Table 3 Unadjusted and adjusted 2-year and 5-year mortality ratio

		2-year mortality ratio Unadjusted hazard ratio (95% CI)	*P*-value	Adjusted hazard ratio (95% CI)**	*P*-value	5-year mortality ratio Unadjusted hazard ratio (95% CI)	*P*-value	Adjusted hazard ratio (95% CI)**	*P*-value
Oral status									
	A	1.0		1.0		1.0		1.0	
	B	1.55 (0.79–3.05)	0.202	1.31 (0.66–2.60)	0.472	1.35 (0.91–2.01)	0.134	1.13 (0.75–1.70)	0.554
	C	3.09 (1.75–5.46)	< 0.001	1.84 (1.01–3.36)	0.047	1.93 (1.38–2.71)	< 0.001	1.30 (0.90–1.88)	0.168
Age*									
	65–74	1.0		1.0		1.0		1.0	
	75–84	1.19 (0.60–2.35)	0.614	1.42 (0.71–2.84)	0.321	1.93 (1.17–3.17)	< 0.001	2.29 (1.38–3.81)	0.001
	85–	2.62 (1.39–4.96)	0.003	2.92 (1.51–5.66)	0.002	3.91 (2.41–6.34)	< 0.001	4.51 (2.74–7.44)	< 0.001
Gender	Male	1.0		1.0		1.0		1.0	
	Female	0.55 (0.37–0.81)	0.003	0.46 (0.30–0.70)	< 0.001	0.71 (0.53–0.96)	0.028	0.54 (0.39–0.73)	< 0.001
Cardiac disease	Absent	1.0		1.0		1.0		1.0	
	Present	0.96 (0.58–1.59)	0.865	1.10 (0.65–1.85)	0.718	0.94 (0.66–1.32)	0.705	1.01 (0.71–1.44)	0.961
Cerebrovascular disease	Absent	1.0		1.0		1.0		1.0	
	Present	1.24 (0.84–1.84)	0.280	0.99 (0.66–1.48)	0.942	1.54 (1.18–2.02)	0.002	1.33 (1.00–1.77)	0.038
Diabetes mellitus	Absent	1.0		1.0		1.0		1.0	
	Present	1.02 (0.52–2.02)	0.954	1.20 (0.60–2.42)	0.601	0.92 (0.56–1.51)	0.921	1.14 (0.69–1.89)	0.612
Cognitive function Dementia	Absent	1.0		1.0		1.0		1.0	
	Present	4.06 (1.66–9.95)	0.002	2.22 (0.87–5.65)	0.095	2.32 (1.46–3.67)	< 0.001	1.58 (0.97–2.58)	0.067
ADL Dependence	Absent	1.0		1.0		1.0		1.0	
	Present	3.32 (1.89–5.81)	< 0.001	2.22 (1.21–4.06)	0.010	1.89 (1.39–2.57)	< 0.001	1.44 (1.02–2.05)	0.040

*Age adjusted odds ratios. Age was fitted 10 years age bands: 65–74; 75–84; 85 or more. **Adjusted model includes the following variables: age; gender and severity of cognitive function and ADL.

Table 4 Underlying and immediate causes of death during 5-year follow up

Causes of death	Group A (n = 99)		Group B (n = 98)		Group C (n = 206)	
	Number of deaths	Mortality rate (n/N,%)	Number of deaths	Mortality rate (n/N,%)	Number of deaths	Mortality rate (n/N,%)
All causes	45	45.5	54	55.1	136	66.0
Respiratory-tract infections	14	14.1	14	14.3	38	18.4
Senility without mention of psychosis	5	5.1	8	8.2	39	18.9
Ischemic heart disease	6	6.1	8	8.2	18	8.7
Cerebrovascular disease	8	8.1	3	3.1	10	4.9
Malignant neoplasms	2	2.0	4	4.1	4	1.9
Other infections*	0	0.0	4	4.1	3	1.5
Gastrointestinal bleeding	0	0.0	0	0.0	5	2.4
Cirrhosis of the liver	0	0.0	1	1.0	1	0.5
Renal failure	0	0.0	0	0.0	1	0.5
External and Unknown causes	10	10.1	12	12.2	17	8.3

*Other infections include septicemia (n = 3) and infections of the kidney and urinary tract (n = 4).

It is well documented that many older patients in nursing homes have poor oral status. Other researchers have shown that such poor dental status was strongly associated with age, cognitive function and ADL.[13,21] Nordenram *et al.* reported significant correlations between the ability to chew and cognitive and functional capacity.[22] These findings may be explained in relation to the character of institutionalized elderly patients. For example, they are unlikely to perform personal oral hygiene care sufficient to keep adequate natural dentition because of impaired cognitive function and lower ADL.[5,6] With progression of the disease, demented patients would not keep their dentures on at ease and physically disabled patients may be recommended not to use dentures to prevent inspiration of the dentures.

Further, oral hygiene in long-term-care institutions has been neglected and there are different explanations for this, such as the difficulty of access to professional dental care,[23] little time to share by the staff,[24] and lack of understanding, knowledge, interest by the staff including primary care physicians and geriatricians.[25,26] On the basis of such conditions for oral health and care, many institutionalized elderly may lose their teeth and may be unsatisfactorily treated.

We found that inadequate dentition for mastication significantly increased the risk of 2-year overall mortality in institutionalized elderly patients. A few reports have shown that edentulous people without dentures are significantly prone to death as compared to those with adequate dentition in community-dwelling elderly people[27,28] or institutionalized elderly people.[15] Appollonio *et al.* reported that edentulous people without dentures had a significant risk for death independent of physical–mental health status at baseline and discussed that poor dental status may have negative effects on mortality through malnutrition.[28] In another report they showed that inadequate dental status and micronutrients such as folate were significant and independent predictors of mortality in community-dwelling elderly women.[29] Moreover, a number of studies have shown that poor dental status is associated with malnutrition.[30,31] Although we did not consider estimating nutritional status such as bodyweight and serum albumin, previous studies strongly suggest that inadequate dental status and the susceptibility to death are partly linked by malnutrition.

In contrast, the present study showed that inadequate dental status did not seem to be an independent prognostic variable of 5-year mortality but an associated variable of other strong predictors of mortality, such as increasing age and low ADL. Adjustment for these factors weakened the predictive power of dental status. A possibility is that longer survival rates may be greatly influenced by age and ADL because these older and frail patients might reach the end points sooner apart from dental status.

In our study, death from respiratory infections was scored as a primary cause of death, and no other terminal diseases entered into this group.[32] Therefore, the prevalence of the death from respiratory infections might have been underestimated because people diagnosed as senility were in poorly defined conditions and likely to have underlying disease conditions such as asymptomatic pneumonia.[32] Although a direct and independent relationship between poor dental state and death from respiratory infections remains unclear, it is recognized that respiratory infections can be the result of infection by anaerobic bacteria, and dental plaque would seem to be a logical source of these bacteria, especially in patients with periodontal disease.[12] Poor

dental health may contribute to the development of pneumonia as an independent or associated prognostic variable.[33] We previously demonstrated that intensive oral care lowered the frequency of pneumonia by 50% in the institutionalized elderly.[14] Therefore, intensive oral care should be recommended for patients who cannot keep their teeth clean by themselves to prevent respiratory infections.[34]

A potential weakness of the study is that patients' concurrent illness that would have affected their prognosis was not fully confirmed or followed up because of limited capacity for objective evaluation in the nursing homes and unwillingness for intensive medical care for relatively old patients. Further, lack of patients' subjective compliant due to limited ADL and cognitive function, especially in Group C, might have made it difficult to identify an underlying fatal disease (Table 4). These provide possible reasons why senility was negatively selected as a cause of death without the supportive evidence.

Another limitation of the present study was that the baseline examination of dental status might not reflect their lifetime dental status, and thus not effectively stratify their risk. This might be true especially for elderly people with comorbidity, causing increased mortality and inadequate dental status when close to death. This factor may explain, hypothetically, the association between inadequate dental status and mortality in institutionalized elderly. Although 30 edentulous patients who had dentures but had not used them were included in Group C, their mortality rates were similar to that of the other edentulous patients who did not have dentures (data not shown); therefore, such a distorting mechanism is unlikely.

In summary, our findings highlight a broader concern about inadequate dental status for mastication in institutionalized elderly people and its relation to poor outcomes. Moreover, the present study provides a basis for keeping an adequate dental status in institutionalized elderly patients in order to minimize poor dental status related deaths. Thus, our findings suggest that systemic attention to dental status should be recommended. However, further study about the relationship between poor dental status and specific causes of mortality is necessary to better elucidate the role of poor dental status both as a precursor and as a sequel of disease states to improve methods for its management.

Acknowledgments

We are very grateful to the Department of Removable Prostholontics, Faculty of Dentistry, Hiroshima University; the Department of Dental and Oral and Maxillofacial Surgery, Hamamatsu University School of Medicine; the Department of Periodontology, Ohu University School of Dentistry; Sendai Dental Association; Futyu City Dental Association; Yono City Dental Association; and the Amagasaki City Dental Association for their generous assistance with this study. This study was supported in part by grants from the Japanese Foundation for Aging and Health from the Ministry of Health, Labor and Welfare (No. 045).

References

1 Gift HC, Atchison KA. Oral health, health and health-related quality of life. *Med Care* 1995; **33** (Suppl): NS57–NS77.
2 Kressin NR, Boehmer U, Nunn MF, Spiro A III. Increased preventive practices lead to greater tooth retention. *J Dent Res* 2003; **82**: 223–227.
3 Hawkins RJ. Functional status and untreated dental caries among nursing home residents aged 65 and over. *Spec Care Dentist* 1999; **19**: 158–163.
4 Steele JG, Sheiham A, Marcenes W, Walls AWG. *National Diet and Nutrition Survey: People Aged 65 Years and Over. Vol. 2: Report of the Oral Health Survey*. London: Stationery Office, 1998.
5 Scannapieco FA, Papandonatos GD, Dunford RG. Associations between oral conditions and respiratory disease in a national sample survey population. *Ann Periodontol* 1998; **3**: 251–256.
6 Simons S, Kidd EAM, Beighton D. Oral health of elderly occupants in residential homes. *Lancet* 1999; **353**: 1761.
7 Miyazaki H, Shirahama R, Ohtani I *et al.* Oral health conditions and denture treatment needs in institutionalized elderly people in Japan. *Community Dent Oral Epidemiol* 1992; **20**: 297–301.
8 Carlsson GE. Masticatory efficiency: the effect of age, the loss of teeth and prosthetic rehabilitation. *Int Dent J* 1984; **34**: 93–97.
9 Leake JL. An index of chewing ability. *J Public Health Dent* 1990; **50**: 262–267.
10 Wayler AH, Chauncey H. Impact of complete dentures and impaired natural dentition on masticatory performance and food choice in healthy aging men. *J Prosthet Dent* 1983; **49**: 427–433.
11 Chauncey HH, Muench ME, Kapur KK, Wayler AH. The effect of the loss of teeth on diet and nutrition. *Int Dent J* 1984; **34**: 93–97.
12 Scannapieco FA, Mylotte JM. Relationships between periodontal disease and bacterial pneumonia. *J Periodontol* 1996; **67**: 1114–1122.
13 Dormenval V, Budtz-Jörgensen E, Mojon P *et al.* Nutrition, general health status and oral health status in hospitalized elders. *Gerodontology* 1995; **12**: 73–80.
14 Yoneyama T, Yoshida M, Ohrui T *et al.* Oral care reduces pneumonia in older patients in nursing home. *J Am Geriatr Soc* 2002; **50**: 430–433.
15 Shimazaki Y, Soh I, Saito T *et al.* Influence of dentition status on physical disability, mental impairment, and mortality in institutionalized elderly people. *J Dent Res* 2001; **80**: 340–345.
16 Yoneyama T, Yoshida M, Matsui T, Sasaki H, Oral Care Working Group. Oral care and pneumonia. *Lancet* 1999; **354**: 515.
17 Folstein MF, Folstein SE, McHugh PR. Mini-Mental State. A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* 1975; **12**: 189–198.
18 Mahoney FI, Barthel DW. Functional evaluation: Barthel Index. *Maryland State Med J* 1965; **14**: 61–65.

