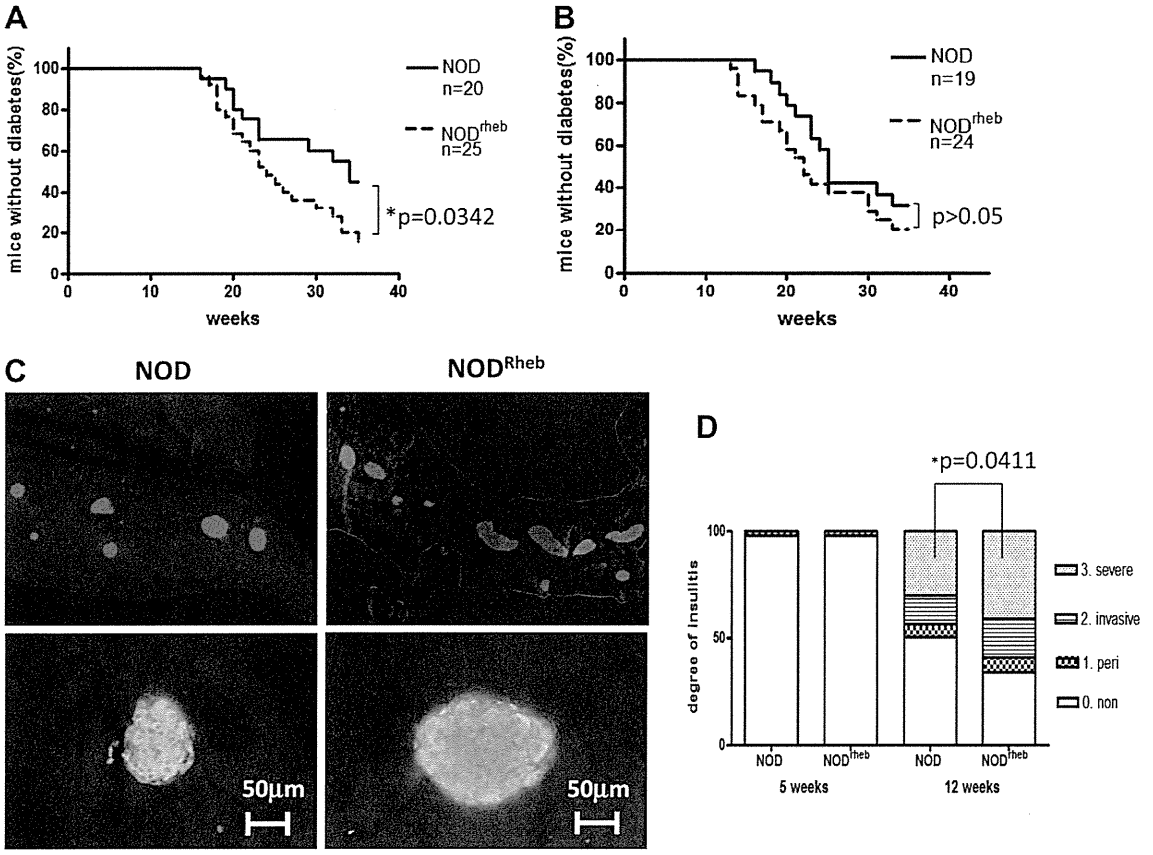


Fig. 2. Diabetes progression and histological analysis in NOD^{Rheb} mice. (A) Diabetes progression of NOD^{Rheb} mice of the R3 line: 84% (21/25) of the NOD^{Rheb} mice and 55% (11/20) of the NOD mice developed diabetes by 35 weeks of age. Diabetes progression was significantly accelerated in NOD^{Rheb} mice compared with NOD mice (log-rank test: $^*P = 0.0342$). (B) Diabetes progression of NOD^{Rheb} mice of the R20 line: 79% (19/24) of the NOD^{Rheb} mice and 68% (13/19) of the NOD mice developed diabetes by 35 weeks of age. Although diabetes progression was not significantly different between NOD^{Rheb} and NOD mice, it tended to be more rapid in the NOD^{Rheb} mice (log-rank test: $P = 0.2366$). (C) Immunostaining of representative pancreas sections from NOD^{Rheb} and NOD mice at 4 weeks of age with anti-insulin (red) and anti-glucagon (green) antibodies (upper panels; low-magnification, lower panels; high-magnification). NOD^{Rheb} mice showed β-cell enlargement compared with NOD mice. (D) Leukocyte infiltration (insulitis) in islets of 5- and 12-week-old NOD^{Rheb} mice. NOD^{Rheb} mice of the R3 line showed significantly more severe insulitis than NOD mice at 12 weeks of age (Mann–Whitney U test: $^*P = 0.0411$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

This study first focused on Rheb in the mTORC1 pathway in a model of autoimmune T1D. We hypothesized that diabetes onset should be prevented in NOD mice overexpressing Rheb in β-cells by mTORC1 activation and promotion of β-cell growth [14]. Unexpectedly, we have found that overexpression of Rheb in β-cells of NOD mice did not prevent but rather accelerated diabetes progression. B6^{Rheb} mice exhibited resistance to obesity-induced hyperglycemia. These mice also showed resistance to hyperglycemia in the MLDS-induced autoimmune diabetes model, in which diabetes is mainly caused in a nitric oxide-dependent fashion and macrophages play an important role in disease progression [18]. Therefore, we considered that overexpression of Rheb in β-cells should suppress diabetes progression in NOD mice, a spontaneous autoimmune diabetes model, by changing the balance between destruction and enlargement of β-cells. We created two NOD^{Rheb} mouse lines by intercrossing NOD mice with B6^{Rheb} mice overexpressing different amounts of Rheb in β-cells. Diabetes progression was more rapid and diabetes incidence was significantly higher in R3 NOD^{Rheb} mice than in NOD mice, while diabetes progression was also accelerated in R20 NOD^{Rheb} mice. Histological verification showed more severe insulitis in NOD^{Rheb} mice than in NOD mice, which is consistent with their accelerated diabetes progression. In addition, an assay of IAA showed more rapid induction of IAA in NOD^{Rheb} mice than in NOD mice, suggesting accelerated and increased severity of autoimmunity and diabetes in NOD^{Rheb} mice. Taken together, the severe insulitis and rapid induction of IAA suggested strong autoimmunity against the islets in NOD^{Rheb} mice. Thus, we found that diabetes progression was accelerated in this spontaneous autoimmune diabetes model overexpressing Rheb in β-cells.

Both MLDS-induced diabetes and spontaneous diabetes in NOD mice are models of autoimmune diabetes. However, the effect of Rheb overexpression on disease progression differed between these two models. In MLDS-induced diabetes, Rheb prevented diabetes, whereas in NOD mice, it accelerated diabetes. This discrepancy could be explained, at least in part, if the β-cell cytotoxicity in the former model mainly depends on macrophages and in the latter model on T-cells. In addition, cytotoxicity in the latter model should be much stronger than in the former, as we have previously reported [18].

