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ORIGINAL ARTICLE

Simple screening test for risk of falls in the elderly

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Background: The aim of this study is to construct a simple screening test for the risk of falls in community-dwelling elder persons.

Methods: A total of 1378 community-dwelling people aged 65 years and older in five different communities in Japan were asked to answer a self rated questionnaire including 22 items covering physical, cognitive, emotional and social aspects of functioning and environmental factors. At a six-month follow-up, the outcome of fall occurrence and the number of falls was ascertained by social workers, health visitors or nurses.

Results: Five out of 22 items were selected using a logistic regression model. Using this five-item version, a screening test was constructed, and at the best cut-off point, the sensitivity and specificity were 68% and 70%, respectively. The validity of this scale was tested on persons with cognitive dysfunction.

Conclusion: The simplicity and the predictive validity of the screening test support the use of this test in health check ups or general outpatient facilities.

Keywords: accidental fall, aged, mass screening, reliability and validity, risk factor.

Introduction

Falls are rated as the third leading cause of a bed-ridden state and are among the principal causes of morbidity in the elderly in Japan.[1] Previous studies evaluating the risk factors for falls have used history of falls, results of physical performance tests,[2] activity of daily living (ADL)[2,3] and balance and gait[4] as predictors.

Early identification of falls risk is likely to result in earlier implementation of interventions and to minimize development of unwanted sequels such as reduced confidence and activity levels.[5]

In Japan, the Ministry of Health, Labour and Welfare has put roughly 6000 local home care support centers around Japan. The task of these centers, according to Long-Term Care Insurance for the elderly, includes screening of the elders at risk of developing disabilities, including risk for falls. In this context, it is critical to develop a simple screening test to adequately evaluate the risk of falls for each elderly person.

The aim of this study is to evaluate predictive validity of a simple questionnaire composed of 22 items, with the intention of constructing a shortened version that would be simple, but effective to assess the future risk of falls during periodic health check-up or outpatient visits.

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All elderly persons who participated in this research gave written informed consent.

Methods

The initial 22-item questionnaire was constructed by the Working Group of Fall Prevention commissioned by the Japanese Ministry of Health, Labour and Welfare. Known risk factors are transformed into comprehensible text for the elderly, as shown in Table 1. These items were selected by studying both international and Japanese research articles on fall risk factors.[6]

The interclass coefficient (ICC) of the one month test-retest reproducibility study of the 22-item questionnaire score was satisfactory (ICC 0.74, 95% CI 0.46–0.89, $n = 21$).[6]

Individuals chosen for this study lived in five different urban and rural communities and they were over 65 years old.

In cases where subjects had cognitive impairment or difficulty answering, a family member acted as a proxy to help answer the questionnaire.

The outcome of fall occurrence and the number of falls were confirmed by social workers, health visitors or nurses six months after baseline measurement. A fall was defined as an unintentional change in position resulting in coming to rest on the ground or other lower positions.[3]

Statistical analysis was performed on subjects who completed the questionnaire both at baseline and at six month follow-up. One half of the subjects were randomly selected, and the relationship between falls and potential predictors was examined by χ^2 test for each predictor separately (developing samples). Items that achieved statistical significance of $P < 0.05$ were incorporated in the logistic regression analysis to identify predictors. Then, the questionnaire items considered to be associated with falls were selected using any falls as an outcome variable, by forward stepwise selection by the logistic regression model ($P < 0.05$).

The predictive power of the set of selected items, adjusted by the odds ratio, was determined using the area under the Receiver-Operating Characteristic (ROC) curve (AUC) on the other half of the subjects as the validating sample. Finally, the sensitivity and specificity of the model were calculated to obtain the cut-off point.

To test the validity of the scale on persons with cognitive dysfunction, different item functioning (DIF) analysis was performed on subgroups with and without cognitive dysfunction using the Rasch measurement

Table 1 The initial 22-item questionnaire constructed by the Working Group of Fall Prevention and commissioned by the Japanese Ministry of Health, Labour and Welfare

Questionnaire items	Answer (%)†	Incidence of fall (%)‡	*P*
Q1. History of fall within one year = yes	107 (16%)	54 (50%)	$P < 0.0001$
Q2. History of stumbling within one year = yes	288 (42%)	75 (42%)	$P < 0.0001$
Q3. Can you climb stairs without help? = no	261 (38%)	65 (25%)	$P = 0.0001$
Q4. Do you feel your walking speed declined recently? = yes	353 (51%)	76 (22%)	$P = 0.0025$
Q5. Can you cross the road within the green signal interval? = no	74 (11%)	25 (11%)	$P = 0.0019$
Q6. Can you walk 1 km continuously? = no	172 (25%)	46 (27%)	$P = 0.0011$
Q7. Can you stand on one foot for about five seconds? = no	180 (26%)	55 (31%)	$P < 0.0001$
Q8. Do you use cane when you walk? = yes	123 (18%)	43 (35%)	$P < 0.0001$
Q9. Can you squeeze the towel tightly? = no	80 (12%)	26 (33%)	$P = 0.0026$
Q10. Do you feel dizzy? = yes	151 (22%)	39 (26%)	$P = 0.0076$
Q11. Is your back bended? = yes	213 (31%)	62 (29%)	$P < 0.0001$
Q12. Do you have knee pain? = yes	264 (38%)	64 (24%)	$P = 0.0005$
Q13. Do you have a vision problem? = yes	292 (42%)	56 (19%)	$P = 0.2794$
Q14. Do you have a hearing problem? = yes	227 (33%)	48 (21%)	$P = 0.0781$
Q15. Do you think you are forgetful? = yes	332 (48%)	73 (22%)	$P = 0.0020$
Q16. Do you feel anxious to fall when you walk? = yes	226 (33%)	60 (27%)	$P = 0.0001$
Q17. Do you take more than five kinds of prescribed medicines? = yes	161 (23%)	39 (24%)	$P = 0.0231$
Q18. Do you feel dark walking within your home? = yes	54 (8%)	18 (33%)	$P = 0.0124$
Q19. Are there any obstacles within the house? = yes	87 (13%)	25 (29%)	$P = 0.0181$
Q20. Is there any level difference within your home? = yes	426 (62%)	79 (19%)	$P = 0.1799$
Q21. Do you have to use stairs in daily living? = yes	129 (19%)	23 (18%)	$P = 0.7951$
Q22. Do you walk steep slope around the house? = yes	202 (29%)	28 (14%)	$P = 0.2517$

†The answers as indicated in the question raw. ‡The incidence of fall among the relevant answer.

technique.[7–9] Three hundred persons were randomly selected to obtain adequate sample size for this analysis.[10]

In addition, results of the ROC curve were stratified by the presence and absence of memory problem using Q15 of the questionnaire to test the validity of the short version on those with cognitive function problems.

Results

Of 1734 elderly, 1378 (79%) completed the questionnaire both at the baseline study and its six month follow-up. The mean age of the subjects was 75.8 (SD 6.8) years. The number of elders by five research centers was, 1050, 104, 82, 81 and 61, respectively. At least one fall had occurred in 208 elderly (15.1%) during the six month follow-up period. Of these, 103 (50%) suffered from multiple falls, ranging in number from 2 to 20.

Of eligible samples, 1026 elders provided information regarding mobility, cognitive status and ADL regarding eating and toileting. In mobility, no disability was seen in 69.8% of them, while mild difficulty in climbing stairs was present in 18.1%, and moderate or severe difficulty required cane or wheel chair for moving around outside in 12.1%.

In cognitive status, no memory disturbance was seen in 62.8%, while mild and severe memory dysfunctions were in 26.0% and 8.0%, respectively.

Regarding eating ADL, 93.4% showed no problem, while 4.6% complained they had a mild problem, and 2.0% required assistance. Toileting related ADL was intact in 89.0% of the elders while mild difficulty and dependent status on toileting were seen in 6.0% and 5.0%, respectively. Although 8.3% of them were living alone, 23.0% were with their spouse, and the rest were with their children.

The samples were then divided into the developing samples ($n = 689$) and validating samples ($n = 689$). There was no statistical significance between these two samples, in distribution of living areas, gender and response pattern to the questionnaire items examined by χ^2 test (data not shown). The average age of the validating samples (75.8) was not significantly different from developing samples (75.7) by t-test.

Table 1 shows the predictors in relation to falls in developing samples. The incidence of at least a single fall and multiple falls were 108 (15.7%) and 55 (8.0%), respectively. Gender did not achieve the statistical significance to single fall ($P = 0.05$) and multiple falls ($P = 0.15$), respectively. Fallers were elder than non-fallers ($P < 0.01$) with average age of 79.1 versus 75.8, respectively.

Questionnaire items, except for Q13, Q14, Q20, Q21 and Q22, achieved statistical significance and were entered into the regression model. Table 2 shows the item selected by the stepwise logistic regression model.

Using the odds ratio at integer level as the weight of these five items, we constructed a screening test whose score ranged from 0 to 14, and the AUC was 74% (95% CI 69–79%) in the validating samples, as shown in Figure 1. This was at the same level as the AUC of initial 22 items score (72%:95% CI 67–79%)

The maximum sum of sensitivity and specificity reached <6 (sensitivity 0.68, specificity 0.70) and <7 (sensitivity 0.67, specificity 0.71). If a cut-off score of <6 was applied, subjects identified as positive had a 27.9% rate of falls (positive predictive value) compared with a

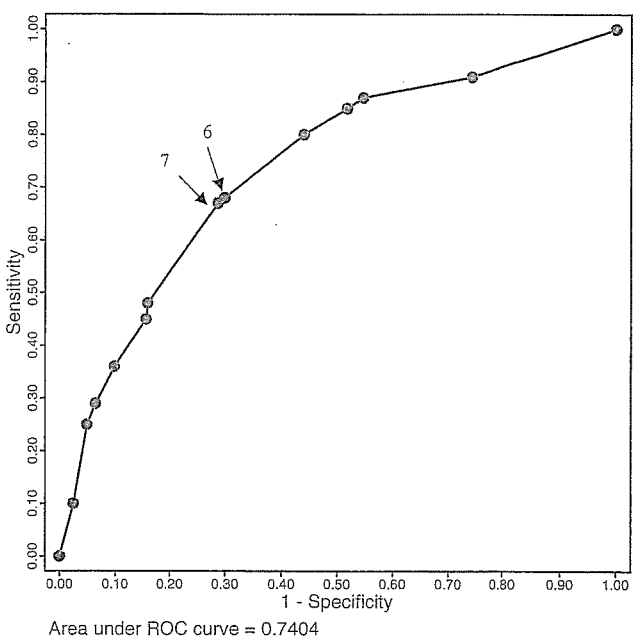

Figure 1 The Receiver-Operating Characteristic (ROC) of the five-item screening test to detect elderly persons at risk of falling.

Table 2 Questionnaire items selected by the stepwise logistic regression model

Questionnaire item selected by step wise logistic regression model	Odds ratio	95%CI	P
Q1. History of fall within one year = yes	4.5	(2.8–7.2)	0.00
Q4. Do you feel your walking speed declined recently? = yes	1.9	(1.0–3.6)	0.04
Q8. Do you use cane when you walk? = yes	1.8	(1.1–2.8)	0.02
Q11. Is your back bended? = yes	1.8	(1.1–2.8)	0.02
Q17. Do you take more than five kinds of prescribed medicines? = yes	1.7	(1.0–2.7)	0.03

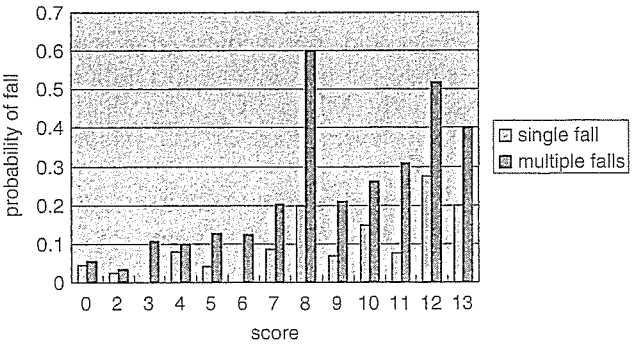

Figure 2 The probability of single and multiple falls by score.