19 WHO. *International Classification of Diseases, Injuries, and Causes of Death*. Geneva, Switzerland: WHO, 1977.
20 Cox DR. Regression models and life-tables. *J Roy Stat Soc B* 1972; **34**: 187–220.
21 Lundgren M, Osterberg T, Emilson G *et al.* Oral complaints and utilization of dental services in relation to general services in relation to general health factors in an 88-year-old Swedish population. *Gerodontology* 1995; **12**: 81–88.
22 Nordenram G, Ljunggren G. Oral status, cognitive and functional capacity versus oral treatment need in nursing home residents: a comparison between assessments by dental and ward staff. *Oral Dis* 2002; **8**: 296–302.
23 Gift HC, Cherry-Peppers G, Oldakowski RJ. Oral health status and related behaviours of US nursing home residents, 1995. *Gerodontology* 1997; **14**: 89–99.
24 Karuza J, Miller WA, Lieberman D *et al.* Oral status and resident well-being in a skilled nursing facility population. *Gerontologist* 1992; **32**: 104–112.
25 Fiske J, Lloyd HA. Dental needs of residents and carers in elderly people's homes and carers' attitudes to oral health. *Eur J Prosthodont Restor Dent* 1992; **1**: 91–95.
26 Baum BJ. Oral health for the older patient. *J Am Geriatr Soc* 1996; **44**: 997–998.
27 Osterberg T, Melstrom D, Sundh V. Dental health and functional ageing: a study of 70-year-old people. *Community Dent Oral Epidemiol* 1990; **18**: 313–318.
28 Appollonio I, Carabellese C, Frattola A, Trabucchi M. Dental status, quality of life, and mortality in an older community population: a multivariate approach. *J Am Geriatr Soc* 1997; **45**: 1315–1323.
29 Appollonio I, Carabellese C, Frattola A, Trabucchi M. Influence of dental status on dietary intake and survival in the community-dwelling elderly. *Age Ageing* 1997; **26**: 445–456.
30 Sullivan DH, Martin W, Flaxman N *et al.* Oral health problems and involuntary weight loss in a population of frail elderly. *J Am Geriatr Soc* 1993; **41**: 725–731.
31 Posner BM, Jette A, Smigelski C *et al.* Nutritional risk in New England elders. *J Gerontol* 1994; **49**: M123–M132.
32 Yamaya M, Yanai M, Ohrui T *et al.* Intervention to prevent pneumonia among older adults. *J Am Geriatr Soc* 2001; **49**: 85–89.
33 Yoshino A, Ebihara T, Ebihara S *et al.* Daily oral care and risk factors for pneumonia among elderly nursing home patients. *JAMA* 2001; **286**: 2235–2236.
34 Terpenning M, Shay K. Oral health is cost-effective to maintain but costly to ignore. *J Am Geriatr Soc* 2002; **50**: 584–585.

ELECTRONIC LETTER

Association of susceptibility to the development of pneumonia in the older Japanese population with haem oxygenase-1 gene promoter polymorphism

H Yasuda, S Okinaga, M Yamaya, T Ohrui, M Higuchi, M Shinkawa, S Itabashi, K Nakayama, M Asada, A Kikuchi, S Shibahara, H Sasaki

J Med Genet 2006;**43**:e17 (http://www.jmedgenet.com/cgi/content/full/43/4/e17). doi: 10.1136/jmg.2005.035824

Background: Oxidative stresses including cigarette smoking are implicated in the pathogenesis of cerebrovascular diseases, which are associated with pneumonia because of frequent aspiration. Haem oxygenase-1 (HO-1) acts in cytoprotection against oxidants, provides anti-inflammatory effects, and inhibits atherogenesis. A $(GT)_n$ dinucleotide repeat in the human *HO-1* promoter modulates *HO-1* gene expression and shows length polymorphism, which is grouped into three classes: class S (<27 repeats), class M (≥27, <33 repeats), and class L (≥33 repeats) alleles.

Objective: To investigate the correlation between the *HO-1* gene polymorphism and development of pneumonia in elderly Japanese.

Methods: The length of the $(GT)_n$ repeats was analysed in 200 elderly patients with pneumonia and 200 control subjects. The association of the *HO-1* gene polymorphism with risk of pneumonia was estimated by logistic regression.

Results: The proportion of allele frequencies in class L, and the proportion of genotypic frequencies in the L-allele carriers (L/L, L/M, and L/S), was significantly higher in patients with pneumonia than in controls (20% *v* 10% in class L, and 34% *v* 18% in L-allele carriers). After adjustment for potentially confounding factors, both cerebrovascular disorders and *HO-1* gene L-allele carriers were significant and independent risk factors for pneumonia. The adjusted odds ratio for L-allele carriers *v* non-L-allele carrier was 2.1 (95% confidence interval, 1.2 to 3.6).

Conclusions: The large size of a $(GT)_n$ repeat in the *HO-1* gene promoter may be associated with susceptibility to pneumonia in the older Japanese population.

Pneumonia is not only a common infection in older people, it is also the most common cause of death from nosocomial infection in the Japanese population.[1] Disorders of the central nervous system are more likely to develop in the elderly, and pneumonia has been estimated to occur in about one third of patients with stroke.[2] Cerebrovascular disease is associated with a high incidence of pneumonia owing to frequent aspiration.[3] As well as factors including diabetes mellitus, hyperlipidaemia, and hypertension, oxidative stresses such as cigarette smoking are also associated with the pathogenesis of cerebrovascular disease.[4] Genetic factors affecting antioxidants may be involved in the susceptibility to atherosclerosis of the cerebral arteries and the subsequent development of pneumonia in the elderly. Although the antioxidant enzymes inhibit the formation of atherosclerosis,[5] the roles of reduced expression of these enzymes on the development of pneumonia in elderly people are still uncertain.

Haem oxygenase (HO) oxidatively degrades haem to biliverdin, which is subsequently reduced to bilirubin, an efficient scavenger of reactive oxygen species (ROS), by biliverdin reductase.[6] HO-1, an inducible form of HO—and also a constitutive form of HO, including HO-2—provides cellular protection against haem mediated and non-haem-mediated oxidant injury.[6] HO-1 is thought to be an essential component in protection against various ROS.

A $(GT)_n$ repeat in the 5′ flanking region of the human *HO-1* gene is polymorphic,[7] and modulates human *HO-1* gene transcription by thermal stress[8] and hydrogen peroxide.[7] The size of the $(GT)_n$ repeat in the *HO-1* gene is associated with the antiapoptotic effects of HO-1 in lymphoblastoid cell lines.[9] We have shown that the size of the $(GT)_n$ repeat in the *HO-1* gene is associated with susceptibility to chronic pulmonary emphysema (CPE)[7] and lung adenocarcinoma,[10] and with longevity[11] in Japanese populations. This *HO-1* gene polymorphism is also associated with coronary artery disease, one of vascular diseases related to ROS.[12] However, the association between the size of the $(GT)_n$ repeat in the *HO-1* gene and the development of pneumonia in older populations is still uncertain.

In the present study, we screened allelic frequencies of the $(GT)_n$ repeats in the *HO-1* gene promoter in elderly people with and without pneumonia, and examined the association between the risk of senile pneumonia and length of the $(GT)_n$ repeats.

METHODS

Clinical protocol and patient characteristics

We studied 200 elderly patients with pneumonia and 200 elderly control subjects without pneumonia, attending the departments of internal medicine in six hospitals in Miyagi prefecture. The hospitals were a university hospital, a Red Cross hospital, three public general hospitals, and a municipal hospital. All participants were Japanese and aged 65 and older. To evaluate whether *HO-1* genotypes are associated with the development of pneumonia in elderly Japanese people, we selected the subjects with a performance status of 2 or better[13] and in a stable state as potential participants, because those with too low a performance status ran a greater risk of infectious disease, which might mask the preventive effect of any genetic factors. Patients were given a score of 0 if they were fully active and asymptomatic, 1 if they were symptomatic but fully ambulatory, 2 if they were

Abbreviations: COPD, chronic obstructive pulmonary disease; CPE, chronic pulmonary emphysema; HO, haem oxygenase; HO-1, inducible haem oxygenase; ROS, reactive oxygen species; TNF, tumour necrosis factor

Table 1 Characteristics of the study subjects

Characteristics	Control subjects (n = 200)	Patients with pneumonia (n = 200)	p Value
Age (years)*	73.8 (0.7)	75.4 (1.0)	NS
Sex			
Male	99 (50%)	101 (50%)	NS
Female	101 (50%)	99 (50%)	
Performance status			
0–1	114 (57%)	108 (54%)	NS
2	86 (43%)	92 (46%)	
Smoking history (pack-year)*	18.2 (2.6)	19.3 (2.8)	NS
Cerebrovascular disease			
Yes	14 (7%)	101 (50%)	<0.0001
No	186 (93%)	99 (50%)	
COPD			
Yes	35 (18%)	38 (19%)	NS
No	165 (82%)	162 (81%)	
Congestive heart failure			
Yes	17 (9%)	28 (14%)	NS
No	183 (91%)	172 (86%)	
Hypertension			
Yes	43 (22%)	59 (30%)	NS
No	157 (78%)	141 (70%)	
Diabetes mellitus			
Yes	21 (10%)	34 (17%)	NS
No	179 (90%)	166 (83%)	
Hyperlipidaemia			
Yes	9 (5%)	10 (5%)	NS
No	191 (95%)	190 (95%)	

Values are n (%) or *mean (SD).
COPD, chronic obstructive pulmonary disease.

symptomatic and confined to bed or chair for less than 50% of their waking hour, 3 if they were symptomatic and confined to bed or chair for more than 50% of their waking hours, and 4 if they were completely bedridden. The study was approved by the Tohoku University ethics committee, and informed consent was obtained from each subject. This study was carried out between April 2002 and December 2004.

During the study period, 264 elderly patients with pneumonia were identified. Pneumonia was defined as pulmonary infiltrate on chest radiograph, cough, and a temperature higher than 38.0°C.[3] All patients with pneumonia had the features of pulmonary infiltrate on chest radiographs, cough, and a temperature above 38.0°C. The patients were enrolled consecutively. Among them, we selected for the case group those with a performance status of 2 or better and in a stable state. We excluded patients who were immunocompromised—for example, those with active malignant disease, on renal dialysis, receiving corticosteroid treatment, or with HIV-1 infection. Patients were also excluded if they had obvious swallowing dysfunction, chronic sepsis in pressure sores, venous ulcers, or an indwelling urinary catheter. After these selections and exclusions were applied, 200 elderly patients with pneumonia were enrolled in the case group.

Potential control subjects were 439 elderly patients who continued attending the departments of hospitals over the study period and who had never had pneumonia at any time in their life including the study period. Control subjects were excluded if their past history relating to pneumonia were unclear. After the same selection and exclusion criteria as in the case group were applied, 383 control subjects were available for frequency matching. To carry out a case–control study, we randomly selected 200 control subjects in a frequency matched manner from the control cohort. They were frequency matched on age (±5 years), sex, smoking history, and performance status with the patients with pneumonia. Physical characteristics, smoking history, and complications in patients with pneumonia and control subjects are shown in table 1.