Recently, overexpression of insulin receptor substrate-2 (Irs2) in β-cells of NOD mice was reported to reduce diabetes incidence [19]. In our study, however, overexpression of Rheb in β-cells of NOD mice resulted in the opposite effect. Transgenic Rheb overexpression and Irs2 overexpression both enlarged β-cell mass, but we have no evidence that Rheb promotes an increase in β-cell number, because we previously showed that staining for Ki-67, a cell proliferation marker, was not increased in B6^{Rheb} islets [15]. In contrast,

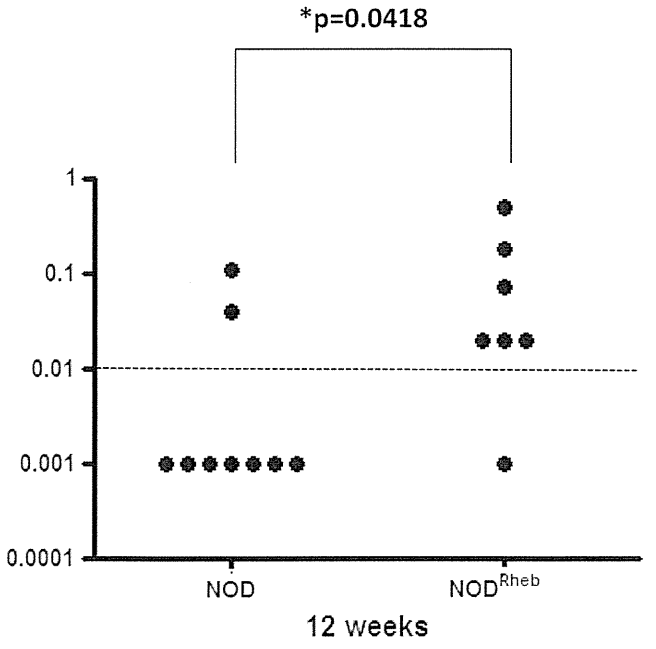


Fig. 3. Serum IAA expression. Serum IAA expression of NOD^{Rheb} mice of the R3 line and wild type littermate NOD mice was measured at 12 weeks of age. IAA expression in NOD^{Rheb} mice was significantly higher than that of NOD mice (Mann–Whitney *U* test: $^*P = 0.0418$).

Irs2 promoted an increase in β-cell number, because the staining of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU), another cell proliferation marker, was increased in NOD^{Irs2} islets in the study reported by Norquay et al. [19]. Taken together, transgenic Irs2 overexpression in NOD mice resulted in enlargement (increased size) with regeneration (increased number) of β-cells, whereas transgenic Rheb overexpression in NOD mice only resulted in β-cell enlargement. This suggests that the effect of Rheb is to shift the balance between destruction and enlargement of β-cells toward destruction compared with the effect of Irs2. We speculate that β-cell enlargement without regeneration may only enhance the autoimmunity of pathogenic T-cells against islets. Interestingly, Shigeyama et al. reported that TSC2 deficiency-associated mTORC1 activation eventually induced decreased β-cell number in spite of an initial increase in β-cell size [20]. Furthermore, it was also reported that mTORC1/ribosomal S6 activation in β-cells caused negative feedback inhibition on IRS signaling in a nonautoimmune mouse model [21]. In this report, they showed that S6K regulated the levels of IRS2, a major β-cell determinant for proliferation and survival, and that the negative feedback of S6K on IRS2 signaling could be a major modulator of β-cell mass and function, leading to increased apoptosis as a result of decreased survival signals from Akt. These data suggested that activation of S6K through mTORC1 activation induced insulin resistance by feedback inhibition of IRS2 signaling. In contrast, our two NOD^{Rheb} mouse lines that had different amounts of Rheb gene expression showed different patterns of diabetes progression. The R20 line of NOD^{Rheb} mice, with the higher transgene expression, showed no significant acceleration of diabetes progression, although it tended to be more rapid compared with NOD mice. However, the R3 line of NOD^{Rheb} mice, with lower transgene expression, developed diabetes significantly earlier than NOD mice [15]. These results suggest that it is not simply the amount of Rheb gene expression in β-cells that determines diabetes progression, but that the balance of the activation between mTORC1 signaling and IRS2 signaling may play a crucial role in disease progression.

Several studies of islet regeneration combined with immunological studies have been reported in the T1D field [9,22–29]. Faustman et al. previously reported that CFA would eliminate anti-islet autoreactive T-cells [22,23], while Singh et al. suggested that CFA might act to stimulate β-cell regeneration through an increase in Reg2 gene expression [29]. These studies suggest that, in addition to its blocking of autoimmunity, reversal of autoimmune NOD diabetes by CFA treatment would be, at least in part, the result of β-cell regeneration. Therefore, we hypothesized that in NOD^{Rheb} mice, CFA treatment with or without GLP-1 analog, which has also been reported to regenerate β-cells, would suppress the pathogenic T-cells that cause the strong autoimmunity against the islets, and reverse hyperglycemia by a combinatorial effect with Rheb overexpression [30]. Interestingly, almost none of the newly-diabetic NOD^{Rheb} mice injected with CFA achieved normoglycemia, although some diabetic NOD mice showed disease reversal and achieved normoglycemia. In addition, GLP-1 analog in combination with CFA could reverse hyperglycemia in NOD mice, but even this combination therapy could not reverse hyperglycemia in NOD^{Rheb} mice. These results indicate that even CFA treatment with GLP-1 analog could not overcome the strong autoimmunity against the islets of NOD^{Rheb} mice.

In summary, Rheb overexpression in β-cells of B6^{Rheb} mice led to β-cell enlargement with increased insulin secretion and resistance to obesity- and MLDS-induced hyperglycemia. However, in NOD mice, a model of spontaneous autoimmune T1D, diabetes progression was significantly accelerated rather than prevented. These findings provide us with important clues for T1D therapy.