7.2% rate in negative individuals (negative predictive power: 93%), with an odds ratio of 3.88 (95% CI 3.16–4.75).

The sensitivity and specificity was 0.63 and 0.67, respectively, for multiple falls. The positive and negative predictive value at this cut off score for multiple falls was 0.12 and 0.96, respectively, with the odds ratio of 3.04. Figure 2 illustrates the probability of fall by score levels.

On Rasch analysis of each item, some items did not fit the Rasch Model (Q16, Q20, Q21 and Q22) and these items were deleted for subsequent DIF analysis. Then no item showed DIF on cognitive functioning after Bonferroni adjustment (data not shown). After stratifying the sample with Q15, the area under ROC curve was 0.74 (95% CI 0.66–0.82) and 0.74 (0.69–0.78) for with and without cognitive dysfunction, respectively.

Discussion

Falls are considered as having multiple risk factors.[11] Previous epidemiological studies have identified the risk for falls, for example, history of falls,[2,3,12–15] activity of daily living (ADL),[2,3,15] cognitive and sensory function,[2,3,12,15] chronic conditions,[12,16,17] and medication use.[3,16–19]

Many studies tried to convert these risk factors for fall risk screening.[3,4,20] These screening tools for elders have been developed for various care settings, including residential,[14,21] intermediate[22] and inpatient care[23–25] as well as for community.[26–28]

Initially, the authors selected a comprehensive questionnaire composed of 22 items that can be answered by yes or no, and then selected several items that can be applied for mass screening or in general practice settings[6] because of the requirement of Japanese long-term care insurance (LTCI) law.

The items selected by the logistic model in this study were history of falls, walking speed, cane use, back deformation and medication use. All of these items were in concordance with the previous reports.

We also included environmental factors as part of the questionnaire. On comparison between fallers and non-fallers, environmental barriers such as level difference, stair and slope were not identified as risk factors, indicating the barrier recognized by the elders may not be associated with falls. All other items, except for vision problems were associated with incidence of falls.

The use of large prospective validating samples adds strength to this study. In most similar studies, the predictive validity is tested only on the developmental sample of the tools, and thus the predictive performance in a new sample is expected to be optimistic.[29] Although the predictive power on the development sample is usually high, the predictive power is usually lower in the validating samples.[30] In addition, the sensitivity of the scale is lower in the validating sample[31] and only a few studies use a large scale validating sample as was used in this study.[26]

Finally, the AUC of the initial 22 items were at the same level of the shortened five-item version. Therefore, the shortened version is preferred for its simplicity. In addition, the five-item scale was validated on the elderly with and without problems of cognitive function.

In the process of item selection using the logistic regression, inclusion criteria were $P < 0.05$, and exclusion criteria were $P > 0.10$. This procedure resulted in inclusion of items with weak association, such as Q4 and Q17. However, the adequacy of including these two items was proved on the validating sample.

In validating samples, the negative predictive value was 0.92 for single falls and 0.96 for multiple falls indicating that those with negative result have very low risk of falling in the next six months. This property of the high negative predictive validity makes the use of the screening test useful in mass screening.

History of fall was one of the most frequently reported risk factor of falls.[32,33] Decline of walking speed was captured with other questionnaire studies, as well as by physiological measurement.[6,18,34] Cane users and kolioskiphosis might have relation to bone abnormalities such as osteoporosis or arthritis.[13] These Q4, Q8 and Q11 compose a spectrum of physiologic decline referred to as frailty.[35,36] The relationship between medication use and falls can be explained by the effects of a drug itself that might cause sensory and balance disturbance, and also decreased metabolism, which relates to the loss of physiologic and metabolic function. Medication review is a possible intervention to prevent falls.[37]

In conclusion, a simple screening tool for falls is constructed using a large scale developing and validating sample. The scale constructed in this study is simple and valid. Therefore, it can be used as a screening tool of falls for community-dwelling elders.

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References

1 Aoyagi K, Ross PD, Davis JW, Wasnich RD, Hayashi T, Takemoto T. Falls among community-dwelling elderly in Japan. *J Bone Miner Res* 1998; **13**: 1468–1474.
2 Stel VS, Pluijm SM, Deeg DJ, Smit JH, Bouter LM, Lips P. A classification tree for predicting recurrent falling in community-dwelling older persons. *J Am Geriatr Soc* 2003; **51**: 1356–1364.
3 Tromp AM, Pluijm SM, Smit JH, Deeg DJ, Bouter LM, Lips P. Fall-risk screening test: a prospective study on predictors for falls in community-dwelling elderly. *J Clin Epidemiol* 2001; **54**: 837–844.
4 Raiche M, Hebert R, Prince F, Corriveau H. Screening older adults at risk of falling with the Tinetti balance scale. *Lancet* 2000; **356**: 1001–1002.
5 Tennant A, Penta M, Tesio L *et al.* Assessing and adjusting for cross-cultural validity of impairment and activity limitation scales through differential item functioning within the framework of the Rasch model: the PRO-ESOR project. *Med Care* 2004; **42**: I37–I48.
6 Toba K, Okochi J, Takahashi T *et al.* Development a portable fall risk index for elderly people living in the community. *Nippon Ronen Igakkai Zasshi.* 2005; **42**: 346–52.
7 Rasch G. *Probablistic Models for Some Intelligence and Attainment Tests* (Expanded Edition). Chicago: The University of Chicago Press (original work published in 1960), 1980.
8 Kucukdeveci AA, Sahin H, Ataman S, Griffiths B, Tennant A. Issues in cross-cultural validity: example from the adaptation, reliability, and validity testing of a Turkish version of the Stanford Health Assessment Questionnaire. *Arthritis Rheum* 2004; **51**: 14–19.
9 Scheuneman JD, Subhiyah RG. Evidence for the validity of a Rasch model technique for identifying differential item functioning. *J Outcome Meas* 1998; **2**: 33–42.
10 Stone M, Yumoto F. The effect of sample size for estimating Rasch/IRT parameters with dichotomous items. *J Appl Meas* 2004; **5**: 48–61.
11 Hoyl MT, Alessi CA, Harker JO *et al.* Development and testing of a five-item version of the Geriatric Depression Scale. *J Am Geriatr Soc* 1999; **47**: 873–878.
12 Salgado RI, Lord SR, Ehrlich F, Janji N, Rahman A. Predictors of falling in elderly hospital patients. *Arch Gerontol Geriatr* 2004; **38**: 213–219.
13 Albrand G, Munoz F, Sornay-Rendu E, DuBoeuf F, Delmas PD. Independent predictors of all osteoporosis-related fractures in healthy postmenopausal women: the OFELY study. *Bone* 2003; **32**: 78–85.
14 Rosendahl E, Lundin-Olsson L, Kallin K, Jensen J, Gustafson Y, Nyberg L. Prediction of falls among older people in residential care facilities by the Downton index. *Aging Clin Exp Res* 2003; **15**: 142–147.
15 Kuriyama S, Koizumi Y, Matsuda-Ohmori K *et al.* Obesity and depressive symptoms in elderly Japanese: the Tsurugaya Project. *J Psychosom Res* 2006; **60**: 229–235.
16 Huang HC, Gau ML, Lin WC, George K. Assessing risk of falling in older adults. *Public Health Nurs* 2003; **20**: 399–411.
17 Lawlor DA, Patel R, Ebrahim S. Association between falls in elderly women and chronic diseases and drug use: cross sectional study. *BMJ* 2003; **327**: 712–717.
18 de Rekeneire N, Visser M, Peila R *et al.* Is a fall just a fall: correlates of falling in healthy older persons. The Health, Aging and Body Composition Study. *J Am Geriatr Soc* 2003; **51**: 841–846.
19 Kelly KD, Pickett W, Yiannakoulias N *et al.* Medication use and falls in community-dwelling older persons. *Age Ageing* 2003; **32**: 503–509.
20 Cwikel JG, Fried AV, Biderman A, Galinsky D. Validation of a fall-risk screening test, the Elderly Fall Screening Test (EFST), for community-dwelling elderly. *Disabil Rehabil* 1998; **20**: 161–167.
21 Lundin-Olsson L, Nyberg L, Gustafson Y. The Mobility Interaction Fall chart. *Physiother Res Int* 2000; **5**: 190–201.
22 Tinetti ME, Williams TF, Mayewski R. Fall risk index for elderly patients based on number of chronic disabilities. *Am J Med* 1986; **80**: 429–434.
23 Brians LK, Alexander K, Grota P, Chen RW, Dumas V. The development of the RISK tool for fall prevention. *Rehabil Nurs* 1991; **16**: 67–69.
24 Morse JM, Black C, Oberle K, Donahue P. A prospective study to identify the fall-prone patient. *Soc Sci Med* 1989; **28**: 81–86.
25 Nyberg L, Gustafson Y. Using the Downton index to predict those prone to falls in stroke rehabilitation. *Stroke* 1996; **27**: 1821–1824.
26 Lajoie Y, Gallagher SP. Predicting falls within the elderly community: comparison of postural sway, reaction time, the Berg balance scale and the Activities-specific Balance Confidence (ABC) scale for comparing fallers and non-fallers. *Arch Gerontol Geriatr* 2004; **38**: 11–26.
27 Chiu AY, Au-Yeung SS, Lo SK. A comparison of four functional tests in discriminating fallers from non-fallers in older people. *Disabil Rehabil* 2003; **25**: 45–50.
28 Assantachai P, Praditsuwan R, Chatthanawaree W, Pisalsarakij D, Thamlikitkul V. Risk factors for falls in the Thai elderly in an urban community. *J Med Assoc Thai* 2003; **86**: 124–130.
29 Altman DG, Royston P. What do we mean by validating a prognostic model? *Stat Med* 2000; **19**: 453–473.
30 Altman DG. Study to predict which elderly patients will fall shows difficulties in deriving and validating a model. *BMJ* 1997; **315**: 1309.
31 Price C, Suddes M, Maguire L, Harrison S, O'Shea D. Single assessment of risk predicted which elderly patients would fall. *BMJ* 1998; **316**: 1750.
32 Gillespie L, Gillespie W, Robertson M, Lamb S, Cumming R, Rowe B. Interventions for preventing falls in elderly people. *Cochrane Database Syst Rev* 2003; **4**: CD000340.
33 Lundin-Olsson L, Jensen J, Nyberg L, Gustafson Y. Predicting falls in residential care by a risk assessment tool, staff judgment, and history of falls. *Aging Clin Exp Res* 2003; **15**: 51–59.
34 Bergland A, Jarnlo GB, Laake K. Predictors of falls in the elderly by location. *Aging Clin Exp Res* 2003; **15**: 43–50.
35 Skelton DA, Beyer N. Exercise and injury prevention in older people. *Scand J Med Sci Sports* 2003; **13**: 77–85.
36 Fried LP, Tangen CM, Walston J *et al.* Frailty in older adults: evidence for a phenotype. *J Gerontol A Biol Sci Med Sci* 2001; **56**: M146–M156.
37 Stevens JA, Olson S. Reducing falls and resulting hip fractures among older women. *MMWR Recomm Rep* 2000; **49**: 3–12.