Analysis of length variability of $(GT)_n$ repeats in HO-1 gene promoter

Genomic DNAs were extracted from leucocytes in peripheral venous blood by conventional procedures. The 5′-flanking region containing a poly $(GT)_n$ repeat of the *HO-1* gene was amplified by polymerase chain reaction (PCR)[7 11] with a fluorescently labelled primer pl-s (5′-AGAGCCTGCAGC TTCTCAGA-3′) and an unlabeled antisense primer pl-as (5′-ACAAAGTCTGGCCATAGGAC-3′), which were designed

Table 2 Allele and genotypic frequencies of HO-1at polymorphic locus

	Control subjects (n = 200)	Patients with pneumonia (n = 200)	OR (95% CI) v all other classes or subjects	p Value
Allele class				
L	38 (10%)	79 (20%)	2.3 (1.5 to 3.5)	<0.0001
M	189 (47%)	159 (40%)	0.7 (0.5 to 0.9)	<0.05
S	173 (43%)	162 (40%)	0.9 (0.7 to 1.2)	NS
Genotype group				
L-allele carrier	36 (18%)	68 (34%)	2.3 (1.5 to 3.7)	<0.001
Non-L-allele carrier	164 (82%)	132 (66%)		

CI, confidence interval; OR, odds ratio.

Table 3 Multivariate analysis of risk factors related to pneumonia in older adults

Variable	OR (95% CI)	p Value
Haem oxygenase-1 genotype subgroup		
L-allele carriers v non-L-allele carriers	2.1 (1.2 to 3.6)*	<0.01
Cerebrovascular disease		
Yes v no	28.0 (13.3 to 58.6)†	<0.0001

*OR was calculated with the non-L-allele carriers as the reference group, and adjusted for age, gender, performance status, smoking history, and complications.
†OR was calculated with the patients without cerebrovascular disease as the reference group, and adjusted for age, gender, performance status, smoking history, *HO-1* genotype, and complications other than cerebrovascular disease.
CI, confidence interval; OR, odds ratio.

according to the published sequence.[7 14] The PCR was carried out over 30 cycles of 20 seconds at 94°C, 10 seconds at 60°C, and 20 seconds at 72°C. The PCR products were analysed in a DNA sequencer (ALF express II DNA sequencer version 2.2, Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). Each size of $(GT)_n$ repeat in the PCR product was calculated with ALFwin fragment analysis version 1.03 (Amersham Pharmacia Biotech) using four cloned alleles as size markers, which were already sequenced with the ABI prism dye terminator sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, California, USA).[7] The repeat numbers of these size markers were 16, 23, 29, and 38, respectively. The investigators of genetic analysis were blinded with respect to the status of the subjects.

Carboxyhaemoglobin concentrations in patients with pneumonia

Blood samples were taken from the radial artery in patients with pneumonia on the first day of hospital admission. The patients for the carboxyhaemoglobin analysis were all non-smokers and consisted of five L-allele carriers and five non-L-allele carriers (L/L genotype and S/S genotype, respectively), who showed a similar C reactive protein concentration (15.0 to 20.0 mg/dl) and white blood cell (WBC) count (9500 to 12 500 cells/μl) at the time of analysis. The carboxyhaemoglobin concentrations were measured with a spectrophotometer (ASL System, Radiometer, Copenhagen, Denmark).[15]

Statistical analysis

In the analysis of *HO-1* gene polymorphism in this study, the patient and control groups were frequency matched by age, sex, performance status, and smoking history. For statistical analysis, age and smoking history (pack-year) between the two groups were compared using Student's *t* test, and sex, performance status, and the frequency of the complications between the two groups were compared using χ^2 tests (table 1), as described previously in coronary artery disease.[12] The proportion of allelic frequencies and genotypic frequencies between the two groups were also compared using the χ^2 test (table 2). Factors associated with the presence of senile pneumonia such as age, sex, performance status, smoking status, complications, and *HO-1* gene polymorphism (L-allele carrier) were examined with multivariate analysis by logistic regression analysis (table 3). Odds ratios (OR) and their 95% confidence intervals (CI) were calculated to assess the relative risk conferred by a particular genotype (L-allele carrier), and adjusted for age, sex, performance status, smoking history, and complications using logistic regression as described previously (table 3).[12] All the statistical analyses were undertaken using SYSTAT (version 10.2; SYSTAT Software, Richmond, California, USA). The values for age and smoking history (pack-year) are reported as means (SD). The *HO-1* genotype distributions were in Hardy–Weinberg equilibrium. Significance was accepted at $p<0.05$.

For statistical analysis in the study on the correlation between carboxyhaemoglobin level and *HO-1* genotype in the patients with pneumonia, the mean values for age (year), smoking history (pack-year), WBC number (cells/μl), C reactive protein (mg/dl), and carboxyhaemoglobin concentration (%) between the five L-allele carriers and the five non-L-allele carriers were compared using Student's *t* test and sex using the χ^2 test (table 4).

RESULTS

Allele frequencies of HO-1 gene in control and patients with pneumonia in older adults

There were between 16 and 39 $(GT)_n$ repeats in the human *HO-1* gene in the study subjects (fig 1). The distribution of the number of $(GT)_n$ repeats was trimodal, as previously reported, with two main peaks located at 23 and 30 GT repeats and another peak located at 33 GT repeats.[7 10 11] We therefore divided the alleles into three subclasses, as previously reported[7]: class S (<27 repeats), class M (≥27 and <33 repeats), and class L (≥33 repeats) alleles.

In the control subjects, the distributions of the 400 alleles were 173 (43%) class S, 189 (45%) class M, and 38 (10%) class L (table 2); in the patients with pneumonia, the distributions were 162 (40%) class S, 159 (40%) class M, and 79 (20%) class L. The proportion of allelic frequencies in class L was significantly higher in all patients with pneumonia (n = 79, 20%) than that in all control subjects (n = 38, 10%)

Table 4 Arterial blood carboxyhaemoglobin in patients with pneumonia

Patient	HO-1 genotype	Age (years)*	Sex†	Smoking history (pack-year)*	WBC (cells/μl)*	CRP (mg/dl)*	Arterial blood Hb-CO (%)‡
L-allele carrier 1	LL	71	M	0	12 300	18.3	0.57
L-allele carrier 2	LL	65	F	0	10 500	15.2	0.20
L-allele carrier 3	LL	79	F	0	9 700	15.7	0.80
L-allele carrier 4	LL	73	F	0	9 600	19.0	0.21
L-allele carrier 5	LL	76	F	0	10 020	19.4	1.20
Non-L-allele carrier 1	SS	65	M	0	12 400	18.5	1.50
Non-L-allele carrier 2	SS	77	M	0	11 000	16.3	1.20
Non-L-allele carrier 3	SS	79	F	0	10 500	19.2	1.02
Non-L-allele carrier 4	SS	65	F	0	9 900	15.6	1.10
Non-L-allele carrier 5	SS	75	F	0	9 600	19.5	0.90

*There was no significant difference in the mean value between L-allele carrier and non-L-allele carrier ($p>0.7$).
†There was no significant difference in the ratio between L-allele carrier and non-L-allele carrier ($p>0.5$).
‡There was a significant difference in the mean value between L-allele carrier and non-L-allele carrier ($p<0.04$).
CRP, C reactive protein; F, female; Hb-CO, carboxyhaemoglobin; M, male; WBC, white blood cell count.

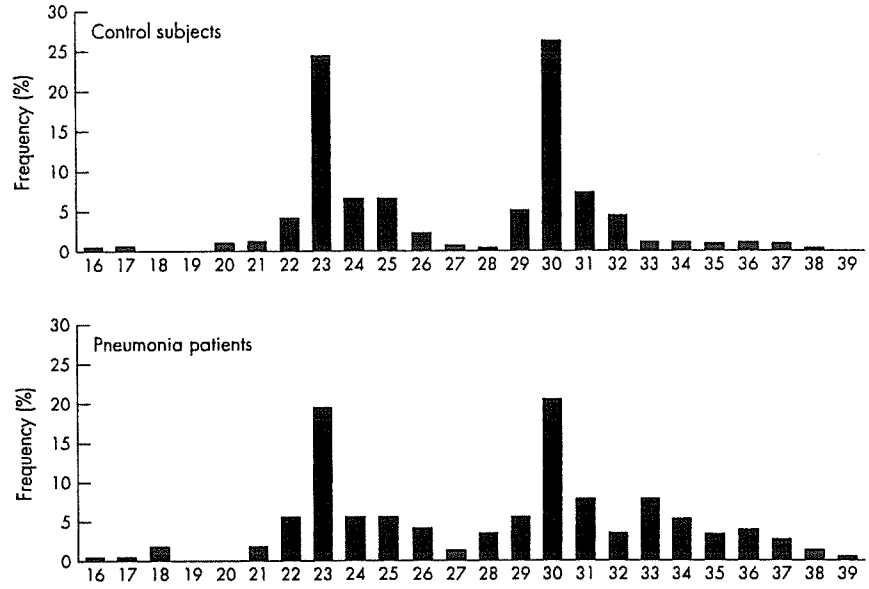

Figure 1 Frequency distribution of the number of $(GT)_n$ repeats in control subjects (n = 400 alleles) and patients with pneumonia (n = 400 alleles).

(p<0.0001). The odds ratio for pneumonia with L alleles *v* non-L alleles (class M allele + class S allele) was 2.3 (95% CI, 1.5 to 3.5) (table 2).

Genotypic frequencies of HO-1 gene in control and patients with pneumonia

Six genotypes (L/L, L/M, L/S, M/M, M/S, and S/S) of $(GT)_n$ repeats in the human *HO-1* gene promoter were divided into two subgroups according to allelic subclasses: L-allele carriers with a class L allele (L/L, L/M, L/S) and non-L-allele carriers without a class L allele (M/M, M/S and S/S).[7] The proportion of genotypic frequencies in L-allele carriers was significantly higher in all patients with pneumonia (n = 68, 34%) than that in all control subjects (n = 36, 18%) (p<0.0001). The odds ratio for patients with pneumonia with L-allele carriers *v* non-L-allele carriers was 2.3 (95% CI, 1.4 to 3.7) (table 2).

Risk factors for pneumonia

On multivariate analysis, cerebrovascular disease (p<0.0001) and *HO-1* genotype (p<0.01) were significantly and independently associated with the development of pneumonia (table 3), when the variables were adjusted by age, sex, performance status, smoking history, and complications including congestive heart failure, COPD, hypertension, diabetes mellitus, and hyperlipidaemia. The adjusted odds ratio (95% CI) was 2.1 (1.2 to 3.6) for *HO-1* genotype and 28.0 (18.3 to 58.6) for cerebrovascular disease (table 3).

Carboxyhaemoglobin concentrations in patients with pneumonia

To show the correlation between *HO-1* genotype and HO-1 activity caused by the inflammation of pneumonia, we examined the carboxyhaemoglobin concentration in several patients with pneumonia on their first day of hospital admission. The subjects for carboxyhaemoglobin analysis were five L-allele carriers and five non-L-allele carriers (L/L genotype and S/S genotype, respectively). There were no significant differences in age, sex, smoking history, WBC count, and C reactive protein concentration level between these two groups. However, the patients without the L-allele showed significantly higher carboxyhaemoglobin levels than those with the L-allele (1.14 (0.23)% *v* 0.5 (0.42)%, respectively; p<0.04) (table 4).