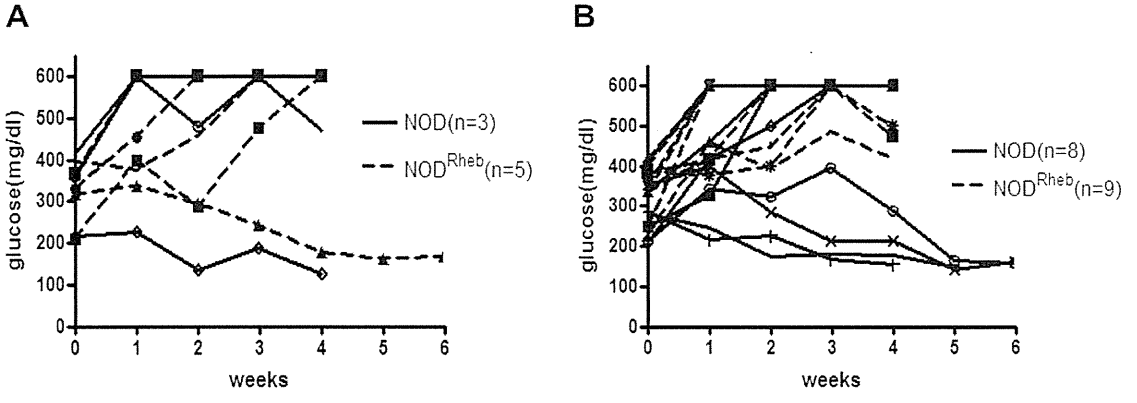


Fig. 4. Reversal of hyperglycemia in newly-diabetic NOD^{Rheb} mice and wild type littermate NOD mice that received CFA treatment with or without GLP-1 analog. (A) Reversal of hyperglycemia in NOD^{Rheb} mice and wild type littermate NOD mice of the R3 line after CFA treatment. One of three NOD mice and one of five NOD^{Rheb} mice achieved normoglycemia. (B) Reversal of hyperglycemia in NOD^{Rheb} mice and wild-type littermate NOD mice of the R20 line after CFA treatment with or without GLP-1 analog. Four of eight NOD mice achieved normoglycemia, whereas zero of nine NOD^{Rheb} mice, including two mice that received combination therapy, achieved normoglycemia.

Increased size, but not number, of β-cells might merely increase the target for pathogenic T-cells and enhance the autoimmunity against islets, resulting in acceleration of diabetes progression. In typical T1D, sufficient insulin replacement is indispensable to maintain a normal serum glucose level, because simply islet hyperplasia and promotion of insulin secretion may not lead to a solution. We conclude that not only enlargement but also regeneration of β-cells in addition to the prevention of β-cell destruction will be required for the ideal therapy of autoimmune T1D.

Acknowledgments

We thank Takashi Hamada and Atsumi Katsuta for excellent technical assistance. We are grateful to Shun-ichiro Asahara and Yoshiaki Kido for helpful discussion and technical assistance. This work was supported, in part, by Grant-in-aid for Research Activity Start-up from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 22890112).

References

[1] G.S. Eisenbarth, Type 1 diabetes mellitus. A chronic autoimmune disease, N. Engl. J. Med. 314 (1986) 1360–1368.
[2] M. Nakayama, N. Abiru, H. Moriyama, et al., Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice, Nature 435 (2005) 220–223.
[3] S. Makino, K. Kunimoto, Y. Muraoka, et al., Breeding of a non-obese, diabetic strain of mice, Jikken Dobutsu 29 (1980) 1–13.
[4] Z.J. Zhang, L. Davidson, G. Eisenbarth, et al., Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin, Proc. Natl. Acad. Sci. USA 88 (1991) 10252–10256.
[5] D. Daniel, D.R. Wegmann, Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9–23), Proc. Natl. Acad. Sci. USA 93 (1996) 956–960.
[6] P.R. Huchings, A. Cooke, Comparative study of the protective effect afforded by intravenous administration of bovine or ovine insulin to young NOD mice, Diabetes 44 (1995) 906–910.
[7] Diabetes prevention Trial-type 1 diabetes Study Group, Effects of insulin in relatives of patients with type 1 diabetes mellitus, N. Engl. J. Med 346 (2002) 1685–1691.
[8] S. Sreenan, A.J. Pick, M. Levisetti, et al., Increased β-cell proliferation and reduced mass before diabetes onset in the nonobese diabetic mouse, Diabetes 48 (1999) 989–996.
[9] T. Nir, D.A. Melton, Y. Dor, Recovery from diabetes in mice by β cell regeneration, J. Clin. Invest. 117 (2007) 2553–2561.
[10] S. Wullschleger, R. Loewith, M.N. Hall, TOR signaling in growth and metabolism, Cell 124 (2006) 471–484.
[11] K. Hara, Y. Maruki, X. Long, et al., Raptor, a binding partner of target of rapamycin (TOR) mediates TOR action, Cell 110 (2002) 177–189.
[12] D.H. Kim, D.D. Sarbassov, S.M. Ali, et al., GbetaL, A positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR, Mol. Cell 11 (2003) 895–904.
[13] K. Hara, K. Yonezawa, M.T. Kozlowski, et al., Regulation of eIF-4EBP1 phosphorylation by mTOR, J. Biol. Chem. 272 (1997) 26457–26463.
[14] J. Avruch, K. Hara, Y. Lin, et al., Insulin and amino-acid regulation of mTOR signaling and kinase activity through the Rheb GTPase, Oncogene 25 (2006) 6361–6372.
[15] S. Hamada, K. Hara, T. Hamada, et al., Upregulation of the mammalian target of rapamycin complex 1 pathway by Ras homolog enriched in brain in pancreatic beta-cells leads to increased beta-cell mass and prevention of hyperglycemia, Diabetes 58 (2009) 1321–1332.
[16] D.V. Serreze, H.D. Chapman, B. Varnum DS, et al., B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "Speed congenic" Stock of NOD. Ig mu null mice, J. Exp. Med. 184 (1996) 2049–2053.
[17] L. Yu, D.T. Robles, N. Abiru, et al., Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes, Proc. Natl. Acad. Sci. USA 97 (2000) 1701–1706.
[18] H. Yasuda, Z. Jin, M. Nakayama, et al., NO-mediated cytotoxicity contributes to multiple low-dose streptozotocin-induced diabetes but not to NOD diabetes, Diabetes Res. Clin. Pract. 83 (2009) 200–207.
[19] L.D. Norquay, K.E. D'Aquino, L.M. Opare-Addo, et al., Insulin receptor substrate-2 in beta-cells decreases diabetes in nonobese diabetic mice, Endocrinology 150 (2009) 4531–4540.
[20] Y. Shigeyama, T. Kobayashi, Y. Kido, et al., Biphasic response of pancreatic beta-cell mass to ablation of tuberous sclerosis complex 2 in mice, Mol. Cell. Biol. 28 (2008) 2971–2979.
[21] L. Elghazi, N. Balcazar, M. Blandino-Rosano, et al., Decreased IRS signaling impairs beta-cell cycle progression and survival in transgenic mice overexpressing S6K in beta-cells, Diabetes 59 (2010) 2390–2399.
[22] S. Ryu, S. Kodama, K. Ryu, et al., Reversal of established autoimmune diabetes by restoration of endogenous beta cell function, J. Clin. Invest. 108 (2001) 63–72.
[23] S. Kodama, S.W. Kuhtreiber, S. Fujimura, et al., Islet regeneration during the reversal of autoimmune diabetes in NOD mice, Science 302 (2003) 1223–1227.
[24] A.S. Chong, J. Shen, J. Tao, et al., Reversal of diabetes in non-obese diabetic mice without spleen cell-derived β cell regeneration, Science 311 (2006) 1774–1775.
[25] J. Nishio, J.L. Gaglia, S.E. Turvey, et al., Islet recovery and reversal of murine type 1 diabetes in the absence of any infused spleen cell contribution, Science 311 (2006) 1775–1778.
[26] A. Suri, B. Calderon, T.J. Esparza, et al., Immunological reversal of autoimmune diabetes without hematopoietic replacement of β cells, Science 311 (2006) 1778–1780.
[27] Y. Okubo, A. Shimada, Y. Kanazawa, et al., Hyperplastic islets observed in "reversed" NOD mice treated without hematopoietic cells, Diabetes Res. Clin. Pract. 79 (2008) 18–23.
[28] Y. Mori, T. Kodaka, T. Kato, et al., Critical role of IFN-gamma in CFA-mediated protection of NOD mice from diabetes development, Int. Immunol. 21 (2009) 1291–1299.
[29] K. Huszarik, B. Wright, C. Keller, et al., Adjuvant immunotherapy increases beta cell regenerative factor Reg2 in the pancreas of diabetic mice, J. Immunol. 185 (2010) 5120–5129.
[30] G. Xu, D.A. Stoffers, J.F. Habener, et al., Exendin-4 stimulates both β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rat, Diabetes 48 (1999) 2270–2276.