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CASE REPORT

Elderly patient presenting with severe thyrotoxic hypercalcemia

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An 81-year-old woman with Graves' disease and osteoporosis was referred to the hospital because of anorexia over one month and impaired consciousness. She also presented with low-grade fever and emaciation. Laboratory tests revealed marked hypercalcemia (corrected serum calcium level of 12.4 mg/dL), which was initially suspected to result from vitamin D toxicity, because she had been taking vitamin D3 (alphacalcidol of 0.5 μg/day) for the treatment of osteoporosis. However, discontinuation of vitamin D3 and fluid infusion did not ameliorate hypercalcemia one week later. After excluding hyperparathyroidism and malignancy-related hypercalcemia, hypercalcemia was considered to be attributable to the exacerbation of hyperthyroidism (free T4 of 6.69 ng/dL, free T3 of 13.27 pg/mL and thyroid stimulating hormone (TSH) <0.015 μIU/mL) with increased bone resorption. Finally, the increased dose of thiamazole (30 mg/day) normalized serum calcium level and thyroid function three months later. Laboratory tests suggested that normal bone formation in spite of increased bone resorption contributed to hypercalcemia in hyperthyroid state.

Keywords: deoxypyridinoline, hypercalcemia, hyperthyroidism, osteoporosis, p-N-telopeptides of collagen cross-links.

Introduction

Hypercalcemia has been associated in approximately 20% of the patients with hyperthyroidism, but is mild in most cases, ranging from the upper normal limit to the slightly elevated level.[1–3] Consequently, we rarely see hyperthyroidism with symptomatic hypercalcemia. Many genotypes have been associated with Graves' disease.[4] Also, a small number of studies have shown that polymorphisms in calcium-regulating genes such as calcium-sensing receptor[5] and vitamin D receptor[6] may influence calcium metabolism in adults. However, no study has reported the association of those polymorphisms with thyrotoxic hypercalcemia. More studies as well as more polymorphisms including haplotype analysis should be performed to clarify the underlying mechanism.

Here, we report an elderly patient presenting with severe symptomatic hypercalcemia resulting from hyperthyroidism.

Case report

An 81-year-old woman was admitted to the Department of Geriatric Medicine, Kyorin University Hospital because of hypercalcemia on February 14 2004. She had Basedow's disease and osteoporosis, and had been taking thiamazole 5 mg/day and alphacalcidol 0.5 μg/day. In January 2004, anorexia had gradually developed followed by gait disturbance. When she was referred to the hospital on February 14, she also presented with confusion and low-grade fever of 37.2°C. Her blood pressure was 122/62 mmHg with a pulse rate of 98 bpm. Physical examination showed a soft diffuse goiter and a systolic ejection murmur of Levine II/VI at the apex, while abdominal and neurological findings were normal.

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Table 1 Laboratory tests on admission

Test	Result
Hb	10.5 g/dL
Ht	32.6%
RBC	$367 \times 10^4/\mu L$
PLT	$22.2 \times 10^4/\mu L$
WBC	3200/μL
Na	144 mEq/L
K	3.1 mEq/L
Cl	100 mEq/L
Ca	11.7 mg/dL
IP	3.4 mg/dL
BUN	19.3 mg/dL
Cr	0.7 mg/dL
TP	6.4 g/dL
Alb	3.3 g/dL
ALP	226 IU/L
AST	37 IU/L
ALT	35 IU/L
LDH	333 U/L
CK	25 IU/L
Glu	126 mg/dL
CRP	0.2 mg/dL

1 Alb, ...; ALP, ...; ALT, ...; AST, ...; BUN, ...; CK, ...; CRP, ...; LDH, ...; PLT, ...; RBC, ...; TP, ...; WBC, ...

Table 2 Results of thyroid function test

Test	Result (normal range)
FreeT4	6.69 ng/dL (0.73–1.53)
FreeT3	13.27 pg/mL (1.63–3.20)
Thyroid stimulating hormone (THS)	0.015 IU/mL (0.41–5.27)
TSH receptor antibody	51.2% (15<)
TSAb (thyroid stimulatory antibody)	540% (180<)
Antithyroid peroxydase antibody	43.8 U/mL (0.3<)
Serum thyroglobulin autoantibodies	0.3 < U/mL (0.3<)

On laboratory tests (Table 1), she showed blood hemoglobin of 10.5 g/dL, white blood cell counts of 3200/μL and serum calcium of 11.7 mg/dL (corrected calcium of 12.4 mg/dL). Other electrolytes as well as liver and kidney function were normal. Thyroid function tests (Table 2) revealed marked hyperthyroidism; free T4 of 6.69 (reference, 0.90–1.70) ng/dL, free T3 of 13.27 (2.3–4.3) pg/mL and thyroid stimulating hormone (TSH) of <0.015 (0.5–5.0) μIU/mL. Plasma levels of TSH receptor antibody, thyroid stimulating antibody and anti-TPO antibody were elevated, compatible with the findings in Graves' disease. Plasma intact PTH was 13 (10–65) pg/mL and PTH-related protein was not detected.

Table 3 Results of markers of bone metabolism

Marker	Result (normal range)
Osteocalcin	9.5 ng/mL (2.5–13)
Bone-specific alkaline phosphatase	24.2 U/L (9.6–35.4)
p-N-telopeptides	43.3 nMBCE/L (10.7–24.0)
Deoxypyridinoline/Cr	43.8 nmol/L/nMCr (2.8–7.6)
calcitonin	33 pg/mL
1–25(OH)VitD_3	6 pg/mL (20–60)

Figure 1 X-ray of lumbar vertebrae.

As shown in Table 3, markers of bone resorption such as deoxypyridinoline (DPD) and N-telopeptides of collagen cross-links (NTx) were elevated, whereas those of bone formation such as osteocalcin and bone-type alkaline phosphatase were not. Bone mineral density of lumbar vertebrae was −3.29 (T score), and that of femur was −3.72 (T score). Multiple compression fractures and remarkable reduction in bone mineral density were found on spinal lateral X-rays and dual energy X-ray absorptiometry, respectively (Fig. 1).

Initially, vitamin D toxicity was suspected as a cause of hypercalcemia; thus, alphacalcidol was ceased with fluid infusion to wash out calcium. However, the

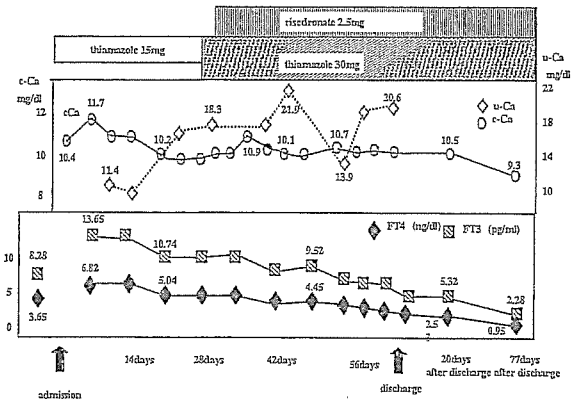

Figure 2 Clinical course of the patient. Thyroid stimulating hormone (TSH) was below the detection limit throughout the clinical course. c-Ca, collected serum calcium; u-Ca, urinary calcium; FT4, free thyroxine; FT3, free triiodothyronine.

hypercalcemia had not improved one week later. Laboratory and imaging tests were carried out to exclude hyperparathyroidism, humoral hypercalcemia of malignancy, osteolytic bone metastases and multiple myeloma. Finally, hypercalcemia was considered to be attributable to the exacerbation of hyperthyroidism with high bone turnover. Consequently, the dose of thiamazole was increased to 30 mg/day to normalize thyroid function. As shown in Figure 2, free T4 and free T3, as well as serum calcium were gradually decreased, and the patient was discharged on May 14 2004. In August 2004, her thyroid function returned to normal (free T4 of 0.95 ng/dL and free T3 of 2.28 pg/mL) with corrected serum calcium concentration of 9.2 mg/dL.

Discussion

Hypercalcemia associated with hyperthyroidism has been reported to occur more frequently in elderly patients than in younger patients; the incidence of hypercalcemia was 2.3% in hyperthyroid patients under 60 years of age and was 18.8% in those over 60 years of age.[2] The severity of hypercalcemia, however, is generally mild, ranging from the upper normal limit to the slightly elevated level,[3] and other complications should be suspected when serum calcium concentration is over 12 mg/dL.[7] Actually, case reports have shown that hyperparathyroidism is uncommonly associated with hypercalcemia in thyrotoxicosis.[8] Only several cases have been reported that hyperthyroidism was considered the only cause of hypercalcemia over 12.0 mg/dL.[9–11] In our case, laboratory tests and diagnostic imaging excluded hyperparathyroidism as well as malignant neoplasms. Furthermore, hypercalcemia was ameliorated in parallel with the improvement of hyperthyroidism, indicating that hypercalcemia resulted from hyperthyroidism.

Thyroid hormones play a critical role in bone development because hypothyroidism in childhood results in the impaired skeletal development.[12] In adults, thyroid hormones are important in the maintenance of bone mass. Thyroid hormone receptors are expressed in bone cells such as osteoblasts and osteoclasts.[12] In adult hyperthyroidism, there is increased bone remodelling, characterized by an increase in both bone resorption and formation, and an imbalance between bone resorption and formation, which results in bone loss and an increased risk for osteoporotic fracture.[12] In our case, however, the markers of bone resorption were elevated but those of bone formation were not. This pattern is consistent with the changes of bone metabolism in older osteoporotic patients,[13] but is different from that in hyperthyroidism as mentioned above. This might be due to the age-related decline in thyroid hormone signaling that leads to bone formation. However, no reports including animal experiments to support this hypothesis can be found so far. This should be investigated in the future.

Anti-thyroid drugs restore not only serum calcium levels[14] but also bone mineral density[15] in patients with thyrotoxic hypercalcemia. It has been also reported that a β blocker, propranolol,[16,17] and radioiodine therapy[10] may ameliorate thyrotoxic hypercalcemia. In our case, an increased dose of thiamazole normalized both thyroid function and serum calcium levels several months later, but bone mineral density was not increased. Longer time periods would be necessary to see the recovery of bone mass if possible.

References

1 Daniel T, Aran B. The skeletal system in thyrotoxicosis. In: Lewis EB, Robert DU, (eds). *Werner and Ingbar's the Thyroid*, 8th edn. Philadelphia, PA: A Wolters Kluwer Co., 2000; 659–666.
2 Szabo ZS, Ritzl F. Hypercalcemia in hyperthyroidism. Role of age and goiter type. *Klin Wochenschr* 1981; 59: 275–279.
3 Mosekilde L, Melsen F, Bagger JP *et al.* Bone changes in hyperthyroidism: interrelationships between bone morphometry, thyroid function and calcium-phosphorus metabolism. *Acta Endocrinol* 1977; 85: 515–525.
4 Dittmar M, Kahaly GJ. Immunoregulatory and susceptibility genes in thyroid and polyglandular autoimmunity. *Thyroid* 2005; 15: 239–250.
5 Cole DE, Vieth R, Trang HM *et al.* Association between total serum calcium and the A986S polymorphism of the calcium-sensing receptor gene. *Mol Genet Metab* 2001; 72: 168–174.
6 Akcay A, Ozdemir FN, Sezer S *et al.* Association of vitamin D receptor gene polymorphisms with hypercalcemia in peritoneal dialysis patients. *Perit Dial Int* 2005; 25: S52–S55.
7 Ryo M, Shigeru Y, Hyo ES *et al.* The parathyroid function in patients with hyperthyroidism. *Nippon Naibunpi Gakkai Zasshi* 1984; 60: 892–898.