DISCUSSION

In this study we analysed *HO-1* gene polymorphism and showed that the proportion of allele frequencies in class L and the proportion of genotypic frequencies in the L-allele carriers (L/L, L/M, and L/S) were significantly higher in elderly people with pneumonia than in control subjects. The proportion of subjects with cerebrovascular disease in the pneumonia group was significantly higher than in the control group. With multivariate analysis, *HO-1* genotype and the presence of cerebrovascular disease were significant and independent risk factors for pneumonia. These findings suggest that the large size of a $(GT)_n$ repeat in the *HO-1* gene promoter may be associated with the development of pneumonia in older Japanese people with cerebral infarction.

Disorders of the central nervous system are more likely to develop in the elderly, and pneumonia has been estimated to occur in about one third of patients with stroke.[2] Basal ganglia infarction is associated with a high incidence of pneumonia owing to frequent aspiration[3] resulting from the reduction in the cough and swallowing reflexes.[16] In fact, in the present study, half these older patients with pneumonia also had cerebrovascular disease.

Oxidative stress such as cigarette smoking[1] is one of the important risk factors for cerebrovascular diseases, including basal ganglia infarction. Various ROS including superoxide and hydrogen peroxide induce lipid peroxide formation, which is a key process in atherosclerotic plaques in hypercholesterolaemia.[17] ROS are also involved in the brain tissue damage in stroke.[18] On the other hand, antioxidant systems such as glutathione, superoxide dismutase, and HO are suggested to protect the vascular disease caused by ROS.[19] The initial degradation of haem by microsomal HO involves the liberation of iron and CO and the formation of biliverdin, which is subsequently reduced to bilirubin by cytosolic biliverdin reductase.[4] Higher intracellular HO-1 activity may increase the content of bilirubin, which is an efficient scavenger of ROS,[6] and a natural inhibitor of intimal hyperplasia after balloon injury.[20] In fact, Ishikawa *et al.*

reported inhibitory effects of HO-1 on the atherogenesis in hyperlipidaemic rabbits.[21] Enhanced endothelial cell injury caused by oxidative stress was observed in a human case of *HO-1* deficiency.[22] Reduced expression of HO-1 might be partly associated with the development of stroke and subsequent pneumonia.

A $(GT)_n$ dinucleotide repeat in the 5′-flanking region of human *HO-1* gene shows length polymorphism.[7] We previously reported the influence of the number of the $(GT)_n$ repeats on the inducibility of the *HO-1* gene promoter under oxidative stimulus by transient transfection assay in human cell lines. The promoter activity of *HO-1* is modulated by the length variability of the $(GT)_n$ repeats, and large $(GT)_n$ repeats have a potent inhibitory activity on H_2O_2 induced gene expression of HO-1.[7] Furthermore, Epstein-Barr virus transformed lymphoblastoid cell lines were established from smokers with class L alleles (L/L) and with class S (S/S). When treated with H_2O_2, lymphoblastoid cells with the L/L genotype showed lower viability than those with the S/S genotype.[9] The GT dinucleotide repeat polymorphism has emerged as a potent genetic risk factor in various diseases, including vascular diseases such as coronary arteriosclerosis[12] and restenosis after balloon angioplasty.[23] These findings are consistent with the view that tissues of the non-L allele carrier could employ the antioxidant activity of HO-1 to a greater extent than that of the L-allele carrier when exposed to reactive oxygen species.[10] Large $(GT)_n$ repeats may affect the protective function against oxidant induced vascular endothelial injury and arteriosclerosis through the inhibition of HO-1 expression.

The results of our study suggest that the *HO-1* genotype is associated with susceptibility to pneumonia independently of cerebrovascular disease. Senile pneumonia is characterised by a high likelihood of aspiration pneumonia.[10] The severity of aspiration pneumonia is associated with the lung inflammation mediated by cytokines such as tumour necrosis factor α (TNFα).[24] On the other hand, it was reported that overexpression of the *HO-1* gene attenuated inflammation and decreased apoptosis of bronchial epithelial cells in a murine model of lung inflammation induced by *Pseudomonas aeruginosa*.[25] Furthermore, overexpression of the *HO-1* gene could reduce TNFα mediated apoptotic cell death in human endothelial cells.[26] These findings suggest that *HO-1* gene expression could be associated with the progress of aspiration pneumonia, and that reduced expression of the *HO-1* gene in elderly L-allele carriers might allow the development of pneumonia independently of cerebrovascular disease.

To examine the association between *HO-1* genotype and HO-1 activity in the pneumonia, we evaluated the carboxyhaemoglobin level in L-allele carriers and non-L-allele carriers with pneumonia. As a result, even after adjustment for the peripheral WBC count and C reactive protein level, patients without the L-allele showed higher carboxyhaemoglobin levels than those with the L-allele. Carbon monoxide (CO) is produced endogenously by HO and combines haemoglobin to form carboxyhaemoglobin complex. Therefore, the carboxyhaemoglobin concentration in the subject is a good marker of endogenous HO activity.[27] Furthermore, it has been reported that HO-1 is strongly induced in patients with bacterial infection.[28] We have already shown that arterial carboxyhaemoglobin increases at the onset of pneumonia in untreated patients returns to baseline on recovery after treatments.[15] We also showed that an increase in arterial carboxyhaemoglobin in pneumonia would be caused by carbon monoxide production in pulmonary inflammation, and that the arterial carboxyhaemoglobin is significantly correlated with disease severity in patients with bacterial pneumonia.[29] A study of lymphoblastoid cell lines by Hirai *et al* showed that mRNA level and activity of HO-1 were significantly higher in lymphoblastoid cells with the S/S genotype than in those with the L/L genotype after oxidant stimulation.[9] Therefore, analysis of the carboxyhaemoglobin level in pneumonia according to *HO-1* genotype would clarify the association between the *HO-1* genotype and HO-1 activity—that is, the HO-1 protein level, resulting from pneumonia. These findings suggest that HO-1 induction might be associated with the *HO-1* genotype (S>M>L).

In contrast to arterial blood carboxyhaemoglobin concentrations, we did not measure HO-1 activity in patients with pneumonia at the onset. However, we obtained new blood samples from eight people in the control group and seven in the pneumonia group after recovery from pneumonia, and analysed the serum HO-1 protein levels using enzyme linked immunosorbent assay methods as previously described.[30] There was no significant difference between these two groups when they were in good physical condition (2.6 (1.2) *v* 2.4 (1.0) ng/ml, $p>0.2$). These values were compatible with the results from a previous report.[30] Because the *HO-1* gene is inducible by inflammation or oxidative stress, the baseline expression of the this gene should be low regardless of the *HO-1* genotype, which was demonstrated in lymphoblastoid cell by Hirai *et al*.[9] Further studies are needed to clarify the relation between HO-1 activity and the *HO-1* genotype at the onset of pneumonia.

Conclusions

This is the first study to show that the 5′-flanking polymorphism in the *HO-1* gene is associated with the development of pneumonia in an older Japanese population with basal ganglia infarction. Increased susceptibility to developing pneumonia may be associated with sclerosis in the cerebral arteries.

ACKNOWLEDGEMENTS

We thank Drs Daisuke Inoue, Hisao Hirai, and Satoru Ebihara for samples, and Mr G Crittenden for English language editing. This study was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture (17790524, 16590732, and 17590591) of the Japanese government to HY, MY, and TO, respectively, and also supported in part by grants from the Japanese Foundation for Aging and Health to KN.

Authors' affiliations

H Yasuda, S Okinaga, M Yamaya, T Ohrui, M Higuchi, M Shinkawa, S Itabashi, K Nakayama, M Asada, A Kikuchi, H Sasaki, Department of Geriatric and Respiratory Medicine, Tohoku University School of Medicine, Sendai, Japan

S Shibahara, Department of Molecular Biology and Applied Physiology, Tohoku University School of Medicine

Conflicts of interest: none declared.

Correspondence to: Dr Mutsuo Yamaya, Department of Geriatric and Respiratory Medicine, Tohoku University School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, 980-8574, Japan; yamaya@geriat.med.tohoku.ac.jp

Received 11 June 2005
Revised version received 11 September 2005
Accepted for publication 17 September 2005

REFERENCES

1 **Niederman MS.** Nosocomial pneumonia in the elderly patient: chronic care facility and hospital considerations. *Clin Chest Med* 1993;14:479–90.
2 **Walker AE,** Robins M, Weinfeld FD. Clinical findings: the National Survey of Stroke. *Stroke* 1981;12(suppl 1):113–44.
3 **Nakagawa T,** Sekizawa K, Arai H, Kikuchi R, Manabe K, Sasaki H. High incidence of pneumonia in elderly patients with basal ganglia infarction. *Arch Intern Med* 1997;157:321–4.
4 **Sacco RL.** Newer risk factors for stroke. *Neurology* 2001;57(suppl 2):S31–4.