INTERNAL MEDICINE

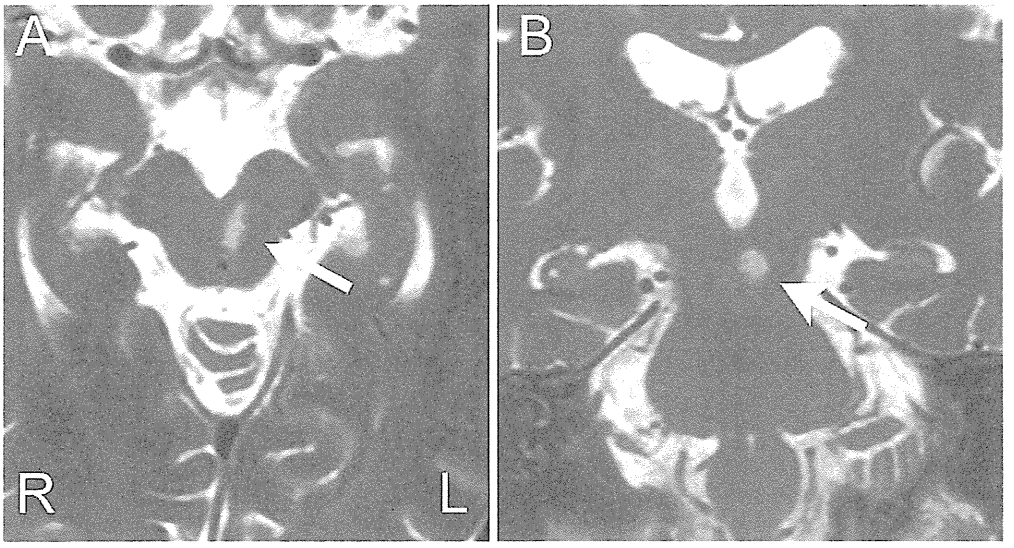
□ PICTURES IN CLINICAL MEDICINE □

Vertical Diplopia due to Midbrain Infarction

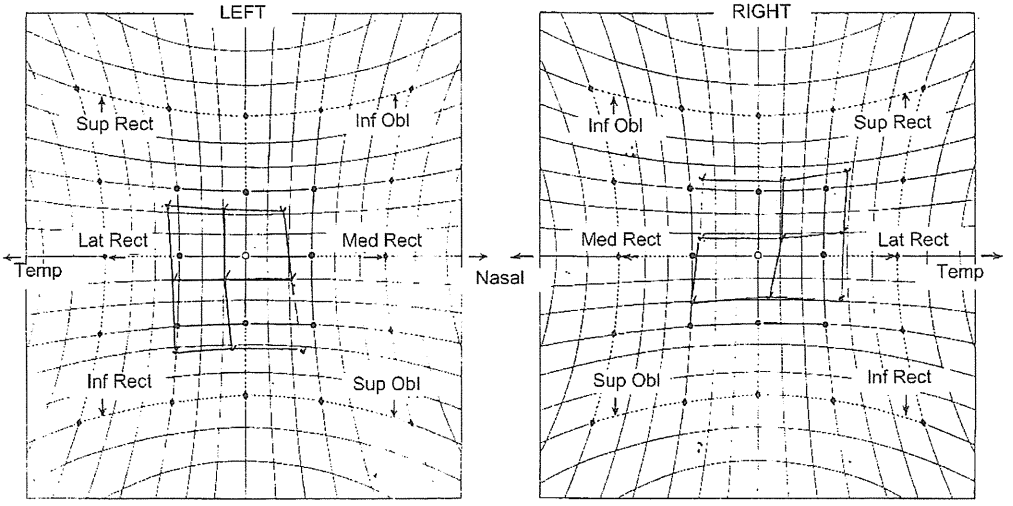
Naoki Saji[1,2], Minoru Tanigawa[3], Yasushi Kita[1] and Koichi Yokono[2]

Key words: Hess chart, inferior oblique palsy, midbrain infarction, Parks' three-step test, vertical diplopia

(Intern Med 50: 531-532, 2011)
(DOI: 10.2169/internalmedicine.50.4687)



Picture 1.



Picture 2.

[1]Department of Neurology, Hyogo Brain and Heart Center at Himeji, Japan, [2]Department of General Internal Medicine, Kobe University Graduate School of Medicine, Japan and [3]Department of Ophthalmology, Nippon Steel Hirohata Hospital, Japan
Received for publication October 14, 2010; Accepted for publication November 16, 2010
Correspondence to Dr. Naoki Saji, sajink@nifty.com

A 78-year-old woman with hypertension and dyslipidemia developed vertical diplopia and gait disturbance. Neurological examination revealed left-sided partial ptosis, skew deviation, dysarthria, right limb ataxia, and body lateropulsion. Magnetic resonance imaging (MRI) showed a tiny midbrain infarction (Picture 1). Parks' three-step test and Hess charts (Picture 2) confirmed isolated left inferior oblique palsy. A paramedian lower midbrain infarction can involve decussation of the superior cerebellar peduncles and the partial fascicular oculomotor fibers (1, 2). This case suggests that fibers controlling the inferior oblique and levator palpebrae muscles might in part be located adjacent to the muscles. This mechanism could explain her clinical symptoms.