8 Maxon HR, Apple DJ, Goldsmith RE. Hypercalcemia in thyrotoxicosis. *Surg Gynecol Obstet* 1987; 147: 694–696.
9 Inaba M, Hamada N, Itoh K *et al.* A case report on disequilibrium hypercalcemia in hyperthyroidism. Comparison of calcium metabolism with other patients with hyperthyroidism. *Endocrinol Jpn* 1982; 29: 389–393.
10 Akihan Z, Singh A. Hyperthyroidism manifested as hypercalcemia. *South Med J* 1996; 89: 997–998.
11 Reular JB, Wise RW, Thorpe JB. Anemia, renal insufficiency, and hypercalcemia in a man with hyperthyroidism. *South Med J* 1985; 78: 59–63.
12 Bassett JH, Williams GR. The molecular actions of thyroid hormone in bone. *Trends Endocrinol Metab* 2003; 14: 356–364.
13 Chan GK, Duque G. Age-related bone loss: old bone, new facts. *Gerontology* 2002; 48: 62–71.
14 Hedman I, Tisell LE. Life-threatening hypercalcemia in a case of thyrotoxicosis: clinical features and management. A case report. *Acta Chir Scand* 1985; 151: 487–489.
15 Diamond T, Julie V, Richard S, Ptrick B. Thyrotoxic bone disease in women: a potentially reversible disorder. *Ann Intern Med* 1994; 120: 8–11.
16 Shahshahani MN, Palmieri GM. Oral propranolol in hypercalcemia associated with apathetic thyrotoxicosis. *Am J Med Sci* 1978; 275: 199–202.
17 Mallette LE, Rubenfeld S, Silverman V. A controlled study of the effects of thyrotoxicosis and propranolol treatment on mineral metabolism and parathyroid hormon immunoreactivity. *Metabolism* 1985; 34: 999–1006.

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Potent free radical scavenger, edaravone, suppresses oxidative stress-induced endothelial damage and early atherosclerosis

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Abstract

Objective: Effects of potent free radical scavenger, edaravone, on oxidative stress-induced endothelial damage and early atherosclerosis were investigated using animal models and cultured cells.
Methods and results: Endothelial apoptosis was induced by 5-min intra-arterial exposure of a rat carotid artery with 0.01 mmol/L H_2O_2. Edaravone treatment (10 mg/kg i.p.) for 3 days suppressed endothelial apoptosis, as evaluated by chromatin staining of *en face* specimens at 24 h, by approximately 40%. Similarly, edaravone dose-dependently inhibited H_2O_2-induce apoptosis of cultured endothelial cells in parallel with the inhibition of 8-isoprostane formation, 4-hydroxy-2-nonenal (4-HNE) accumulation and VCAM-1 expression. Next, apolipoprotein-E knockout mice were fed a high-cholesterol diet for 4 weeks with edaravone (10 mg/kg i.p.) or vehicle treatment. Edaravone treatment decreased atherosclerotic lesions in the aortic sinus (0.18 ± 0.01 to 0.09 ± 0.01 mm^2, $P < 0.001$) and descending aorta (5.09 ± 0.86 to 1.75 ± 0.41 mm^2, $P < 0.05$), as evaluated by oil red O staining without influence on plasma lipid concentrations or blood pressure. Dihydroethidium labeling and cytochrome *c* reduction assay showed that superoxide anions in the aorta were suppressed by edaravone. Also, plasma 8-isoprostane concentrations and aortic nitrotyrosine, 4-HNE and VCAM-1 contents were decreased by edaravone treatment.
Conclusions: These results suggest that edaravone may be a useful therapeutic tool for early atherosclerosis, pending the clinical efficacy.

Keywords: Atherosclerosis; Reactive oxygen species; Free radical scavenger; Edaravone; 4-HNE; Apolipoprotein E knockout mouse

1. Introduction

Accumulating evidence has shown that stress-induced injury of vascular endothelial cells (ECs) is an initial event in the development of atherosclerosis [1]. In particular, oxidative stress has been implicated in endothelial injury caused by oxidized LDL and smoking as well as hypertension, diabetes and ischemia-reperfusion [1–3]. This notion is supported by the findings that the production of reactive oxygen species (ROS) is upregulated in vascular lesions [4,5], and that lesion formations such as endothelial dysfunction [6] and atherosclerosis [7] are accelerated by superoxide anion ($O_2^{\bullet -}$).

Experimental studies have shown the protective effects of antioxidants on atherosclerosis and endothelial injury. Dietary antioxidants were reported to preserve endothelial function [8,9] and inhibit atherosclerosis [10] in cholesterol-fed rabbits. In a well employed animal model of atherosclerosis, apolipoprotein E knockout (ApoE-KO) mouse fed a high fat diet, it has been shown that there was a significant increase in basal superoxide products [11,12], and that both $O_2^{\bullet -}$ levels and aortic lesion areas were attenuated by treatment with Vitamin E [11] or superoxide dismutase [13]. By contrast, it has been reported that elimination of NAD(P)H oxidase [14] or disruption of its subunit p47phox [15] had no effect on lesion size in ApoE-KO mice. Clinical experiments have

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also shown that antioxidants such as Vitamins C and E can ameliorate endothelial dysfunction in patients with hypercholesterolemia or atherosclerosis [16,17], although recent clinical trials have failed to prove the protective effects of Vitamin E on cardiovascular events in patients with risk factors [18] and in healthy subjects [19].

Edaravone is a potent free radical scavenger that has been clinically used to reduce the neuronal damage following ischemic stroke [20]. Edaravone has promising property to quench hydroxyl radical (•OH) and show inhibitory effects on peroxynitrite ($ONOO^-$) and both water-soluble and lipid-soluble peroxyl radical ($LOO^•$) [21,22]. Accordingly, this compound exerts a wide range of antioxidant activity on ROS beyond the effects of water-soluble or lipid-soluble antioxidant vitamins. Based on this idea, we hypothesized that edaravone would inhibit the process of atherosclerosis.

To test this hypothesis, we investigated the effects of edaravone in two experimental models. First, we examined whether edaravone could inhibit hydrogen peroxide (H_2O_2)-induced EC apoptosis in a rat model [23] and cultured ECs. Second, we examined whether edaravone could suppress the atherosclerotic lesion formation in ApoE-KO mice.

2. Methods

2.1. Animals

Male Wistar rats aged 10–12 weeks (Japan Clea), and male C57BL/6 mice and ApoE-KO mice on C57BL/6 background aged 4–6 weeks (Jackson Laboratory) were used in this study. All of the experimental protocols were approved by the Animal Research Committee of the Kyorin University School of Medicine.

2.2. H_2O_2-induced EC apoptosis in rats and in culture

EC apoptosis was induced by 5-min intra-arterial treatment of a rat carotid artery with 0.01 mmol/L H_2O_2 as previously described [23]. Briefly, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; 3 or 10 mg/kg; donated by Mitsubishi Pharma Corporation, Japan) or its vehicle was intraperitoneally injected daily for 3 days before H_2O_2 treatment. A catheter was placed in the common carotid artery via the external carotid artery. The lumen was flushed with saline, replaced with 0.01 mmol/L H_2O_2 diluted with saline for 5 min and recovered. At 24 h after H_2O_2 treatment, EC apoptosis was evaluated by chromatin staining of *en face* specimens of the carotid artery using Hoechst 33342 dye. Apoptotic cells were identified by their typical morphological appearance; chromatin condensation, nuclear fragmentation, or apoptotic bodies. The numbers of apoptotic cells and intact cells were counted in 10 high-power fields for each specimen by an observer blinded to the treatment group.

Apoptosis of ECs isolated from a bovine carotid artery was induced as previously described [24]. Briefly, subconfluent ECs were pretreated for 24 h with culture medium containing edaravone or vehicle. After washing twice with Hank's balanced salt solution, the cells were exposed to H_2O_2 (0.2 mmol/L) diluted in Hank's balanced salt solution for 1.5 h at 37 °C to induce apoptosis. Then ECs were cultured in culture medium containing edaravone or vehicle until assay. Apoptosis was evaluated at 24 h after H_2O_2 treatment as histone-associated DNA fragments using a photometric enzyme immunoassay (Cell Death Detection ELISA, Roche), according to the manufacturer's instructions.

2.3. Atherosclerosis in ApoE-KO mice

ApoE-KO mice received a high-cholesterol diet (1% cholesterol, 10% fat in CE-2 standard diet; Japan Clea) for 4 weeks. Simultaneously, edaravone (10 mg/kg) or its vehicle was intra-peritoneally injected daily throughout the experiments. Body weight and systolic blood pressure were recorded every week in a conscious state by the tail cuff method (BP-98A; Softron, Tokyo).

At 4 weeks of treatment, mice were sacrificed with an overdose of diethyl ether and perfusion-fixed. Atherosclerotic lesions in the aortic sinus were quantified according to the method described previously [25]. We also measured the surface area of atherosclerotic lesions in the whole descending aorta including the abdominal aorta just proximal to the iliac bifurcation. *En face* specimens of the descending aorta were stained with oil red O, photographed and analyzed using the NIH image software. Total cholesterol, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol in mice plasma were determined by a commercial laboratory (SRL, Japan).

2.4. Measurement of ROS

Aortic samples for ROS measurements were prepared separately from those for atherosclerosis evaluation. At 4 weeks of treatment, ApoE-KO mice were sacrificed with CO_2 inhalation. Descending aortas were rapidly removed and placed into chilled modified Krebs/HEPES buffer. C57BL/6 mice fed a standard diet were also used as the control. To determine superoxide production *in situ*, frozen cross-sections of the aorta were stained with 10 μmol/L dihydroethidium (DHE; Molecular Probes), followed by fluorescent microscopy [26]. Also, superoxide production in aortic rings was quantified using the superoxide dismutase-inhibitable cytochrome *c* reduction assay as previously described [27]. Immunohistochemical detection of 3-nitrotyrosine in the aorta was visualized by diaminobenzidine as reported previously [28].

Intracellular production of superoxide anions was measured using DHE as described previously [29], and the intensity values were calculated using the Metamorph software [24]. Concentrations of 8-isoprostane (8-iso prostaglandin

$F_{2\alpha}$) in the culture supernatants and mouse plasma were measured using a commercially available EIA kit (Cayman Chemical). Culture supernatants were directly applied to EIA, while plasma was applied to EIA after solid phase extraction purification according to the manufacturer's instructions.

2.5. Western blotting

Western blotting was performed as previously described [30], to detect the expression of VCAM-1 and 4-HNE in cultured ECs and mouse aortas. Descending aortas were prepared as described in ROS measurements. The antibodies used in this study were anti-4-HNE monoclonal antibody (JaICA, Shizuoka, Japan), anti-VCAM-1 polyclonal antibody (Santa Cruz Biotechnology) and anti-3-nitrotyrosine monoclonal antibody (Upstate). Densitometric analysis was performed using an image scanner and the NIH software.