5 **Fukai T**, Folz RJ, Landmesser U, Harrison DG. Extracellular superoxide dismutase and cardiovascular disease. *Cardiovasc Res* 2002;**55**:239–49.
6 **Maines MD**. The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol* 1997;**37**:517–54.
7 **Yamada N**, Yamaya M, Okinaga S, Nakayama K, Sekizawa K, Shibahara S, Sasaki H. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. *Am J Hum Genet.* 2000;66: 187–95, [Erratum, *Am J Hum Genet* 2001;**68**:1542.].
8 **Okinaga S**, Takahashi K, Takeda K, Yoshizawa M, Fujita H, Sasaki H, Shibahara S. Regulation of human heme oxygenase-1 gene expression under thermal stress. *Blood* 1996;**87**:5074–84.
9 **Hirai H**, Kubo H, Yamaya M, Nakayama K, Numasaki M, Kobayashi S, Suzuki S, Shibahara S, Sasaki H. Microsatellite polymorphism in heme oxygenase-1 gene promoter is associated with susceptibility to oxidant-induced apoptosis in lymphoblastoid cell lines. *Blood* 2003;**102**:1619–21.
10 **Kikuchi A**, Yamaya M, Suzuki S, Yasuda H, Kubo H, Nakayama K, Handa M, Sasaki T, Shibahara S, Sekizawa K, Sasaki H. Association of susceptibility to the development of lung adenocarcinoma with the heme oxygenase-1 gene promoter susceptibility. *Hum Genet* 2005;**116**:354–60.
11 **Yamaya M**, Nakayama K, Ebihara S, Hirai H, Higuchi S, Sasaki H. Relationship between microsatellite polymorphism in the haem oxygenase-1 gene promoter and longevity of the normal Japanese population. *J Med Genet* 2003;**40**:146–8.
12 **Chen YH**, Lin SJ, Lin MW, Tsai HL, Kuo SS, Chen JW, Charng MJ, Wu TC, Chen LC, Ding PYA, Pan WH, Jou YS, Chau LY. Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. *Hum Gen* 2002;**111**:1–8.
13 **Oken MM**, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, Carbone PP. Toxicity and response criteria of the eastern cooperative oncology group. *Am J Clin Oncol* 1982;**5**:649–55.
14 **Shibahara S**, Sato M, Muller RM, Yoshida T. Structural organization of the human heme oxygenase gene and the function of its promoter. *Eur J Biochem* 1989;**179**:557–63.
15 **Yasuda H**, Yamaya M, Yanai M, Ohrui T, Sasaki H. Increased blood carboxyhemoglobin concentrations in inflammatory pulmonary diseases. *Thorax* 2002;**57**:779–83.
16 **Yamaya M**, Yanai M, Ohrui T, Arai H, Sasaki H. Interventions to prevent pneumonia among older adults. *J Am Geriatr Soc* 2001;**49**:85–90.
17 **Nilsson J**, Regnstrom J, Frostegard J, Stiko A. Lipid oxidation and atherosclerosis. *Herz* 1992;**17**:263–9.
18 **Braughler JM**, Hall, eds. Central nervous system trauma and stroke. I.Biochemical considerations for oxygen radical formation and lipid peroxidation. *Free Radic Biol Med* 1989;6:289–301.
19 **Maytin M**, Leopold J, Loscalzo J. Oxidant stress in the vasculature. *Curr Atherosclerosis Rep* 1999;**1**:156–64.
20 **Öllinger R**, Bilban M, Erat A, Froio A, McDaid J, Tyagi S, Csizmadia E, Graça-Souza AV, Liloia A, Soares MP, Otterbein LE, Usheva A, Yamashita K, Bach FH. Bilirubin: a natural inhibitor of vascular smooth muscle cell proliferation. *Circulation* 2005;**112**:1030–9.
21 **Ishikawa K**, Sugowara D, Goto J, Watanabe Y, Kawamura K, Shiomi M, Itabe H, Maruyama Y. Heme oxygenase-1 inhibits atherosclerosis in Watanabe heritable hyperlipidemic rabbits. *Circulation* 2001;**104**:1831–6.
22 **Yachie A**, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, Koizumi S. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J Clin Invest* 1999;**103**:129–35.
23 **Exner M**, Schillinger M, Minar E, Mlekusch W, Schlerka G, Haumer M, Mannhalter C, Wagner O. Heme oxygenase-1 gene promoter microsatellite polymorphism is associated with restenosis after percutaneous transluminal angioplasty. *J Endovasc Ther* 2001;**8**:433–40.
24 **Davidson BA**, Knight PR, Helinski JD, Nader ND, Shanley TP, Johnson KJ. The role of tumor necrosis factor-alpha in the pathogenesis of aspiration pneumonitis in rats. *Anesthesiology* 1999;**91**:486–99.
25 **Tsuburai T**, Kaneko T, Nagashima Y, Ueda A, Tagawa A, Shinohara T, Ishigatsubo Y. Pseudomonas aeruginosa-induced neutrophilic lung inflammation is attenuated by adenovirus-mediated transfer of the heme oxygenase 1 cDNA in mice. *Hum Gene Ther* 2004;**15**:273–85.
26 **Kushida T**, Li Volti G, Quan S, Goodman A, Abraham NG. Role of human heme oxygenase-1 in attenuating TNF-alpha-mediated inflammation injury in endothelial cells. *J Cell Biochem* 2002;**87**:377–85.
27 **Marks GS**, Vreman HJ, McLaughlin BE, Brien JF, Nakatsu K. Measurement of endogenous carbon monoxide formation in biological systems. *Antioxid Redox Signal* 2002;**4**:271–7.
28 **Yachie A**, Toma T, Mizuno K, Okamoto H, Shimura S, Ohta K, Kasahara Y, Koizumi S. Heme oxygenase-1 production by peripheral blood monocytes during acute inflammatory illnesses of children. *Exp Biol Med* 2003;**228**:550–6.
29 **Yasuda H**, Sasaki T, Yamaya M, Ebihara S, Maruyama M, Kanda A, Sasaki H. Increased arteriovenous carboxyhemoglobin differences in patients with inflammatory pulmonary diseases. *Chest* 2004;**125**:2160–8.
30 **Kirino Y**, Takeno M, Iwasaki M, Ueda A, Ohno S, Shirai A, Kanamori H, Tanaka K, Ishigatsubo Y. Increased serum HO-1 in hemophagocytic syndrome and adult-onset Still's disease: use in the differential diagnosis of hyperferritinemia. *Arthritis Res Ther* 2005;**7**:R616–24.

Address correspondence and reprint requests to Dr. G.I. Wolfe, Department of Neurology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-8897; e-mail: gil.wolfe@utsouthwestern.edu

References

1. Griggs RC, Mendell JR, Miller RG. The muscular dystrophies. In: Griggs RC, Mendell JR, Miller RG, eds. Evaluation and treatment of myopathies. Philadelphia: Davis, 1995:93–153.
2. Wahl M. When form meets function: exploring surgery to restore muscle power in FSH dystrophy. Quest 2003;2:22–27.
3. Copeland SA, Howard RC. Thoracoscapular fusion for facioscapulohumeral dystrophy. J Bone Joint Surg Br 1978;60:547–551.
4. Twyman RS, Harper GD, Edgar MA. Thoracoscapular fusion in facioscapulohumeral dystrophy: clinical review of a new surgical method. J Shoulder Elbow Surg 1996;5:201–205.
5. Kocialkowski A, Frostick SP, Wallace WA. One-stage bilateral thoracoscapular fusion using allografts. Clin Orthop 1991;273:264–267.
6. Sakurai M. Thoracic scapulopexy for restoration of arm elevation in facioscapulohumeral type muscular dystrophy. In: Bateman JE, Welsh PE, eds. Surgery of the shoulder. Philadelphia: Becker, 1984:255–258.
7. Matsumura JS, Rilling WS, Pearce WH, et al. Helical computed tomography of the normal thoracic outlet. J Vasc Surg 1997;26:776–783.

ACE inhibitors and protection against pneumonia in elderly patients with stroke

T. Arai, MD; K. Sekizawa, MD; T. Ohrui, MD; H. Fujiwara, MD; N. Yoshimi, MD; H. Matsuoka, MD; and H. Sasaki, MD

Pneumonia is the most common cause of death from nosocomial infection in the elderly. The increased incidence of pneumonia and the high mortality are consequences of a number of age-related factors, including coexisting illnesses, therapeutic interventions, and the aging process itself.[1] Pneumonia has been estimated to occur in about one third of patients with stroke.[2] The most important factor contributing to the risk of pneumonia in patients with stroke is suggested to be dysphagia with aspiration.[1]

Angiotensin-converting enzyme (ACE) inhibitors have been shown to improve silent aspiration[3] and prevent pneumonia in elderly patients with stroke.[4] However, little is known about whether ACE inhibitors have a beneficial role in reducing the risk of pneumonia as compared to other classes of antihypertensive drugs in elderly patients with stroke. Thus, we investigated whether ACE inhibitors can reduce the risk of pneumonia as compared to other antihypertensive drugs.

Methods. We recruited patients with stroke who were followed up for more than 6 months after their ictus from eight outpatient clinics. We enrolled 1,190 patients in April 1999, and prospectively followed them for 35 months. The criteria for diagnosis of pneumonia and the patients' inclusion and exclusion criteria were described previously.[5] Eligible patients were those who received antihypertensive therapy, had a history of stroke, but were not bedridden.

We analyzed the incidence of pneumonia in three groups of hypertensive patients with stroke who were classified on the basis of treatment with antihypertensive drugs as follows: patients who received ACE inhibitors, calcium-channel blockers, and diuretics. Our hypertensive patients received only the same class of antihypertensive drugs. The control group consisted of non-hypertensive patients with stroke who did not receive any antihypertensive drugs. Follow-up data were available for all participants.

For the main analyses, we used the log-rank procedure and Cox's proportional hazards model to calculate the CI. Cumulative incidence curves were generated by the Kaplan-Meier method for endpoints in the ACE inhibitors, calcium-channel blockers, diuretics, and control groups. Significance was set at $p < 0.05$.

Results. There were no significant differences in age, sex, stroke severity as assessed by NIH Stroke Scale,[6] and poststroke duration among the four groups (table). During the follow-up, new pneumonia was diagnosed in 12 (2.8%) of the 430 patients in the ACE inhibitors group, 36 (8.8%) of the 409 patients in the calcium-channel blockers group, 29 (8.3%) of the 351 patients in the diuretics group, and 14 (8.8%) of the 160 patients in the control group. The patients in the ACE inhibitors group had a lower risk of pneumonia than those in the control group; the hazard ratio was 0.30 (95% CI 0.14 to 0.66, $p = 0.0013$). However, the risk in the calcium-channel blockers group (1.01, 95% CI 0.53 to 1.92, $p > 0.40$) or the diuretics group (0.94, 95% CI 0.48 to 1.83, $p > 0.30$) did not differ from that in the control group.

Discussion. We found a significantly reduced risk of pneumonia in patients receiving ACE inhibitors vs control patients. No such decreased risk was noted in users of calcium-channel blockers or diuretics. Silent aspiration reportedly disappears by treatment with ACE inhibitors in association with an increase in the serum substance P levels in hypertensive patients with stroke.[3] ACE inhibitors may increase the serum substance P levels, thereby reducing aspiration pneumonia in elderly patients with stroke.

A recent large-scale randomized trial has demonstrated that treatment with ACE inhibitors significantly reduced the risk of pneumonia among the participants of Asian ethnicity, although the protective effects of ACE inhibitors against pneumonia were not observed in the non-Asian participants.[7] However, this trial[7] included patients with a history of transient ischemic attacks and the mean Barthel index score of the patients was quite high. Since the incidence of pneumonia increased in association with a decrease in the Barthel index score,[5] the effects of ACE inhibitors against pneumonia might be underestimated by a population of patients with a high activity of daily life. Our present study only included patients with well-documented cerebral hemispheric strokes.

The present study supports the hypothesis that treatment with ACE inhibitors may be beneficial in reducing the risk of pneumonia in elderly patients with stroke.

From the Arai Clinic (Dr. Arai), Gifu; Department of Respiratory Medicine (Dr. Sekizawa), Institute of Clinical Medicine, University of Tsukuba, Ibaraki; Department of Geriatric and Respiratory Medicine (Drs. Ohrui and Sasaki), Tohoku University School of Medicine, Sendai; Second Department of Internal Medicine (Dr. Fujiwara), Gifu University School of Medicine, Gifu; Tumor Pathology (Dr. Yoshimi), University of Ryukyu Faculty of Medicine, Okinawa; and Department of Internal Medicine (Dr. Matsuoka), Dokkyo University School of Medicine, Tochigi, Japan.

Received July 2, 2004. Accepted in final form October 8, 2004.

Table Characteristics and clinical features of the four groups

	ACE inhibitors	Calcium-channel blockers	Diuretics	Control
No.	430	409	351	160
Female/male	224/206	213/196	183/168	78/82
Mean age, y	75 (1)	75 (1)	75 (1)	76 (1)
Stroke severity	6 (1)	6 (1)	6 (1)	6 (2)
Poststroke duration, y	3.1 (1.1)	3.3 (0.9)	3.4 (1.1)	3.3 (1.2)

Values in parentheses are SD.

ACE = angiotensin-converting enzyme.

Address correspondence and reprint requests to Dr. Kiyohisa Sekizawa, Department of Respiratory Medicine, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennoudai, Ibaraki 305-8575, Japan; e-mail: kiyo-se@md.tsukuba.ac.jp

References

1. Ely EW, Haponik EF. Pneumonia in the elderly. J Thorac Imaging 1991; 6:45–61.
2. Walker AE, Robins M, Weinfeld FD. Clinical findings: the National Survey of Stroke. Stroke 1981;12:I-13–I-37.
3. Arai T, Yoshimi N, Fujiwara H, Sekizawa K. Serum substance P concentrations and silent aspiration in elderly patients with stroke. Neurology 2003;61:1625–1626.
4. Sekizawa K, Matsui T, Nakagawa T, Nakayama K, Sasaki H. ACE inhibitors and pneumonia. Lancet 1998;352:1069.
5. Nakagawa T, Sekizawa K, Arai H, Kikuchi R, Manabe K, Sasaki H. High incidence of pneumonia in elderly patients with basal ganglia infarction. Arch Intern Med 1997;157:321–324.
6. Goldstein LB, Bertels C, Davis JN. Interrater reliability of the NIH stroke scale. Arch Neurol 1989;46:660–662.
7. Ohkubo T, Chapman N, Neal B, et al. Effects of an angiotensin-converting enzyme inhibitor-based regimen on pneumonia risk. Am J Respir Crit Care Med 2004;169:1041–1045.