It is possible that MRI can be used to analyze morphological changes in the brain, but functional change might not be analyzed, whereas ophthalmological assessments by Parks' three-step test and Hess charts are possible. In cases with vertical diplopia, not only brain MRI but also these ophthalmological methods are useful in detecting ocular motor impairment.

The authors state that they have no Conflict of Interest (COI).

References

1. Lee H. Body lateropulsion as a presenting symptom of rostral paramedian midbrain syndrome. Eur Neurol **60**: 101-103, 2008.
2. Castro O, Johnson LN, Mamourian AC. Isolated inferior oblique paresis from brain-stem infarction. Perspective on oculomotor fascicular organization in the ventral midbrain tegmentum. Arch Neurol **47**: 235-237, 1990.

Geriatrics Gerontology INTERNATIONAL 1st Impact Factor in June 2010 Medline Indexed

Geriatr Gerontol Int 2011; **11:** *297–303*

ORIGINAL ARTICLE: EPIDEMIOLOGY, CLINICAL PRACTICE AND HEALTH

Causes of decreased activity of daily life in elderly patients who need daily living care

Hiroshi Yoshino,[1] Takashi Sakurai,[1] Kazuo Hasegawa[2] and Koichi Yokono[1]

[1]*Department of Internal and Geriatric Medicine, Kobe University Graduate School of Medicine, Kobe, and* [2]*Inamino Hospital, Kakogawa, Japan*

Aim: The causes of decreased activity of daily life (ADL) in elderly patients include cerebrovascular diseases, bone fracture by falls, and dementia. The present study was conducted among elderly patients with decreased ADL who were hospitalized in nursing wards in order to investigate the causes of becoming early bedridden and to determine precautionary measures against decreased ADL.

Methods: The study subjects were 224 elderly patients with decreased ADL (mean age: 83.3 ± 8.0 years) and 49 outpatients without decreased ADL (mean age: 76.8 ± 5.3 years). Current age, age at the start of ADL decrease, medical history and history of smoking were investigated.

Results: In the groups with decreased ADL, current age and the age of becoming bedridden in non-diabetic versus diabetic groups were 84.7 ± 7.9 versus 80.3 ± 7.5 and 82.7 ± 8.3 versus 77.6 ± 8.0 years, respectively, both showing significantly lower values in the diabetic group ($P < 0.05$). Multiple regression analysis revealed that sex difference and diabetes were the factors determining the age of becoming early bedridden. Diabetic patients with smoking habit were significantly younger than diabetic and non-diabetic patients without smoking habit.

Conclusion: Sex difference, smoking habit and presence of diabetes mellitus are independent risk factors of becoming early bedridden. Therefore, the major targets of medical care among elderly should be diabetic men with a smoking habit to lower the risks of decreased ADL. **Geriatr Gerontol Int 2011; 11: 297–303.**

Keywords: activity of daily life, bedridden, diabetes mellitus, elderly, smoking habit.

Introduction

In our country, an aging population is already prominent and we will face further increase in the elderly population who need daily living care. The financial and psychological burden of families as well as the rise of medical expenditure in the national budget have become serious social problems demanding urgent countermeasures.

Accepted for publication 29 November 2010.

Correspondence: Dr Hiroshi Yoshino MD PhD, Department of Internal Medicine, Kohnan Hospital, 1-5-16 Kamokogahara, Higashinada-ku, Kobe 658-0064, Japan. Email: ko_h.yoshino@kohnan.or.jp

The causes of decreased activities of daily living (ADL) of Japanese elderly include cerebrovascular diseases (27.7%), bone fracture by falls (11.8%) and dementia (10.7%), all of which result from complicated or overlapped lifestyle diseases.[1]

On the other hand, the incidence of metabolic syndrome, which is a combination of lifestyle diseases, has continued to increase with age in Japan, with high rates among men (29.7%) and women (19.3%) alike after 70 years of age.[2] Failure to intervene in metabolic syndrome is usually followed by the onset of type 2 diabetes mellitus in a short time. It has been reported that, once the diagnosis of diabetes mellitus is made, overall life expectancy is shortened by approximately 7 years.[3]

doi: 10.1111/j.1447-0594.2010.00683.x

In older populations, failure of independent living or self-support increases with disturbed ADL or cognitive functions due to major and minor vascular diseases.

Because these conditions significantly compromise quality of life (QOL), early and vigorous control of lifestyle diseases is required to maintain QOL among the elderly.

According to the World Health Organization, the health age, which refers to the age without decreased ADL, of the Japanese is 74.1 years, while the average life expectancy is approximately 80 years (men 78.6, women 85.6 years).[4] In particular, falls among the elderly is one of the important causes of decreased ADL, which is experienced by 30% of the US population aged 75 years or older.[5,6] Investigation on the risk factors of falls, therefore, would be helpful in reducing mortality and morbidity in this age group. The National Service Framework for the elderly also emphasizes the prevention of falls, especially in the high-risk group.[7–10]

However, comprehensive studies have rarely been conducted on the causes of decreased ADL such as nutritional status and atherosclerotic conditions as well as the presence of lifestyle diseases including type 2 diabetes among the elderly. It is well-known that patients with diabetes mellitus develop complications such as retinopathy at late stage, neuropathy and nephropathy, which may lead to decreased ADL. Therefore, we hypothesized that age of becoming bedridden in diabetic patients is younger than non-diabetic patients.

Consequently, this study was conducted on elderly patients with decreased ADL who were hospitalized in nursing wards in order to investigate the causes of decreased ADL, to evaluate nutritional status and atherosclerotic conditions, and to determine precautionary measures against decreased ADL.

Methods

The study subjects consisted of 224 elderly patients (mean age: 83.3 ± 8.0 years) with decreased ADL who were hospitalized in Inamino Hospital, Hyogo, Japan (Table 1). A total of 155 patients were non-diabetic (113 female) and 69 patients had diabetes mellitus (47 female). Sixty non-diabetic and 29 diabetic patients with decreased ADL were excluded from the analysis of age of decreased ADL, because of the lack of exact information concerning the age at decreased ADL from their families.