2.6. Data analysis

All values are express as mean ± S.E.M. Data were analyzed using one-factor ANOVA. If a statistically significant effect was found, Newman–Keuls' test was performed to isolate the difference between the groups. Differences with a value of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of edaravone on H_2O_2-induced EC apoptosis and ROS

As shown in Fig. 1A, edaravone dose-dependently inhibited EC apoptosis in culture, which was induced 24 h after H_2O_2 treatment. Edaravone was then employed in a rat model of H_2O_2-induced EC apoptosis. Consistent with the *in vitro* experiment, edaravone of 10 mg/kg/day decreased EC apoptosis of the rat carotid artery by approximately 40% (Fig. 1B).

We next examined whether edaravone decreased ROS production in the process of H_2O_2-induced EC apoptosis. For this purpose, DHE fluorescent, a marker of intracellular production of superoxide anions, release of 8-isoprostane into the culture supernatants and accumulation of 4-HNE, a pivotal end-product of lipid peroxidation [31], were measured using cultured ECs. We also examined the expression of VCAM-1 as a marker of endothelial injury or activation [32]. Edaravone decreased DHE fluorescent, 8-isoprostane formation and VCAM-1 expression at 3 h after H_2O_2 treatment in a dose-dependent manner (Fig. 2A–C). As shown in Fig. 2D, multiple bands showing 4-HNE-Michael protein adducts [33,34] were accumulated after H_2O_2 treatment in a time-dependent manner. Consequently, the effect of edaravone on 4-HNE expression was examined at 3 h after H_2O_2 treatment (4.5 h after H_2O_2 was initially added). Edaravone decreased 4-HNE expression in a dose dependent manner.

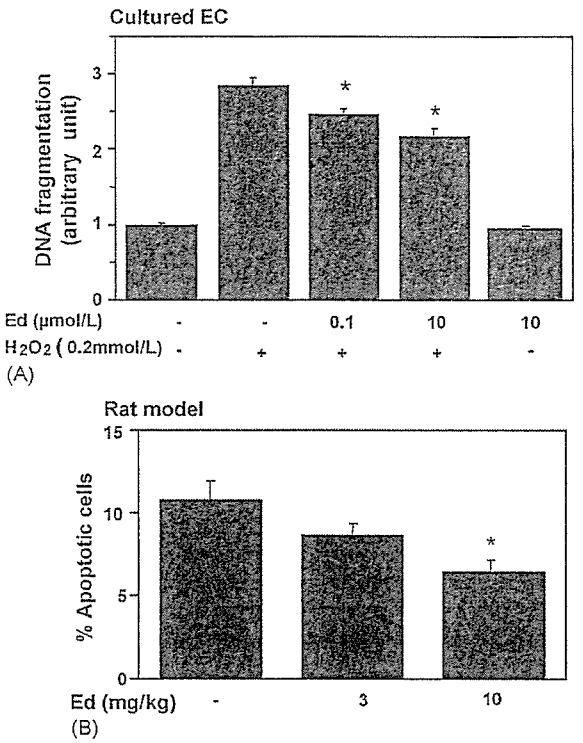

Fig. 1. Effects of edaravone (Ed) on H_2O_2-induced EC apoptosis in culture (A) and in a rat model (B). (A) Ed or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. EC apoptosis was evaluated 24 h after H_2O_2 treatment (0.2 mmol/L) by means of DNA fragmentation. Values are expressed as mean ± S.E.M. ($n = 3$). $^*P < 0.05$ vs. H_2O_2 (+) + Ed (−). (B) Ed or its vehicle was intraperitoneally injected once a day for 3 days before H_2O_2 treatment. At 24 h after H_2O_2 treatment, apoptotic ECs were counted per high power field and the ratio of the apoptotic cell number to the intact cells was calculated using *en face* specimens of the carotid artery stained with Hoechst 33342. Values are expressed as mean ± S.E.M. ($n = 7$). $^*P < 0.05$ vs. vehicle.

3.2. Effects of edaravone on atherosclerotic lesions and ROS in ApoE-KO mice

In the next set of experiments, we examined whether edaravone could suppress the atherosclerotic lesions in ApoE-KO mice fed a high cholesterol diet for 4 weeks. As shown in Fig. 3A and B, atheromatous lesions both in the aortic sinus and the descending aorta were smaller in mice treated with 10 mg/kg/day edaravone than in those with vehicle. This dose of edaravone did not influence body weight, blood pressure or plasma LDL and HDL cholesterol levels (Table 1).

Then, we examined whether the anti-atherogenic effects of edaravone were associated with the decrease in ROS production. Peroxynitrite formation was assessed as 3-nitrotyrosine accumulation in the aorta [28]. Both immunohistochemistry and Western blotting showed that edaravone inhibited nitrotyrosine accumulation in the aorta of ApoE-KO mice (Fig. 4A(a) and A(b)). Superoxide production *in situ* was examined using DHE staining of the descend-

Table 1
Body weight, blood pressure and plasma lipid levels in ApoE-KO mice treated with edaravone or vehicle

	Vehicle	Edaravone
Body weight (g)	21.4 ± 0.5	21.0 ± 0.5
Systolic blood pressure (mmHg)	106 ± 2	103 ± 3
Total cholesterol (mg/dL)	1967 ± 38	1872 ± 66
HDL cholesterol (mg/dL)	66 ± 6	82 ± 9
LDL cholesterol (mg/dL)	602 ± 24	602 ± 12

The values are shown as mean ± S.E. ($n = 14$). There were no significant differences in the values between the two groups.

ing aorta. As shown in Fig. 4B, ethidium fluorescence, which was amplified in ApoE-KO mice, was decreased by edaravone treatment. A quantitative analysis by the superoxide dismutase-inhibitable cytochrome *c* reduction assay revealed that $O_2^{\bullet -}$ levels in aortic rings of ApoE-KO mice were decreased by 43% in edaravone-treated ApoE-KO mice compared to those in vehicle-treated mice (Fig. 4C). Consistent with these results, plasma 8-isoprostane levels and 4-HNE expression in the descending aorta, both of which were elevated in ApoE-KO mice compared to

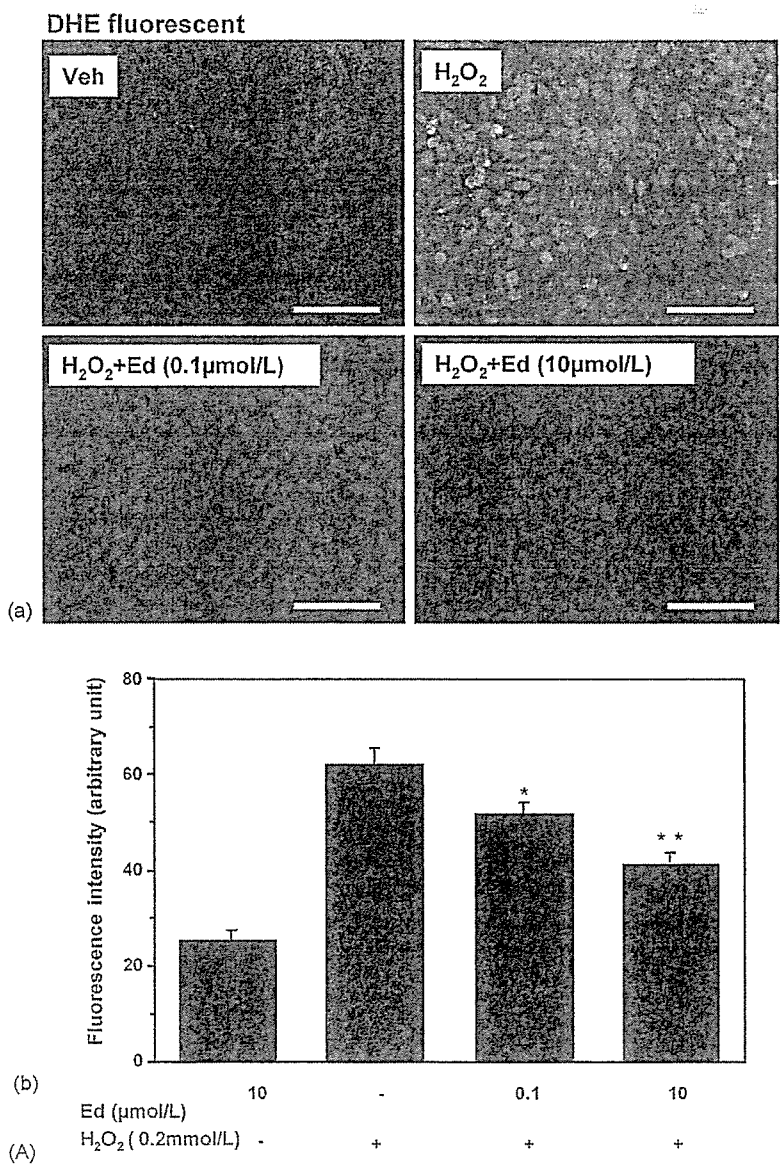

Fig. 2. Effects of edaravone (Ed) on DHE fluorescent (A) and 8-isoprostane formation (B), VCAM-1 expression (C) and 4-HNE expression (D) in cultured EC. Ed or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. DHE fluorescent ($n = 6$), 8-isoprostane concentration ($n = 3$) and VCAM-1 expression ($n = 3$) in the cell lysate were measured 3 h after H_2O_2 treatment. Values are expressed as mean ± S.E.M. Time dependent changes of 4-HNE expression after H_2O_2 treatment was detected by Western blotting. Representative image showed that 4-HNE-Michael protein adducts were accumulated after treatment (D(a)). The major 97 kDa band was measured 4.5 h after H_2O_2 treatment in the presence or absence of edaravone (D(b)). Values are expressed as mean ± S.E.M. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. H_2O_2 (+) + Ed (−).

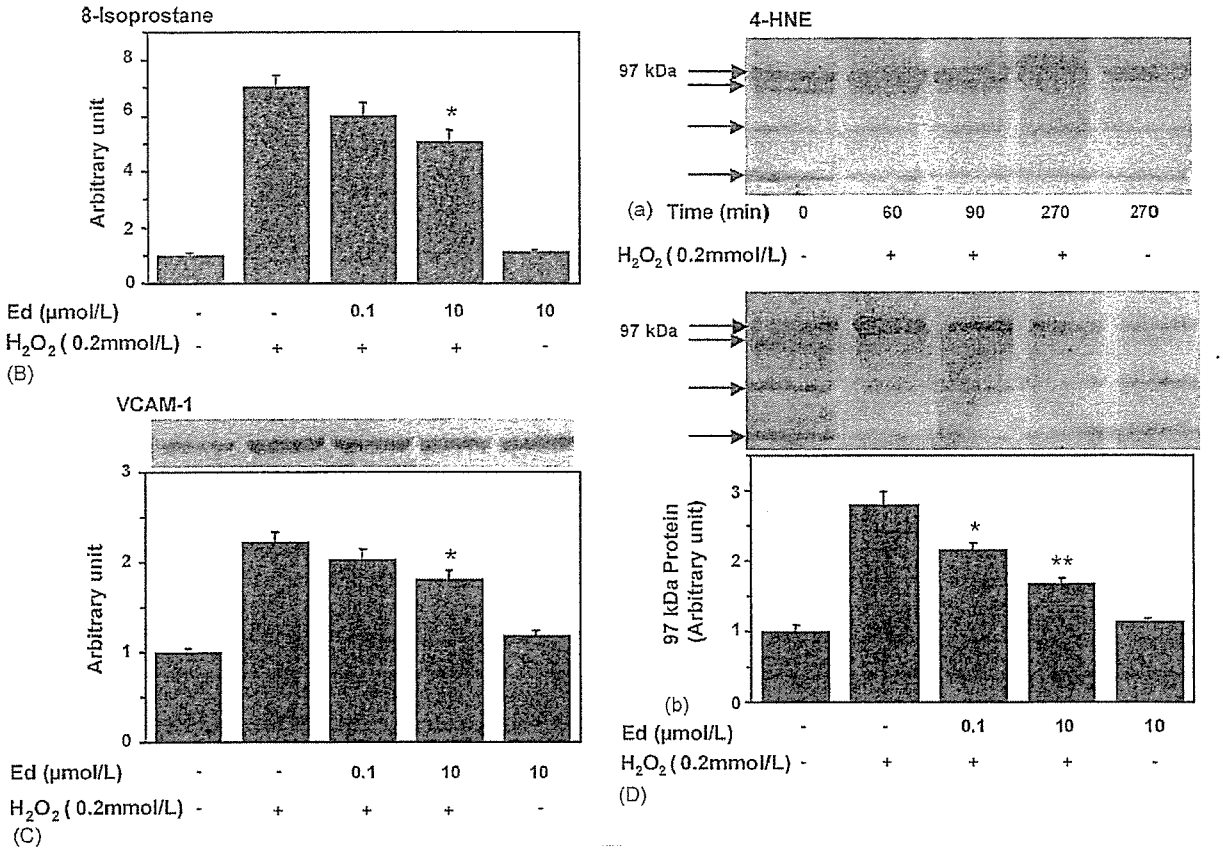

Fig. 2. (*Continued*).