Hypernatremia from a hunger strike as a cause of osmotic myelinolysis

Annette H.M. van der Helm-van Mil, MD; Jeroen P.P. van Vugt, MD, PhD; Gert Jan Lammers, MD, PhD; and Hubertus I.J. Harinck, MD, PhD

Too rapid correction of hyponatremia often causes osmotic myelinolysis. A rapid shift from normal to hypernatremia may also be dangerous. We report a hunger striker that developed an extreme serum sodium concentration followed by coma and radiologic abnormalities characteristic of osmotic myelinolysis.

Case report. A 19-year-old Algerian asylum seeker started a hunger strike after his residence permit had been rejected. His medical history was unremarkable. He used no medications, including lithium. One month before fasting, he was placed in isolation because of behavioral disturbances. At this time, the weather was hot, and the patient refused sufficient intake of water and food. One day after he stopped eating and drinking, he became confused; after another 5 days, he became somnolent. He was transferred to a penitentiary hospital. On admission, his serum sodium level was 187 mmol/L, creatinine 213 μmol/L, and glucose 6.8 mmol/L. Urine osmolality was not measured. A feeding tube rehydration regimen was started with 2 L/day of water. His sodium level was 172 mmol/L the next day. After 2 days, he became comatose and was referred to our intensive care unit. On admission, his blood pressure was 105/55 mm Hg, temperature was 38.5°C, Glascow Coma Scale score was 6 (E1M4V1), the pupillary light reflex was delayed, and the Achilles tendon reflexes were absent. Sodium level was 152 mmol/L, potassium 2.5 mmol/L, creatinine 91 μmol/L, urea 7.3 mmol/L, phosphate 0.41 mmol/L, magnesium 0.96 mmol/L, and albumin 26 g/L. CT of the brain and CSF analysis were normal. EEG showed diffuse slowing. A chest radiograph showed bilateral infiltrates. The patient was intubated and treated for aspiration pneumonia. Potassium and phosphate were replaced.

Because of the extreme hypernatremia, osmotic myelinolysis was considered. Brain MRI 5 days after admission was consistent with pontine and extrapontine myelinolysis (figure, A and B). MRI also revealed acute hydrocephalus (see the figure, B) and posterior fossa edema (see the figure, C). An external ventricular drain was inserted. The intracranial pressure proved normal. As the patient did not respond to 6 days of drainage, the drain was removed. Over the next days, the pupillary light reflex normalized and the patient regained consciousness. When asked, he was able to open, close, and move his eyes and slightly move his fingers. No other voluntary movements were possible. After 1 month, his neurologic condition gradually improved. After 4 months, he was able to speak and walk short distances. After 7 months, he was fully recovered but needed a cane while walking.

Discussion. We present a hunger striker that developed osmotic myelinolysis due to extreme hypernatremia from dehydration. The clinical presentation with confusion and coma several days after onset of the severe electrolyte disturbance followed by spontaneous recovery in the course of months is consistent with osmotic myelinolysis.[1]

Central pontine myelinolysis was first described in 1959, associated with alcoholism and malnutrition.[2] In 1976, it was first linked to hyponatremia.[3] In hypotonic hyponatremia, water initially enters brain cells, resulting in cerebral edema. The brain cells adapt by losing electrolytes and organic osmolytes, thus arresting a further influx of water. If chronic hyponatremia is cor-

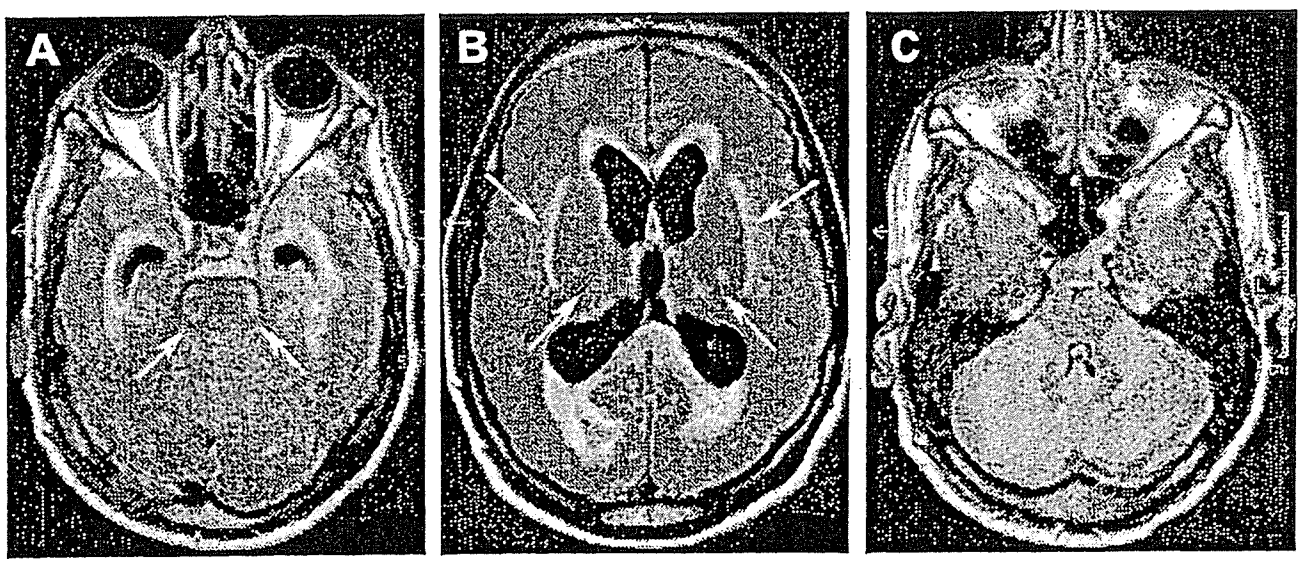

Figure. Axial fluid-attenuated inversion recovery MRI of the brain showing hyperintensities in the dorsolateral regions of the pons (A) and bilaterally in the thalamus, globus pallidus, and capsula extrema (B), consistent with osmotic myelinolysis. Also note the enlargement of the lateral and third ventricles with periventricular hyperintensities (B). This acute triventricular hydrocephalus was presumably caused by posterior fossa edema, yielding impaired CSF circulation (C).

High Glucose-Induced Upregulation of Osteopontin Is Mediated via Rho/Rho Kinase Pathway in Cultured Rat Aortic Smooth Muscle Cells

Harukiyo Kawamura, Koutaro Yokote, Sunao Asaumi, Kazuki Kobayashi, Masaki Fujimoto, Yoshiro Maezawa, Yasushi Saito, Seijiro Mori

Objective—Osteopontin is upregulated in the diabetic vascular wall and in vascular smooth muscle cells cultured under high glucose concentration. In the present study, we analyzed the mechanism of high glucose-induced upregulation of osteopontin in cultured rat aortic smooth muscle cells.

Methods and Results—We found that an inhibitor of Rho-associated protein kinase, Y-27632, suppressed osteopontin mRNA expression under high glucose concentration. Transfection of cells with a constitutive active Rho mutant, pSRα-myc-RhoDA, enhanced osteopontin mRNA expression. Furthermore, incubation of cells under high glucose concentration activated Rho, indicating that Rho/Rho kinase pathway mediates high-glucose–stimulated osteopontin expression. Treatment of cells with an inhibitor of protein kinase C, GF109203X, and azaserine, an inhibitor of the hexosamine pathway, suppressed high glucose-induced Rho activation. Glucosamine treatment was shown to activate Rho. Treatment of cells with an inhibitor of MEK1, PD98059, suppressed osteopontin mRNA expression under high glucose concentration. Incubation of cells under high glucose concentration activated ERK. Finally, transfection of cells with pSRα-myc-RhoDA also activated ERK.

Conclusions—In conclusion, our present findings support a notion that Rho/Rho kinase pathway functions downstream of protein kinase C and the hexosamine pathways and upstream of ERK in mediating high-glucose–induced upregulation of osteopontin expression. (*Arterioscler Thromb Vasc Biol.* 2004;24:276-281.)

Key Words: osteopontin ■ Rho ■ glucose ■ atherosclerosis ■ smooth muscle cells

Osteopontin (OPN)[1] is a multifunctional phosphoprotein secreted by many cell types such as osteoclasts, lymphocytes, macrophages, epithelial cells, and vascular smooth muscle cells (SMC).[1,2] Overexpression of OPN has been found in several physiological and pathological conditions, including immunologic disorders,[3] neoplastic transformation,[4] progression of metastasis,[5] formation of urinary stones,[6] and wound healing.[7]

It was reported that OPN protein and mRNA were expressed in the neointima and in calcified atheromatous plaque.[8] A neutralizing antibody against OPN was found to inhibit rat carotid neointimal formation after endothelial denudation.[9] These results have suggested that OPN promotes the development of atherosclerosis. Recently, we found upregulation of OPN expression in diabetic human and rat vascular walls.[10] It was also noted that high glucose concentrations stimulated OPN expression via a protein kinase C (PKC)-dependent pathway and the hexosamine pathway in cultured rat aortic SMC.[11] Furthermore, OPN was found to stimulate migration and enhance platelet-derived growth factor-mediated DNA synthesis of cultured rat aortic SMC.[10] Based on these data, we suggest that OPN plays a role in accelerated atherogenesis in diabetes mellitus.

In the present study, we further analyzed the mechanism of high glucose-induced upregulation of OPN in cultured rat aortic SMC. We show that Rho/Rho kinase pathway functions downstream of PKC and the hexosamine pathways and upstream of ERK in mediating high glucose-stimulated OPN expression.

Methods

Reagents

GGTI-298, an inhibitor of geranylgeranyltransferase I, FTI-277, an inhibitor of farnesyltransferase, Y-27632, an inhibitor of Rho-associated protein kinase, GF109203X, an inhibitor of PKC, PD98059, an inhibitor of MEK1, SB203580, an inhibitor of p38 mitogen-activated protein (MAP) kinase, and SP600125, an inhibitor of c-Jun N-terminal kinase (JNK), were purchased from Calbiochem (La Jolla, CA). Azaserine, an inhibitor of glutamine:fructose-6-phosphate amidotransferase (GFAT) was from Sigma (St. Louis, MO). The p44/42 MAP kinase assay kit, p38 MAP kinase assay kit, and SAPK/JNK assay kit were from Cell Signaling Technology (Beverly, MA). Rho activation assay kit was from UBI (Lake Placid,

Received July 1, 2003; revision accepted November 19, 2003.

From the Department of Clinical Cell Biology and Medicine, Chiba University Graduate School of Medicine, Inohana, Chiba, Japan

Correspondence to Koutaro Yokote Department of Clinical Cell Biology and Medicine, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chiba 260-8670, Japan. E-mail kyokote-cib@umin.ac.jp

Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

DOI: 10.1161/01.ATV.0000112012.33770.2a

NY). pSRα-myc-RhoDA, an expression vector containing a constitutive active Rho mutant, was kindly provided by Dr Yoshimi Takai (Osaka University, Osaka, Japan). Rat OPN cDNA was from Dr Mark Thiede (Pfizer, Groton, CT). Rat glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA was from Dr Masashi Yamazaki (Chiba University, Chiba, Japan). Pitavastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, was from Dr Masaki Kitahara (Nissan Chemical, Saitama, Japan).

Cell Culture

Primary cultures of rat aortic SMC were isolated as described[12] by the explant method from adult male Wistar rats weighing ≈200 grams. Cells were maintained in Dulbeco modified Eagle medium containing 5.5 mmol/L glucose, 10% fetal bovine serum, and 40 μg/mL gentamicin (Schering-Plough, Kenilworth, NJ) in a humidified atmosphere at 37°C in 5% CO_2. Cells at passages 7 to 9 were used for the present experiments.