On the other hand, 49 outpatients (mean age: 76.8 ± 5.3 years) at Kobe University Hospital with favorable ADL were enrolled as the control group, of which 22 patients were non-diabetic (15 female) and 27 patients had diabetes mellitus (10 female). Informed consent was signed by the families of all hospitalized

Table 1 Characteristics of 2 study groups

	Decreased ADL (n = 224)	Favorable ADL (n = 49)
Age (years)	83.3 ± 8.0	76.8 ± 5.3
Age of decreased ADL (years)	81.2 ± 8.5 (n = 135)	
BMI (kg/m^2)	18.4 ± 3.4	21.0 ± 2.9
Alb (g/dL)	3.4 ± 0.4	4.1 ± 0.3
TC (mg/dL)	171.3 ± 37.6	202.4 ± 34.6
TG (mg/dL)	90.5 ± 40.9	126.8 ± 70.1
HDL-C (mg/dL)	53.0 ± 16.9	64.4 ± 21.6
LDL-C (mg/dL)	99.9 ± 30.7	113.9 ± 27.8
SBP (mmHg)	122.2 ± 20.6	131.7 ± 17.4
DBP (mmHg)	67.2 ± 11.8	67.4 ± 11.0
IMT (mm)	1.2 ± 0.5 (n = 112)	
HDS-R	10.8 ± 8.1 (n = 117)	
CVD	39.2% (n = 135:yes 53, no 82)	
Fall fracture	24.4% (n = 135:yes 33, no 102)	
Dementia	9.6% (n = 135:yes 13, no 122)	
Infection	9.6% (n = 135:yes 13, no 122)	
Smoking	24.0% (n = 104:yes 25, no 79)	

ADL, activity of daily life; Alb, serum albumin; BMI, body mass index; CVD, cerebrovascular disease; DBP, diastolic blood pressure; DM, diabetes mellitus; HDL-C, high-density lipoprotein cholesterol; HDS-R, Hasegawa dementia scale – Revised; IMT, intima-media thickness; LDL-C, low-density lipoprotein cholesterol; NDM, non-diabetic; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride.

patients and by the outpatients themselves. This study was approved by each local ethics committee. This study was performed from April 2005 to March 2008.

With regard to independent living of the disabled, we used the classification of the Japanese long-term care insurance, patients were classified as chair-bound (B) (39.3%) and the others were classified as bed-bound (C) (60.7%).[11]

According to medical record information provided by the families, the causes of decreased ADL were categorized into cerebrovascular diseases, bone fracture by fall, dementia, infection and others. Current age, age at the start of ADL decrease, intima-media thickness (IMT) measured by carotid artery ultrasonography, medical history, body mass index (BMI), blood pressure, blood glucose, HbA1c, lipid profiles (total cholesterol [TC], low-density lipoprotein cholesterol [LDL-C], high-density lipoprotein cholesterol [HDL-C], small dense LDL cholesterol [sLDL-C], triglyceride [TG]) and serum albumin were investigated. History of cigarette smoking was also taken. The definition of "smoking habit" indicates patients who had experienced smoking. All patients with impaired ADL were not current smokers because smoking was prohibited in the hospital.

The severity of dementia was evaluated using the Hasegawa Dementia Scale – Revised (HDS-R). Blood sugar, HbA1c, lipid profiles and sLDL-C were measured using the hydrogen peroxide electrode method, high-performance liquid chromatography, an automated lipid analyzer and the method reported by Hirano *et al.*,[12] respectively.

Simple regression analysis for age at the start of ADL reduction was performed with respective risk factors as independent variables (sex, diabetes mellitus, BMI, cerebrovascular diseases and serum albumin levels). Thereafter, multivariate regression analysis was performed using StatView ver. 5.0 for Windows in order to find the independent association of lifestyle risk factors with the age of becoming bedridden. Hypertension and dyslipidemia were entered as covariates besides variables that were shown to have significant simple correlation with the age at the start of ADL reduction. ANOVA followed by Scheffe's multiple comparison test was used for analysis between four study groups. The χ^2-test was also employed for comparison of frequency of bone fracture between the non-diabetic and diabetic groups of decreased ADL. Data were expressed as mean ± standard deviation.

Results

In the groups with decreased ADL, current age and the age at the start of ADL decrease of the diabetic group were lower than non-diabetic patients ($P < 0.05$ by ANOVA, Table 2). BMI and serum albumin tended to be higher in diabetic patients with decreased ADL. The levels of LDL-C and TG were higher in groups with favorable ADL and without diabetes mellitus. Blood pressure was not significantly different between any group. In the decreased ADL group, lipid parameters (except for TG) and IMT on carotid artery ultrasonography did not show any significant differences between

Table 2 Characteristics of four study groups

	Decreased ADL Non-diabetics ($n = 155$)	Diabetics ($n = 69$)	Favorable ADL Non-diabetics ($n = 22$)	Diabetics ($n = 27$)
Age (years)	84.7 ± 7.9†	80.3 ± 7.5‡	77.0 ± 5.9‡	76.7 ± 4.9‡
Age of decreased ADL (years)	82.7 ± 8.3† ($n = 95$)	77.6 ± 8.0‡ ($n = 40$)		
BMI (kg/m^2)	17.9 ± 3.3†	19.6 ± 3.2‡	20.5 ± 3.0‡	21.4 ± 2.8‡
Alb (g/dL)	3.3 ± 0.4†	3.5 ± 0.4‡	4.1 ± 0.3§	4.1 ± 0.3§
HbA1c (%)		6.4 ± 1.1		7.2 ± 1.2
TC (mg/dL)	170.1 ± 37.0†	174.1 ± 37.4†	212.8 ± 39.5‡	193.9 ± 27.9‡
TG (mg/dL)	88.0 ± 41.0†	95.9 ± 40.6‡	133.5 ± 64.0§	121.4 ± 75.5§
HDL-C (mg/dL)	54.6 ± 17.2†	52.3 ± 16.2†	70.0 ± 17.1‡	60.1 ± 14.0§
LDL-C (mg/dL)	99.1 ± 30.7†	102.6 ± 31.3†	119.3 ± 31.6‡	110.0 ± 23.9‡
SBP (mmHg)	120.5 ± 20.5†	125.7 ± 20.3†	133.6 ± 19.3†	130.2 ± 16.0†
DBP (mmHg)	67.6 ± 12.0†	66.4 ± 11.4†	65.3 ± 12.5†	69.1 ± 9.6†
IMT (mm)	1.2 ± 0.3†	1.2 ± 0.6†		
HDS-R	9.8 ± 7.6† ($n = 66$)	12.2 ± 8.6‡ ($n = 51$)		

†‡§There are significant differences between the groups not sharing the same symbol by ANOVA ($P < 0.05$). ADL, activity of daily living; Alb, serum albumin; BMI, body mass index; DBP, diastolic blood pressure; DM, diabetes mellitus; HDL-C, high-density lipoprotein cholesterol; HDS-R, Hasegawa Dementia Scale – Revised; IMT, intima-media thickness; LDL-C, low-density lipoprotein cholesterol; NDM, non-diabetic; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride.