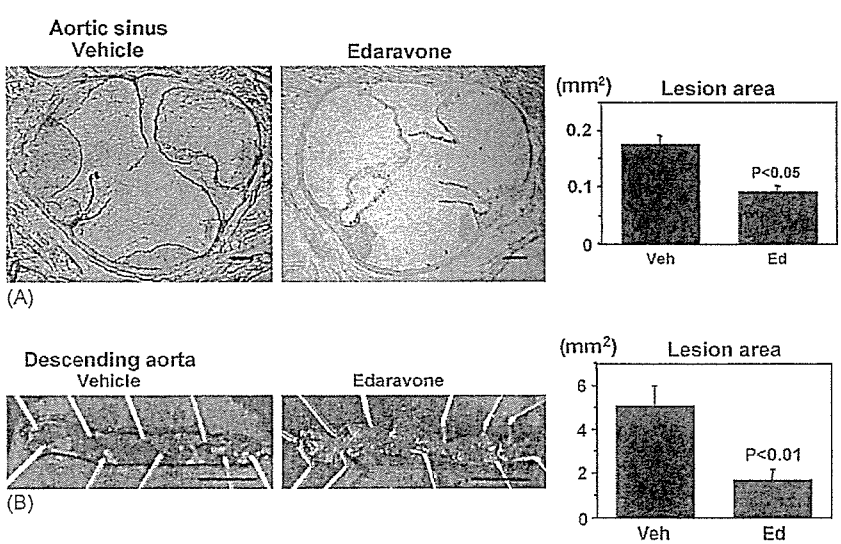

Fig. 3. Effects of edaravone on atherosclerotic lesion in ApoE-KO mice. ApoE-KO mice were fed a high-cholesterol diet for 4 weeks with the administration of edaravone (10 mg/kg daily) or its vehicle by i.p. injection. (A) Oil red O-stained cross-sections of the aortic sinus (bar = 100 μm) and morphometric analysis of the lesions are shown. (B) Oil red O-stained *en face* specimens of the descending aorta (bar = 5 mm) and morphometric analysis of the lesions are shown. Values are expressed as mean ± S.E.M. ($n = 14$).

those in wild-type C57BL/6 mice fed a normal chow, were decreased by edaravone treatment (Fig. 4D and E). Finally, the increase in VCAM-1 expression in the aorta of ApoE-KO mice was attenuated by edaravone as well (Fig. 4F).

4. Discussion

A number of studies have shown that ROS contribute to the pathogenesis of endothelial dysfunction and atherosclerosis formation. In addition to $O_2^{\bullet -}$ that is predominantly pro-

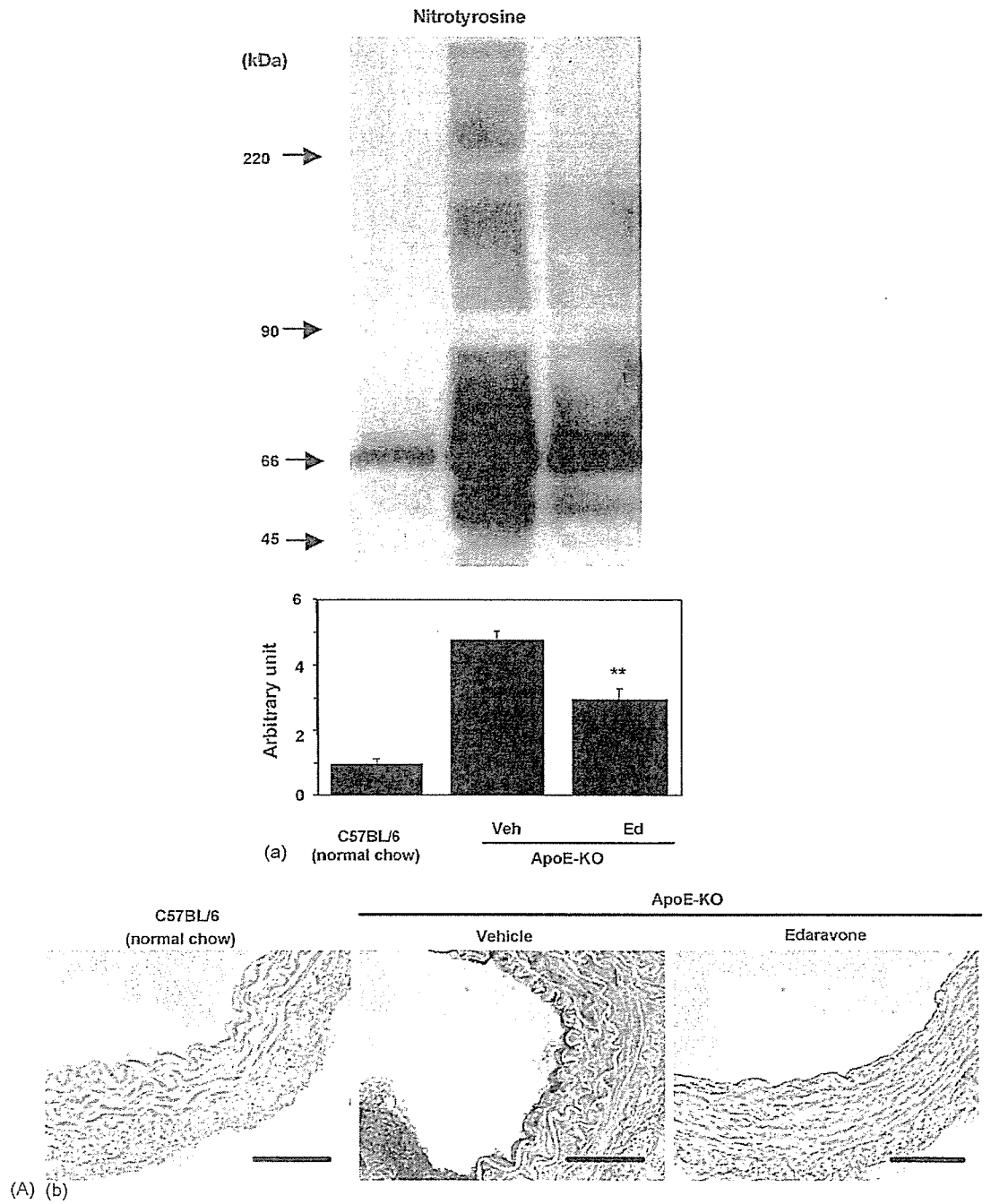

Fig. 4. Effects of edaravone (Ed) on ROS production (A–E) and VCAM-1 expression (F) in ApoE-KO mice. (A) Nitrotyrosine contents in the aorta was examined by Western blot analysis (A(a), $n=6$) and immunohistochemistry (A(b)). Bar = 50 μm. (B) Fresh-frozen cross-sections of the aorta were stained with DHE, and representative fluorescent micrographs are shown (bar = 100 μm). (C) Superoxide anion in aortic rings was determined using SOD inhibitable-cytochrome *c* reduction assay ($n=6$). (D) 8-Isoprostane level in mouse plasma was measured with EIA ($n=6$). (E and F) Representative Western blotting for 4-HNE (97 kDa band) and VCAM-1 expression in the aorta and densitometric analysis are shown ($n=3$). Values are expressed as mean ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ vs. vehicle (Veh). C57/BL6 mice fed a normal chow serve as the control.

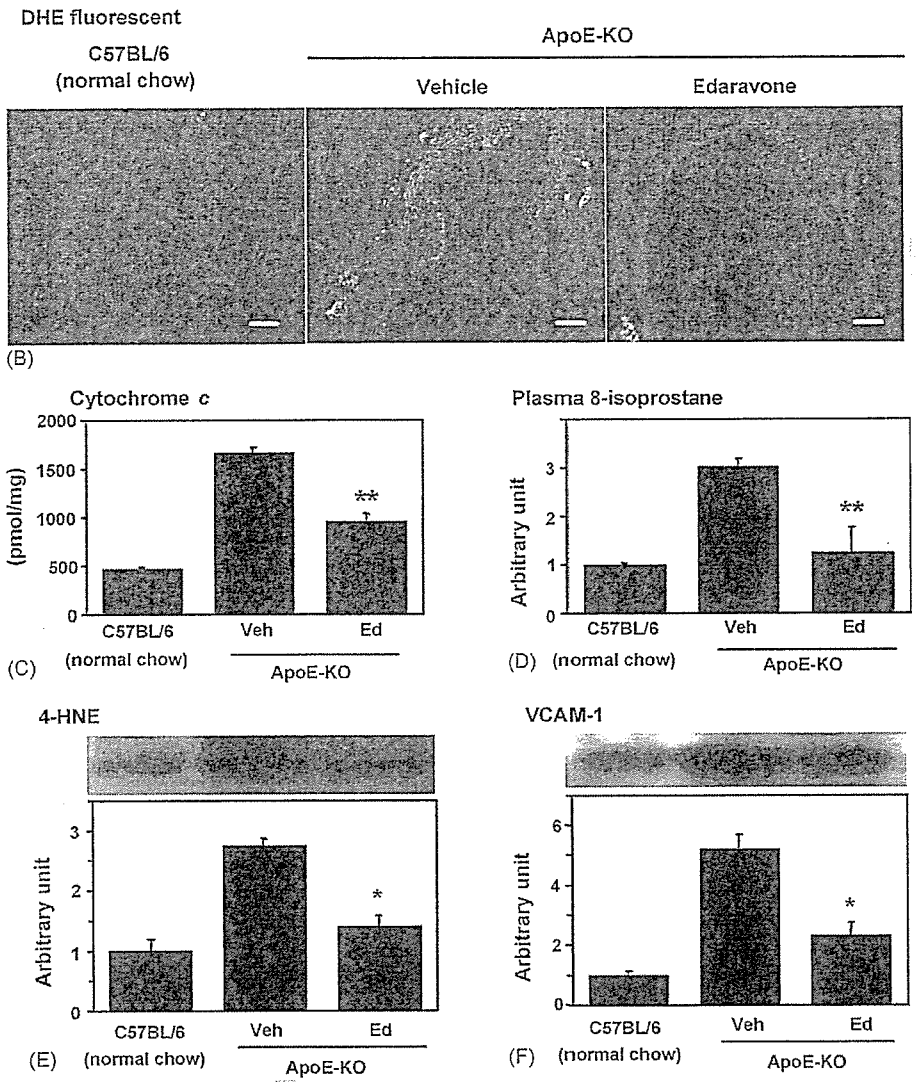

Fig. 4. (*Continued*).

duced via NAD(P)H oxidase [35], •OH as well as LOO• [36] and $ONOO^-$ [37] play a role in atherogenesis. In particular, •OH is extremely strong in terms of oxidative activity and cellular damage [38]. Therefore, it might be essential to scavenge the wide range of ROS for the prevention of atherosclerosis. As a matter of fact, recent clinical trials have denied the protective effects of Vitamin E, which predominantly reacts with LOO• [39], on cardiovascular events [18,19].