Transient Transfection

At 50% confluency in 100-mm dishes, cells were transfected with pSRα-myc-RhoDA by using Fugene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). pSRα-myc-RhoDA was mixed with Fugene 6 transfection reagent at the ratio of 1:3 and incubated at room temperature for 15 to 40 minutes. Then, cells were transfected by incubation with the mixture for 24 hours. After additional 48 hours of incubation under normal glucose concentration (5.5 mmol/L glucose), cells were processed for Northern blotting and MAP kinase activity assays.

Northern Blotting

Subconfluent cells growing in 100-mm dishes were treated with the indicated concentrations of specific inhibitors under normal or high (30 mmol/L) glucose concentrations. After 48 hours of incubation, total RNA was isolated from cells using ISOGEN (Nippon Gene, Tokyo, Japan). Northern hybridization was performed essentially as described[11] using ^{32}P-labeled rat OPN cDNA probe. The blots were stripped and subsequently re-hybridized with ^{32}P-labeled rat GAPDH cDNA probe to assess the amount of RNA loaded in each lane, or with ^{32}P-labeled Rho cDNA probe to estimate the efficiency of transfection with pSRα-myc-RhoDA. Densitometric analysis of fluorograms and autoradiograms were performed using the imaging scanner (EPSON ES 8000) with the NIH Image 1.44 software.

Assay of ERK1/2, p38 MAP Kinase and SAPK/JNK Activities

Subconfluent cells growing in 100-mm dishes were serum-starved for 24 hours and then incubated under different glucose concentrations for the indicated times. After conditioning, activities of ERK1/2 and p38 MAP kinase in cell lysates were measured by immune complex kinase assay using the p44/42 MAP kinase assay kit with an immobilized phospho p44/42 MAP kinase antibody and Elk-1 protein as substrate, or using the p38 MAP kinase assay kit with an immobilized phospho p38 MAP kinase antibody and ATF-2 protein as substrate, respectively, according to the manufacturer's instructions. After phosphorylation reactions, samples were processed for Western blotting with phospho Elk-1 antibody or phospho ATF-2 antibody. After transfection with pSRα-myc-RhoDA, JNK activity was also evaluated by immune complex kinase assay using the SAPK/JNK assay kit with an c-Jun fusion protein beads followed by Western blotting with phospho c-Jun antibody, according to manufacturer's instructions.

Rho Activation Assay

Subconfluent cells growing in 150-mm dishes were treated with the indicated concentrations of GF109203X or azaserine under high glucose concentration, or with the indicated concentrations of glucosamine under normal glucose concentration for 24 hours. Thereafter, Rho activity was measured using the Rho activation assay kit according to the manufacturer's instructions. GTP-Rho in cell lysates was adsorbed to GST-Rhotekin Rho binding domain, which binds selectively to GTP-Rho, not GDP-Rho. After precipitation, samples were processed for Western blotting with a specific anti-Rho antibody.

Western Blotting

Samples were dissolved in SDS sample buffer and boiled for 5 minutes, and the proteins were separated by SDS-PAGE on 15% (wt/vol) polyacrylamide resolving gels and electrophoretically transferred to nitrocellulose membranes (Hybond-ECL; Amersham Biosciences, Piscataway, NJ). For blocking nonspecific binding, membranes were incubated in Block Ace (Dainippon Chemicals, Tokyo, Japan) at room temperature for 1 hour. Then, the membranes were probed with the phospho Elk-1 antibody (dilution 1:1000), the phospho ATF-2 antibody (dilution 1:1000), or the anti-Rho antibody (3 μg/mL) in a dilution buffer consisting of phosphate-buffered saline containing 10% Block Ace at 4°C overnight. After being washed with phosphate-buffered saline containing 0.1% Tween-20, the membranes were incubated with an anti-rabbit IgG horseradish peroxidase-linked whole antibody (dilution 1:1000, Amersham Biosciences) in the dilution buffer at room temperature for 1 to 2 hours. After washing, the antibody binding bands were detected using an enhanced chemiluminescence system (ECL Western blotting detection reagents and analysis system; Amersham Biosciences) and visualized by exposure to Hyperfilm-ECL (Amersham Biosciences). Each experiment presented in this study was repeated at least twice under the identical conditions to confirm the reproducibility of the observations.

Results

Pitavastatin Suppresses OPN Expression Under High Glucose Concentration

Recently, we found upregulation of OPN expression in diabetic human and rat vascular walls.[10] Furthermore, oral administration of Pitavastatin, an HMG-CoA reductase inhibitor, effectively suppressed abnormally upregulated expression of OPN mRNA in the aorta and kidney of streptozotocin-induced diabetic rats.[13] These findings prompted us to examine in vitro effect of Pitavastatin on high glucose-induced upregulation of OPN expression in cultured rat aortic SMC. Cells were incubated with different concentrations of Pitavastatin at 37°C for 48 hours under high glucose concentration (30 mmol/L glucose). After incubation, the cells were processed for Northern blotting. As shown in Figure 1A, Pitavastatin dose-dependently decreased OPN mRNA level. Pitavastatin did not show cytotoxic effect at the examined doses as evaluated by trypan blue dye exclusion assay (data not shown).

Isoprenylation Is Required for OPN Expression

Inhibition of HMG-CoA reductase prevents the biosynthesis of isoprenoids, such as geranylgeranylpyrophosphate and farnesylpyrophosphate, and thereby inhibits subsequent isoprenylation. It is thus conceivable that the observed effect of Pitavastatin may result from inhibition of isoprenylation. To prove this assumption, we examined effects of inhibitors for geranylgeranyltransferase I and farnesyltransferase, GGTI-298 and FTI-277, respectively, on high glucose-induced upregulation of OPN expression in cultured rat aortic SMC. As shown in Figure 1B and C, GGTI-298 and FTI277 dose-dependently decreased OPN mRNA level under high glucose concentration, as expected.

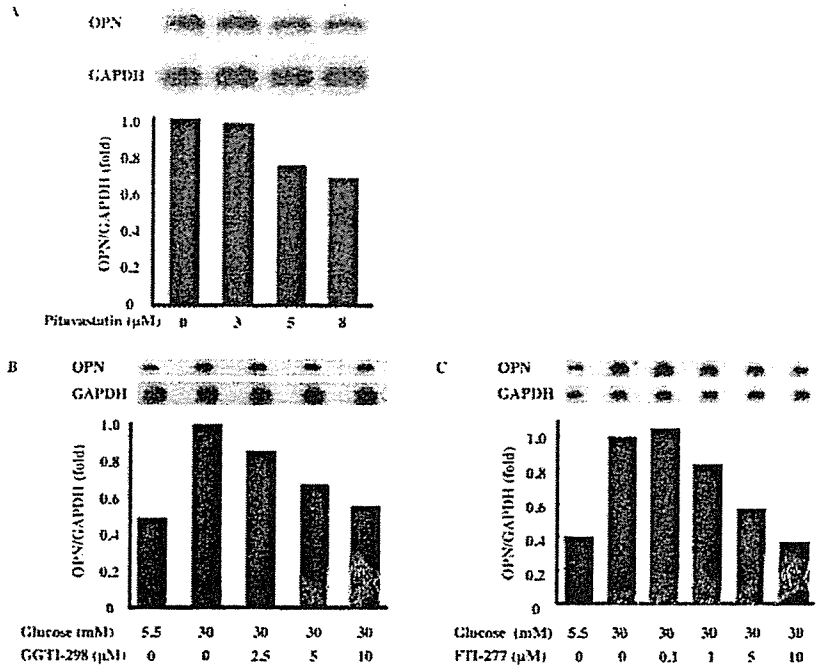

Figure 1. Effects of inhibitors for HMG-CoA reductase, geranylgeranyltransferase, and farnesyltransferase on OPN expression in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of Pitavastatin (A), GGTI-298 (B), or FTI-277 (C) in serum-free medium containing either 5.5 mmol/L or 30 mmol/L glucose for 48 hours. After incubation, cells were processed for Northern blotting with ^{32}P-labeled rat OPN and GAPDH cDNA probes. The level of OPN mRNA expression was estimated by the ratio of OPN signal to GAPDH signal. Data are expressed as fold increase relative to the value obtained in 30 mmol/L glucose without inhibitors. Data shown in this figure are representative of at least 2 independent experiments providing essentially similar results.

Rho/Rho Kinase Pathway Mediates High Glucose-Induced Upregulation of OPN Expression

It is well known that geranylgeranylation is prerequisite for Rho, a small GTP-binding protein, to exert its cellular function. Therefore, Rho seemed to be a possible candidate involved in mediating a positive signal for OPN expression. To evaluate a role of Rho, we first examined effect of an inhibitor of Rho-associated protein kinase, Y-27632, on high glucose-induced upregulation of OPN expression in cultured rat aortic SMC. As shown in Figure 2A, Y-27632 dose-dependently decreased OPN mRNA level under high glucose concentration, suggesting a critical role of Rho kinase activity in OPN expression.

Next, we examined effect of transient transfection of a constitutive active Rho mutant, pSRα-myc-RhoDA, on OPN expression in cultured rat aortic SMC. As shown in Figure 2B, transfection of pSRα-myc-RhoDA enhanced OPN mRNA expression in proportion to the efficiency of its transfection, confirming that Rho mediates a positive signal for OPN expression.

Finally, we examined effect of high glucose on Rho activation in cultured rat aortic SMC. As shown in Figure 2C, the amount of GTP-Rho in cells cultured in 30 mmol/L glucose was found to be much higher than that in 5.5 mmol/L glucose. No difference was found in total Rho protein levels between 5.5 mmol/L glucose and 30 mmol/L

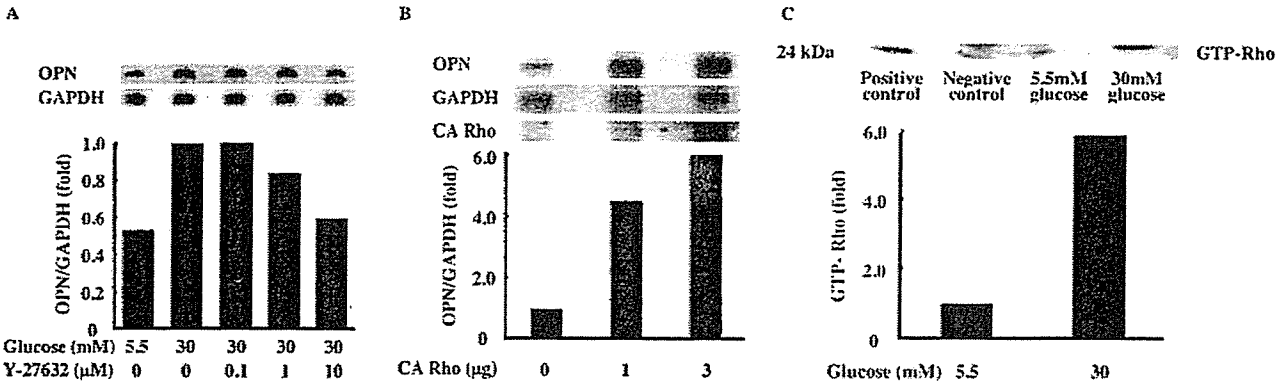

Figure 2. A, Effect of a Rho kinase inhibitor on OPN expression in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of Y-27632 in serum-free medium containing either 5.5 mmol/L or 30 mmol/L glucose for 48 hours. After incubation, cells were processed for Northern blotting as described in the legend to Figure 1. B, effect of transient transfection of a constitutive active Rho mutant (CA Rho) on OPN expression in cultured rat aortic SMC. At 50% confluency, cells were transfected with 1 to 3 μg of pSRα-myc-RhoDA and incubated for 48 hours, as described in Methods. After incubation, cells were processed for Northern blotting. The blots were re-proved with ^{32}P-labeled Rho cDNA probe to estimate the efficiency of transfection. Data are expressed as fold increase relative to the value obtained in the absence of CA Rho. C, High glucose-induced Rho activation in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated in serum-free medium containing either 5.5 mmol/L or 30 mmol/L glucose for 24 hours. After incubation, GTP-Rho in cell lysates was adsorbed to GST-Rhotekin Rho-binding domain and subjected to Western blotting with an anti-Rho antibody. Data are expressed as fold increase relative to the value obtained in 5.5 mmol/L glucose. Data shown in this figure are representative of at least 2 independent experiments providing essentially similar results.

glucose (data not shown). In contrast, treatment of cells with osmotic controls (5.5 mmol/L D-glucose plus 24.5 mmol/L L-glucose or 5.5 mmol/L D-glucose plus 24.5 mmol/L D-mannitol) providing an equivalent osmolarity as 30 mmol/L glucose, did not change Rho activity (data not shown), indicating that the observed enhanced effect on Rho activity is specific to glucose. Taken together, these data strongly support a notion that Rho/Rho kinase pathway mediates high glucose-induced upregulation of OPN expression.