Table 3 Simple regression analysis to explore the determinant of disabled age of decreased ADL patients

	Regression coefficient	95% CI, upper	95% CI, lower	*P*-value
Sex (M 1, F 0)	−4.78	−1.33	−8.22	<0.05
BMI	−0.54	−0.12	−0.96	<0.05
Alb	−5.13	−1.89	−8.36	<0.05
DM (yes 1, no 0)	−5.19	−2.13	−8.25	<0.05
CVD (yes 1, no 0)	−3.16	−0.23	−6.09	<0.05
Fall fracture (yes 1, no 0)	1.72	5.24	−1.80	<0.05
Smoking (yes 1, no 0)	−0.01	−0.001	−0.02	0.07
HT (yes 1, no 0)	1.78	4.68	−1.12	0.23
DL (yes 1, no 0)	−1.84	−1.41	−5.09	0.26

ADL, activities of daily living; Alb, serum albumin; BMI, body mass index; CI, confidence interval; CVD, cerebral vascular disease; DL, dyslipidemia; DM, diabetes mellitus; HT, hypertension.

non-diabetic and diabetic groups (Table 1). sLDL-C level was measured in 24 non-diabetic and 18 diabetic patients, which were significantly higher in the diabetic group (16.7 ± 11.2 vs 26.2 ± 15.5 mg/dL, $P < 0.05$). Cognitive function was evaluated by HDS-R in 66 of 155 (42.5%) and 51 of 69 (73.9%) patients in non-diabetic and diabetic patients of the decreased ADL group, respectively, which is significantly higher in the diabetic patients of the decreased ADL group. However, the scores were 9.8 ± 7.6 and 12.2 ± 8.6, respectively, and the difference was not significant.

In the group with decreased ADL, 95 (20 male, 75 female) and 40 (11 male, 29 female) patients were non-diabetic and diabetic, respectively, at the age of becoming bedridden, while 22 patients were non-diabetic (seven male, 15 female) and 27 patients had diabetes mellitus (17 male, 10 female) in the favorable ADL group.

In the decreased ADL group, the coronary risk levels were categorized according to the number of risk factors (hypertension, dyslipidemia, diabetes mellitus) into three groups: the group with three risk factors, the group with two risk factors, the group with a single risk factor and the group with no risk factor. The age at the start of ADL decrease of the group with three risk factors was 77.2 ± 10.5 years, the group with two risk factors, with a single risk factor and with no risk factor were 80.5 ± 8.6, 81.3 ± 9.0 and 82.5 ± 7.3 years, respectively.

Causes of decreased ADL were clarified in 95 non-diabetic and 40 diabetic patients. The incidence of cerebrovascular diseases was 47.5% and 35.5% in diabetic and non-diabetic participants, respectively, and diabetic bedridden patients after cerebrovascular diseases were younger than non-diabetic individuals (75.1 ± 8.0 vs 82.0 ± 10.0 years, $P < 0.05$). The frequency of patients with bone fracture by fall in the diabetic group was higher than in the non-diabetic (32.5% vs. 21.1%) but the difference was not significant by the χ^2-test. The prevalence of dementia as a reason for ADL reduction was 7.5% and 16.1% in the diabetic and non-diabetic groups, respectively. While 10.5% of non-diabetic patients were bedridden after some serious infection such as pneumonia, no bedridden case after infection was found in the diabetic group.

Simple regression analysis for the age at the start of ADL reduction were performed with respective risk factors as independent variables. Male sex ($P = 0.01$), presence of diabetes mellitus ($P = 0.01$), higher BMI ($P = 0.01$), cerebrovascular diseases ($P = 0.03$) and higher levels of serum albumin ($P = 0.002$) were significantly associated with younger age of becoming bedridden (Table 3). To find the independent association of lifestyle risk factors with the age of becoming bedridden, hypertension and dyslipidemia were entered as covariates besides variables that were shown to have significant correlation ($P < 0.05$) in subsequent multivariate regression analysis. As a result, male sex, higher BMI, higher levels of serum albumin and presence of diabetes mellitus were the independent factors determining the age of becoming bedridden, while hypertension and dyslipidemia were not selected as an independent determinant (Table 4). These results showed the pronounced effects of diabetes on the severe impairment of ADL.

Because smoking habit seemed to have a substantial impact on the age at the start of ADL reduction, we further compared the additive effects of diabetes and smoking on the age of becoming bedridden. As shown in Figure 1, diabetic patients with smoking habit were significantly younger than diabetic and non-diabetic patients without smoking habit.

Discussion

As already mentioned, the population requiring daily living care in Japan has been steadily increasing. The

Table 4 Multiple regression analysis to explore the determinant of disabled age of decreased ADL patients

	Regression coefficient	95% CI, upper	95% CI, lower	*P*-value
Sex (M 1, F 0)	−4.40	−1.10	−7.69	<0.05
BMI	−0.43	−0.02	−0.84	<0.05
Alb	−4.16	−1.05	−7.27	<0.05
DM (yes 1, no 0)	−3.69	−0.68	−6.70	<0.05
CVD (yes 1, no 0)	−2.00	−0.86	−4.86	0.17
HT (yes 1 no 0)	2.00	4.64	−0.72	0.15
DL (yes 1, no 0)	−0.05	3.12	−3.22	0.98

ADL, activities of daily living; Alb, serum albumin; BMI, body mass index; CI, confidence interval; CVD, cerebrovascular disease; DL, dyslipidemia; DM, diabetes mellitus; HT, hypertension.

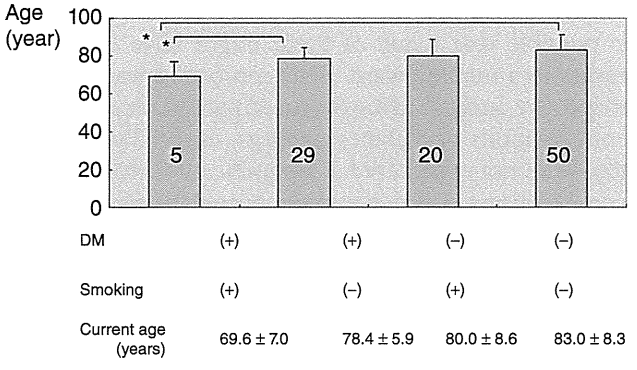


Figure 1 Mean age of bedridden of diabetic and non-diabetic patients with and without smoking habit. The number in the column represents the number in each group. *There are significant differences between each group by ANOVA ($P < 0.05$). DM, diabetes mellitus.

causes of this status are considered to include stroke, dementia and bone fracture by falls, all of which are closely associated with the progression of cerebral atherosclerosis. We conducted this study to investigate the causes of decreased ADL and the bedridden status, as well as to determine precautionary measures for shortening the bedridden period.