Edaravone, a potent free radical scavenger with unique properties, works by donating an electron from edaravone anion to free radicals [22]. Edaravone quenches •OH and inhibits both •OH-dependent and •OH-independent lipid peroxidation [22]. Edaravone shows inhibitory effects on both water-soluble and lipid-soluble LOO-induced peroxidation systems [22]. Edaravone also inhibits $ONOO^-$-induced tyrosine nitration [22]. These properties are different from those of water-soluble Vitamin C and lipid-soluble Vitamin E.

In the present study, we demonstrated that edaravone suppressed endothelial apoptosis and fatty streak formation. Reduced expression of VCAM-1, a marker of vascular injury and activation [32], were corroborated with these results. In cultured ECs, protein expression of VCAM-1 was induced as early as 3 h after H_2O_2 treatment (actually 4.5 h after addition of H_2O_2, Fig. 2C). This is reasonable based on our time course experiments (data not shown), and is consistent with the previous reports that VCAM-1 protein has been induced 4–6 h after cytokine stimulation through an antioxidant-sensitive mechanism [40,41]. Although the experimental conditions were different between the cell culture and animal studies, edaravone inhibited both the rapid induction of VCAM-1 in cultured ECs and the chronic upregulation of VCAM-1 in the aorta of ApoE-KO mice, further supporting the vasoprotective effects of edaravone.

Edaravone has been clinically used as a neuroprotectant in the treatment of ischemic stroke in Japan from 2001. The dose of edaravone used in this study (intraperitoneal injection of 10 mg/kg) has been reported to be comparable to that of intravenous injection in clinical use in terms of plasma concentration [42]. This compound has been reported to preserve endothelial function in ischemic brain [43] and ameliorate ischemia-reperfusion injury in various organs such as kidney [44] and heart [45]. Also, edaravone has been shown to inhibit pressure overload-induced cardiac hypertrophy [42]. To our knowledge, however, the effect of edaravone on atherosclerosis has never been reported till now.

The effects of edaravone on endothelial injury and atherosclerosis were associated with the decrease in ROS production including peroxynitrite, superoxide anion and 8-isoprostane, suggesting the mechanistic role of antioxidation in vascular protection. Edaravone also inhibited the expression of 4-HNE in vascular tissues, further indicating the antioxidant activity and suggesting the signaling cascade leading to endothelial injury, because 4-HNE triggers cellular damages through the MAP kinase pathway as an end-product of ROS [34]. Antioxidant effects of edaravone on lipoproteins were not determined in the present study because of the methodological limitation in mice. It has been reported, however, that edaravone can inhibit oxidative modification of low-density lipoprotein *in vitro* and in rats [46]. Consequently, it is likely that reduced lipoprotein oxidation would have played a role in the anti-atherosclerotic effects of edaravone in ApoE-KO mice. Furthermore, edaravone has been reported to stimulate the expression of endothelial nitric oxide synthase in cultured ECs [46] and the artery [47], leading to the increased production of nitric oxide. Taken together with the effects on peroxynitrite formation, edaravone might synergistically increase the availability of nitric oxide, which exerts vasoprotective and anti-atherosclerotic action.

The effects of edaravone on advanced and complicated lesions of atherosclerosis were not investigated in this study. Neither, the effects on plaque ruptures nor consequent cardiovascular events are known. This study demonstrated that edaravone might be a potential new therapeutic agent for the prevention and treatment of early atherosclerosis. For the purpose of chronic use, however, the innovation of drug preparation for oral administration is necessary. Another application of edaravone might be the prevention of restenosis after percutaneous coronary interventions, since ROS plays an important role in neointimal formation after angioplasty [48]. Intravenous injection of edaravone for several days might inhibit neointimal formation in addition to ischemia reperfusion injury of cardiomyocytes [45]. Taken together, edaravone is expected to show protective effect on ROS-related vascular diseases beyond cerebral infarction.

In summary, edaravone, a free radical scavenger with unique properties, attenuated oxidative stress-induced endothelial damage in rats and early atherosclerosis in ApoE-KO mice in association with the inhibition of ROS formation. These findings provide new information on the role of ROS in atherogenesis and the therapeutic strategy for atherosclerosis.

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References

[1] Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med 1999;340:115–26.
[2] Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol 2000;20:2175–83.
[3] Zalba G, San Jose G, Moreno MU, et al. Oxidative stress in arterial hypertension: role of NAD(P)H oxidase. Hypertension 2001;38:1395–9.
[4] Sorescu D, Weiss D, Lassegue B, et al. Superoxide production and expression of nox family proteins in human atherosclerosis. Circulation 2002;105:1429–35.
[5] Spiekermann S, Landmesser U, Dikalov S, et al. Electron spin resonance characterization of vascular xanthine and NAD(P)H oxidase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Circulation 2003;107:1383–9.
[6] Rey FE, Li XC, Carretero OA, Garvin JL, Pagano PJ. Perivascular superoxide anion contributes to impairment of endothelium-dependent relaxation: role of gp91(phox). Circulation 2002;106:2497–502.
[7] Barry-Lane PA, Patterson C, van der Merwe M, et al. p47phox is required for atherosclerotic lesion progression in ApoE(−/−) mice. J Clin Invest 2001;108:1513–22.
[8] Keaney Jr JF, Gaziano JM, Xu A, et al. Dietary antioxidants preserve endothelium-dependent vessel relaxation in cholesterol-fed rabbits. Proc Natl Acad Sci (USA) 1993;90:11880–4.
[9] Keaney Jr JF, Xu A, Cunningham D, Jackson T, Frei B, Vita JA. Dietary probucol preserves endothelial function in cholesterol-fed rabbits by limiting vascular oxidative stress and superoxide generation. J Clin Invest 1995;95:2520–9.
[10] Lamb DJ, Reeves GL, Taylor A, Ferns GA. Dietary copper supplementation reduces atherosclerosis in the cholesterol-fed rabbit. Atherosclerosis 1999;146:33–43.
[11] Pratico D, Tangirala RK, Rader DJ, Rokach J, FitzGerald GA. Vitamin E suppresses isoprostane generation in vivo and reduces atherosclerosis in ApoE-deficient mice. Nat Med 1998;4:1189–92.
[12] Li Z, Iwai M, Wu L, et al. Fluvastatin enhances the inhibitory effects of a selective AT1 receptor blocker, valsartan, on atherosclerosis. Hypertension 2004;44:758–63.
[13] Fennell JP, Brosnan MJ, Frater AJ, et al. Adenovirus-mediated overexpression of extracellular superoxide dismutase improves endothelial dysfunction in a rat model of hypertension. Gene Ther 2002;9:110–7.
[14] Kirk EA, Dinauer MC, Rosen H, Chait A, Heinecke JW, LeBoeuf RC. Impaired superoxide production due to a deficiency in phagocyte NADPH oxidase fails to inhibit atherosclerosis in mice. Arterioscler Thromb Vasc Biol 2000;20:1529–35.

[15] Hsich E, Segal BH, Pagano PJ, et al. Vascular effects following homozygous disruption of p47(phox): an essential component of NADPH oxidase. Circulation 2000;101:1234–6.
[16] Fang JC, Kinlay S, Beltrame J, et al. Effect of Vitamins C and E on progression of transplant-associated arteriosclerosis: a randomised trial. Lancet 2002;359:1108–13.
[17] Engler MM, Engler MB, Malloy MJ, et al. Antioxidant Vitamins C and E improve endothelial function in children with hyperlipidemia: endothelial assessment of risk from lipids in youth (EARLY) trial. Circulation 2003;108:1059–63.
[18] Lonn E, Bosch J, Yusuf S, et al. Effects of long-term Vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. JAMA 2005;293:1338–47.
[19] Lee IM, Cook NR, Gaziano JM, et al. Vitamin E in the primary prevention of cardiovascular disease and cancer: the Women's Health Study: a randomized controlled trial. JAMA 2005;294:56–65.
[20] Study-Group E. Effect of a novel free radical scavenger, edaravone (MCI-186), on acute brain infarction. Randomized, placebo-controlled, double-blind study at multicenters. Cerebrovasc Dis 2003;15:222–9.
[21] Abe S, Kirima K, Tsuchiya K, et al. The reaction rate of edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186)) with hydroxyl radical. Chem Pharm Bull (Tokyo) 2004;52:186–91.
[22] Watanabe T, Yuki S, Egawa M, Nishi H. Protective effects of MCI-186 on cerebral ischemia: possible involvement of free radical scavenging and antioxidant actions. J Pharmacol Exp Ther 1994;268:1597–604.
[23] Sudoh N, Toba K, Akishita M, et al. Estrogen prevents oxidative stress-induced endothelial cell apoptosis in rats. Circulation 2001;103:724–9.
[24] Akishita M, Nagai K, Xi H, et al. Renin–angiotensin system modulates oxidative stress-induced endothelial cell apoptosis in rats. Hypertension 2005;45:1188–93.
[25] Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis 1987;68:231–40.
[26] Miller Jr FJ, Gutterman DD, Rios CD, Heistad DD, Davidson BL. Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. Circ Res 1998;82:1298–305.
[27] Landmesser U, Dikalov S, Price SR, et al. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J Clin Invest 2003;111:1201–9.
[28] Francia P, Delli Gatti C, Bachschmid M, et al. Deletion of p66shc gene protects against age-related endothelial dysfunction. Circulation 2004;110:2889–95.
[29] Carter WO, Narayanan PK, Robinson JP. Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. J Leuk Biol 1994;55:253–8.
[30] Xi H, Shin WS, Suzuki J, et al. Dystrophin disruption might be related to myocardial cell apoptosis caused by isoproterenol. J Cardiovasc Pharmacol 2000;36(Suppl 2):S25–9.
[31] Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 1991;11:81–128.
[32] Meigs JB, Hu FB, Rifai N, Manson JE. Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. JAMA 2004;291:1978–86.
[33] Uchida K, Toyokuni S, Nishikawa K, et al. Michael addition-type 4-hydroxy-2-nonenal adducts in modified low-density lipoproteins: markers for atherosclerosis. Biochemistry 1994;33:12487–94.
[34] Usatyuk PV, Natarajan V. Role of mitogen-activated protein kinases in 4-hydroxy-2-nonenal-induced actin remodeling and barrier function in endothelial cells. J Biol Chem 2004;279:11789–97.
[35] Jiang F, Guo Y, Salvemini D, Dusting GJ. Superoxide dismutase mimetic M40403 improves endothelial function in apolipoprotein (E)-deficient mice. Br J Pharmacol 2003;139:1127–34.
[36] O'Donnell VB, Chumley PH, Hogg N, Bloodsworth A, Darley-Usmar VM, Freeman BA. Nitric oxide inhibition of lipid peroxidation: kinetics of reaction with lipid peroxyl radicals and comparison with alpha-tocopherol. Biochemistry 1997;36:15216–23.
[37] White CR, Brock TA, Chang LY, et al. Superoxide and peroxynitrite in atherosclerosis. Proc Natl Acad Sci USA 1994;91:1044–8.
[38] Zheng H, Dimayuga C, Hudaihed A, Katz SD. Effect of dexrazoxane on homocysteine-induced endothelial dysfunction in normal subjects. Arterioscler Thromb Vasc Biol 2002;22:E15–8.
[39] Terentis AC, Thomas SR, Burr JA, Liebler DC, Stocker R. Vitamin E oxidation in human atherosclerotic lesions. Circ Res 2002;90:333–9.
[40] Marui N, Offermann MK, Swerlick R, et al. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. J Clin Invest 1993;92:1866–74.
[41] Park SH, Park JH, Kang JS, Kang YH. Involvement of transcription factors in plasma HDL protection against TNF-alpha-induced vascular cell adhesion molecule-1 expression. Int J Biochem Cell Biol 2003;35:168–82.
[42] Tsujimoto I, Hikoso S, Yamaguchi O, et al. The antioxidant edaravone attenuates pressure overload-induced left ventricular hypertrophy. Hypertension 2005;45:921–6.
[43] Amemiya S, Kamiya T, Nito C, et al. Anti-apoptotic and neuroprotective effects of edaravone following transient focal ischemia in rats. Eur J Pharmacol 2005;516:125–30.
[44] Doi K, Suzuki Y, Nakao A, Fujita T, Noiri E. Radical scavenger edaravone developed for clinical use ameliorates ischemia/reperfusion injury in rat kidney. Kidney Int 2004;65:1714–23.
[45] Tsujita K, Shimomura H, Kawano H, et al. Effects of edaravone on reperfusion injury in patients with acute myocardial infarction. Am J Cardiol 2004;94:481–4.
[46] Yoshida H, Sasaki K, Namiki Y, Sato N, Edaravone TN. a novel radical scavenger, inhibits oxidative modification of low-density lipoprotein (LDL) and reverses oxidized LDL-mediated reduction in the expression of endothelial nitric oxide synthase. Atherosclerosis 2005;179:97–102.
[47] Zhang XH, Matsuda N, Jesmin S, et al. Normalization by edaravone, a free radical scavenger, of irradiation-reduced endothelial nitric oxide synthase expression. Eur J Pharmacol 2003;476:131–7.
[48] Cipollone F, Fazia M, Iezzi A, et al. High preprocedural non-HDL cholesterol is associated with enhanced oxidative stress and monocyte activation after coronary angioplasty: possible implications in restenosis. Heart 2003;89:773–9.

早期診断の進歩とその活用法

頭部MRI画像における大脳白質病変の意義

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背　景

近年の医療機械の発達は医学の発展に寄与してきた．computed tomography（CT）およびmagnetic resonance imaging（MRI）により頭蓋内の微細な構造を知ることができるようになった結果，健常高齢者や認知症高齢者において大脳深部の白質にさまざまな程度の白質病変が認められることが明らかとなった．

1986年Hachinskiらは頭部CT画像にて低吸収，頭部MRI T2強調画像にて高信号として描出される白質の変化をleukoaraiosisと呼ぶことを提唱したが[1]，同病変はほかに白質病変white matter lesions（WMLs），periventricular lucency（PVL），脳室周囲高信号域periventricular hyperintensity（PVH），subcortical white matter lesions，深部白質病変deep white matter hyperintensity（DWMH）と呼ばれることもあり，いまだ用語ならびに定義は統一されていない．

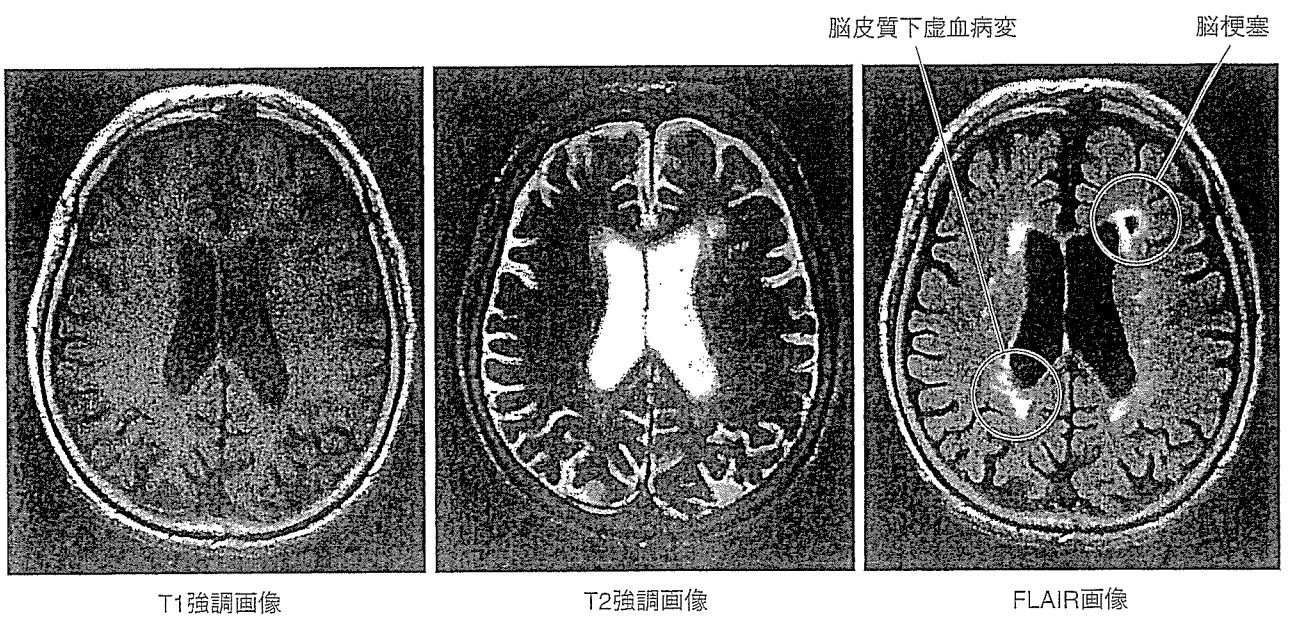
なお，本文中では白質病変を「脳皮質下に存在する虚血性変化に基づく病変」との意義を込めて，脳皮質下虚血病変と呼称する．

脳皮質下虚血病変の定義

1.　脳梗塞と脳皮質下虚血病変の鑑別

頭部MRI画像において，脳皮質下虚血病変はT1強調画像にて等信号域かつT2強調画像（またはFLAIR画像）にて高信号域を示す病変，脳梗塞はT1強調画像にて低信号域かつT2強調画像にて高信号域を示す病変と定義される（図1）．

また脳皮質下虚血病変は加齢，高血圧，動脈硬



頭部MRI画像	T1	T2またはFLAIR
脳梗塞	低信号	高信号
脳皮質下虚血病変	等信号	高信号

図1　脳皮質下虚血病変と脳梗塞の鑑別

●脳皮質下虚血病変は認知機能障害，意欲低下，歩行機能低下，排尿障害と関連がある．

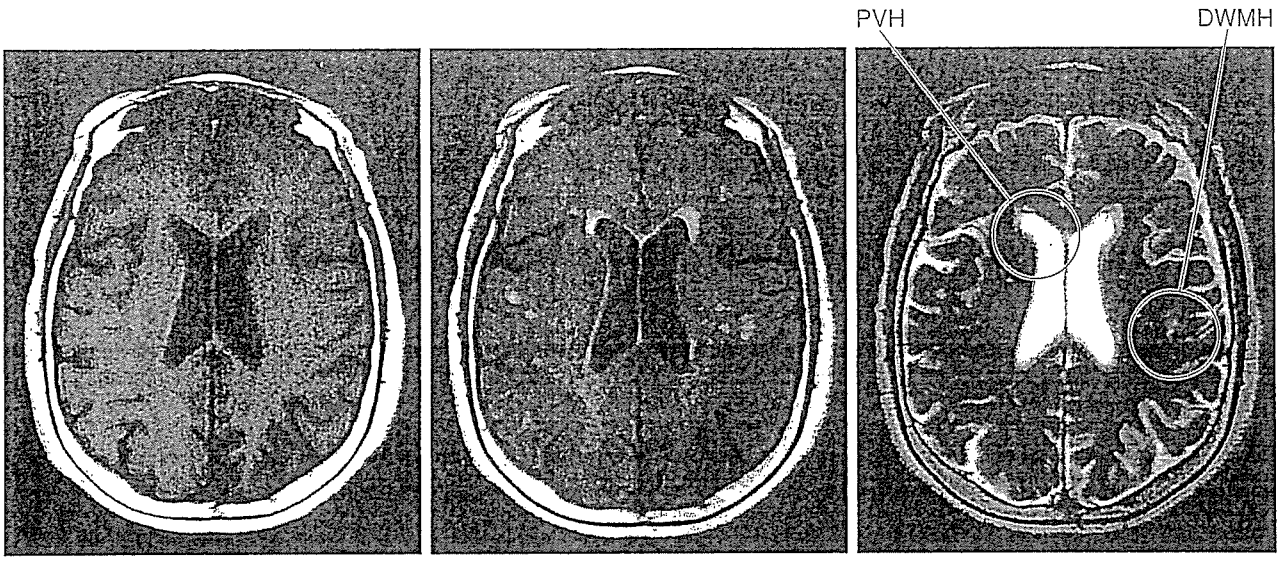


図2　PVHとDWMHの鑑別

化，脳血管障害，遺伝性素因(Notch3 gene, Apolipoprotein E, angiotensin-converting enzyme(ACE)など)と関連することが報告されており[2]，脳皮質下虚血病変の危険因子として脳血管障害の危険因子の存在が示唆されている．

2.　脳皮質下虚血病変の分類

脳皮質下虚血病変は脳室周囲と連続するPVHと，脳室周囲と連続性のないDWMHに分類される(図2)．

これらの病変は加齢に伴い増加し，PVHとDWMHは互いに関連を認めるものの[3]，その特徴や差異については明確にされていない．

脳皮質下虚血病変と臨床症候の関係●

1.　脳皮質下虚血病変と認知機能障害

脳皮質下虚血病変は加齢との関連が強く，認知症高齢者のみならず健常高齢者においても同病変を認めることから，過去においては同病変が正常な脳の加齢変化に伴って出現する無症候性の放射線学的所見であるのか，脳血管性認知症をはじめとする認知機能低下の発現あるいは増悪に関与しているのかについて一定した見解はなかった．

しかし，de Grootらによる1,077名の健常高齢者を対象とした検討において，脳皮質下虚血病変がmini-mental state examination(MMSE)を含めた認知機能の低下と関連したとの報告をはじめとして[4]，認知症高齢者や健常高齢者における認知機能低下と脳皮質下虚血病変との間の関連を指摘する報告が多くなされ，近年においては脳皮質下虚血病変が認知機能低下の増悪因子としてきわめて重要であることが示唆されている．

認知機能とは外界からの情報を選択的に取り入れ，新たな情報を生体内に蓄積し，適切な行為の選択を行うための生体の能動的な情報収集・情報処理活動の総称であり，記憶，見当識，理解，思考，判断，言語，学習，計算，遂行機能が含まれている．

脳皮質虚血病変は記憶力の低下と関連するのみ