In the two groups with decreased ADL, the mean ages of the diabetic group were significantly younger than those of the non-diabetic groups. In other words, elderly diabetics will reach bedridden status approximately 5 years earlier than non-diabetics. Indeed, it is obvious that aging is one of the most important factors for decreased ADL. However, another group of old outpatients with comparable age to that of the decreased ADL group was not available this time. The diabetic patients with impaired ADL were under strict energy control in the hospital. On the other hand, the diabetic patients without impaired ADL were all outpatients and therefore they had free access to any food. Thus, diabetic patients without impaired ADL showed a higher HbA1c level than that of those with impaired ADL.

Roliz-Cruz *et al.* have reported that metabolic syndrome carries a 2.2-times higher risk for decreased ADL than the non-metabolic elderly population.[13] Because hyperglycemia is a major component of metabolic syndrome, the results of our study support this estimate.

The numbers of patients who were able to be evaluated using HDS-R were 51 of 69 (73.9%) and 66 of 155 (42.5%), which was obviously higher in the diabetic group. Furthermore, the mean HDS-R score did not differ between the two groups. It was suggested that the diabetic group was younger and the their periods after becoming bedridden were shorter than the non-diabetic group, and consequently, patients with more severe dementia were fewer in the diabetic group. The frequency of cerebrovascular diseases in the diabetic group was higher than that of the non-diabetic group. Also, diabetic patients who had decreased ADL by cerebrovascular disease were significantly younger than non-diabetics. From these results, it can be concluded that diabetics have a higher risk of becoming bedridden due to stroke. In support of this, it has been widely reported that diabetics have a higher mortality, with cerebrovascular diseases being an independent risk factor.[14–18]

Dementia is known to be one of the complications of the cerebrovascular disease. According to the Copenhagen Stroke Study, it was proven that the mean age of patients with cerebrovascular disease complications was younger in the diabetic group than in the non-diabetic group by 3.2 years.[16] On the other hand, in this study the frequency of dementia was not higher in the diabetic group than the non-diabetic group. The influence of older mean age in the non-diabetic group than in the diabetic group was suggested with regard to dementia.

A recent Taiwanese study on diabetes mellitus and bone fracture has reported a higher risk of femoral fracture in diabetic patients.[19] Functional impairment of osteoblasts[20] and apoptosis of osteoblasts induced by enhanced gluconeogenesis[21] have been suggested as the underlying mechanisms. Menz *et al.* have reported that diabetic individuals with peripheral neuropathy had

impaired peripheral sensation and reaction time, and had impaired ability to stabilize their body when walking on irregular surfaces.[22] They also had reduced walking speed and step length, and less rhythmic acceleration patterns at the head and pelvis compared with controls.[23] In this study, the experience of bone fracture in diabetic subjects with decreased ADL was more frequent than that of non-diabetics, but the difference was not significant. Further study will contribute to better understanding of the influence of bone fracture on decreased ADL of diabetic patients.

Infection was considered to be the cause of decreased ADL in 12.9% and 0% of the patients in the non-diabetic and diabetic groups, respectively. This is contrary to the fact that the defense mechanism against infection is weakened in diabetics. We believe that further research is needed to clarify this finding. With regard to sex, men showed an odds ratio of 2.11 on diabetes and fracture, which are both associated with decreased ADL.[24] Furthermore, because increased BMI may lead to failure of independent living, men over 50 years should particularly be paid attention to in this index.[25]

In this study, the levels of sLDL-C were significantly higher in the diabetic group than in the non-diabetic group (17.0 ± 11.4 vs 25.2 ± 10.6 mg/dL; $P < 0.05$ by ANOVA). The atherogenic phenotype, which refers to a tendency to demonstrate a predominant sLDL-C, has been reported to have a higher risk of myocardial infarction.[26] Increased sLDL-C has also been reported in diabetics.[27] Increased sLDL-C in the diabetic group suggests susceptibility to cerebrovascular diseases in elderly diabetics, and consequently, lower age at becoming bedridden than in the non-diabetic group.

In spite of the overt higher risk in diabetics, plaque scores on carotid artery ultrasonography were not significantly different between the two groups. This may be due to the younger mean age of patients in the diabetic group.

Simple regression analysis on age of becoming bedridden suggested a correlation with sex, BMI, diabetes mellitus and serum albumin. Multiple regression analysis revealed that sex, BMI, serum albumin and the presence of diabetes mellitus were the factors determining the age of becoming bedridden. However, because BMI scores used in this study were determined from weights measured during the observation period, which might differ from those measured at hospitalization from decreased ADL, BMI cannot be considered as one of the causes of the bedridden status. In addition, multiple regression analysis using age at the bedridden status as a dependent variable and the presence of diabetes mellitus and smoking history as independent variables suggested that both diabetes mellitus and smoking history were correlated with the age of becoming bedridden. Therefore, it can be concluded that diabetic men with a smoking history among the elderly become bedridden at the youngest age.

The limitations of the present study are as follows: First, because this investigation is a cross-sectional study of a number of severely demented patients with a mean HDS-R score of 10.8, the causes of decreased ADL were estimated from medical records instead of being directly obtained from the patients. Second, because the range of the subjects examined was limited to patients hospitalized in a nursing ward, it was difficult to compare the examined groups to a healthy elderly group. Third, with regard to diabetes mellitus, interpretation was not performed regarding types (two type I vs 67 type II patients) and treatments (32% with insulin vs 36% with oral hypoglycemic agents). Fourth, the number of bedridden diabetics with smoking habit was only five. Because this patient group was very young, it is possible that many of them might have been dead earlier in a nursing ward. This conjecture warrants retrospective analysis using deceased patient records. Fifth, complications of diabetes mellitus, especially retinopathy, were not considered as a significant factor. Because complications of diabetes mellitus such as visual disturbance, peripheral neuropathy and nephropathy have been reported to be risk factors of falls in the elderly,[28] this area needs further studies. Finally, causes of dependence should be multi-factorial and heterogeneous. However, undernutrition cannot be the main cause of dependence in our wards although undernutrition can be the results of bedridden status.

In conclusion, among diabetes mellitus, hypertension and dyslipidemia, this study showed that diabetes mellitus is an independent risk factor of becoming bedridden. In the diabetic groups, cerebrovascular diseases were the major causes of becoming bedridden at a younger age. Also smoking habit was an independent determinant of becoming bedridden at a younger age. Therefore, the major targets of medical care among elderly should be diabetic male patients with a smoking habit in order to lower the risk of becoming bedridden at a younger age.

Acknowledgments

There is no financial support.

References

1 The Ministry of Health Labour and Welfare. Vital stastics 2001 situation of nursing care. [Cited 1 Mar 2009.] Available from URL: http://www.mhlw.go.jp/toukei/saikin/hw/k-tyosa/k-tyosa01/3-2.html
2 The Ministry of Health and Welfare. Health Station General Administration Division measures:Nation health nourishment investigation 2006 P36-39. [Cited 6 Mar