Rho/Rho Kinase Pathway Is a Common Downstream of PKC and Hexosamine Pathways

It was previously noted that high glucose concentrations stimulated OPN expression via a PKC-dependent pathway and the hexosamine pathway in cultured rat aortic SMC.[11] Therefore, our next question was whether Rho/Rho kinase pathway functions downstream of these pathways. As shown in Figure 3A, treatment of cells with GF109203X, an inhibitor of PKC, dose-dependently inhibited high glucose-stimulated increase in Rho activity, suggesting the involvement of PKC activation in the process. Likewise, treatment with azaserine, an inhibitor of GFAT, the key enzyme of the hexosamine pathway, dose-dependently inhibited high glucose-stimulated increase in Rho activity. Total Rho protein levels were unchanged by addition of high glucose, 1 μM GF109203X or 5 μmol/L azaserine (data not shown). Furthermore, as shown in Figure 3B, glucosamine dose-dependently enhanced Rho activity. These data also suggest the involvement of the hexosamine pathway in the process.

ERK Functions Downstream of Rho in Mediating High Glucose-Induced Upregulation of OPN Expression

Small GTP-binding proteins have been demonstrated to induce a variety of responses, including activation of MAP kinase cascades in various cells. Therefore, to trace a signaling pathway that mediates OPN expression downstream of Rho, we first examined effects of inhibitors for MEK1 (PD98059), p38 MAP kinase (SB203580), and JNK (SP600125) on high glucose-induced upregulation of OPN expression in cultured rat aortic SMC. As shown in Figure 4A, PD98059 and SB203580 dose-dependently decreased OPN mRNA level under high glucose concentration, whereas SP600125 had no effect.

Next, we examined whether high glucose induces activation of ERK and p38 MAP kinase in cultured rat aortic SMC. After incubation of cells under normal (5.5 mmol/L) or high (30 mmol/L) glucose concentrations for 24 to 48 hours, activities of ERK1/2 and p38 MAP kinase were determined by immune complex kinase assay. As shown in Figure 4B, exposure to high glucose for 48 hours led to the increase in ERK activity, as assessed by phosphorylation of Elk-1, whereas activity of p38 MAP kinase, as assessed by phosphorylation of ATF-2, did not change under high glucose condition. Treatment with osmotic control (24.5 mmol/L L-glucose + 5.5 mmol/L D-glucose) had no effect on ERK activity (data not shown), indicating that the observed enhanced effect on ERK activity is specific to glucose.

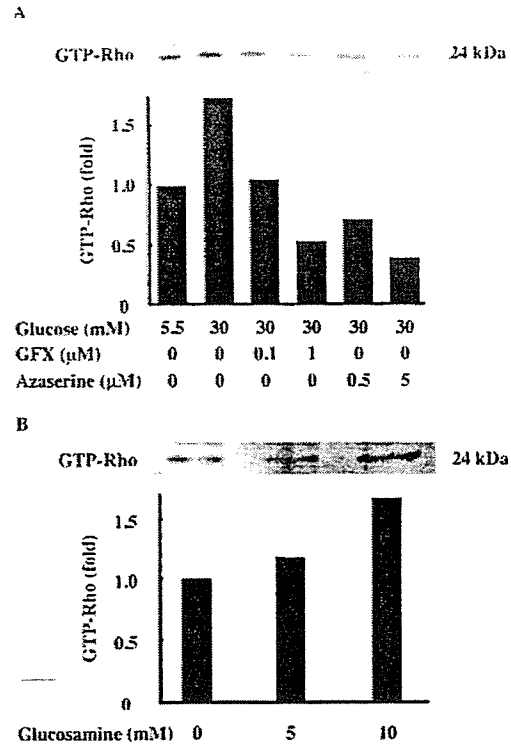

Figure 3. A, Effects of GF109203X (GFX) and azaserine on high glucose-induced Rho activation in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of GFX or azaserine in serum-free medium containing either 5.5 mmol/L or 30 mmol/L glucose for 24 hours. After incubation, cells were processed for Rho activation assay as described in the legend to Figure 2. B, Glucosamine-induced Rho activation in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of glucosamine in serum-free medium containing 5.5 mmol/L glucose for 24 hours. After incubation, cells were processed for Rho activation assay. Data are expressed as fold increase relative to the value obtained in the absence of glucosamine. Data shown in this figure are representative of at least 2 independent experiments providing essentially similar results.

Finally, to confirm that ERK functions downstream of Rho, we examined ERK activity after transient transfection of cultured rat aortic SMC with a constitutive active Rho mutant. As shown in Figure 4C, transfection of pSRα-myc-RhoDA dramatically enhanced ERK activity, whereas transfection of pSRα-myc-RhoDA did not increase either p38 MAP kinase or JNK activities. Based on these data, we concluded that ERK functions downstream of Rho in mediating high glucose-induced upregulation of OPN expression.

Discussion

In the present study, we demonstrate that Rho/Rho kinase pathway functions downstream of PKC and the hexosamine pathways and upstream of ERK in mediating high glucose-induced upregulation of OPN expression. Involvement of Rho in mediating a positive signal for OPN expression has also been reported by Chaulet et al.[14] They showed that extracellular UTP increased OPN expression in cultured rat aortic SMC and thereby induced migration of the cells. Blockade of ERK1/2 or Rho pathways led to the inhibition of

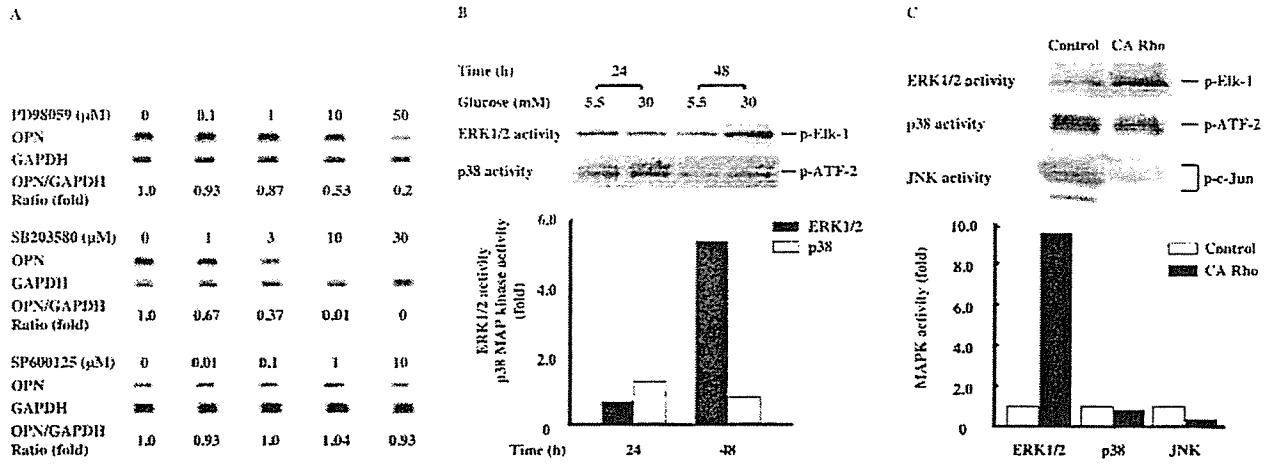

Figure 4. A, Effects of MAP kinase inhibitors on OPN expression in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of PD98059, SB203580, or SP600125 in serum-free medium containing 30 mmol/L glucose for 48 hours. After incubation, cells were processed for Northern blotting as described in the legend to Figure 1. B, High glucose-induced ERK activation in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated in serum-free medium containing either 5.5 mmol/L or 30 mmol/L glucose for 24 to 48 hours. After incubation, activities of ERK1/2 and p38 MAP kinase in cell lysates were measured by immune complex kinase assay with an immobilized phospho p44/42 MAP kinase antibody and Elk-1 protein as substrate, or with an immobilized phospho p38 MAP kinase antibody and ATF-2 protein as substrate, respectively. After phosphorylation reactions, samples were processed for Western blotting with phospho Elk-1 antibody or phospho ATF-2 antibody. Data are expressed as fold increase relative to the value obtained in 5.5 mmol/L glucose at the indicated times. C, Effect of transient transfection of a constitutive active Rho mutant (CA Rho) on activation of MAP kinases in cultured rat aortic SMC. Cells were transfected with 3 μg of pSRα-myc-RhoDA and incubated for 48 hours as described in the legend to Figure 2. After incubation, MAP kinase activities in cell lysates were determined. Data are expressed as fold increase relative to the value obtained in the absence of CA Rho. Double bands in the JNK activity assay correspond to 37- and 35-kilodalton forms of phosphorylated c-Jun fusion proteins. Data shown in this figure are representative of at least 2 independent experiments providing essentially similar results.

UTP-induced OPN increase and migration, demonstrating the central role of OPN in this process. The finding, together with our present observation, underscores the importance of Rho in OPN expression.

Our present finding that high glucose induces Rho activation sheds new light on the mechanism of the accelerated atherogenesis in diabetes mellitus, because involvement of Rho/Rho kinase pathway has been implicated in a wide variety of atherosclerotic processes, including neointimal formation,[15] vasospastic response,[16,17] proliferation,[18,19] migration,[19,20] and anti-apoptosis[20,21] of vascular SMC, and vascular gene expression of monocyte chemoattractant protein-1,[22] transforming growth factor-β1,[22] and inducible nitric oxide synthase.[23] Besides our present study using rat aortic SMC, high glucose-induced Rho activation was also observed in cultured rat mesangial cells[24] and in basilar artery derived from streptozotocin-induced diabetic rats.[25] It is thus conceivable that high glucose promotes diabetic vascular complications not only by upregulation of OPN but also by more diverse effects resulting from Rho activation.

It was reported that transfection of vascular SMC with the c-Ha-rasEJ oncogene induced overexpression of OPN.[26] It is well known that farnesylation is prerequisite for Ras to exert its cellular effect; therefore, our present finding that the inhibitor of farnesyltransferase, FTI-277, suppressed OPN expression might be ascribed to the inhibition of Ras function by the drug. In our previous study, however, the inhibitory effect of Pitavastatin on OPN expression in cultured rat aortic SMC was almost completely reversed by the addition of mevalonate or geranylgeranylpyrophosphate but not by farnesylpyrophosphate.[13] Studies using other types of cells, fibroblasts,[27] or keratinocytes[28] showed that transfection of dominant-negative Rho or dominant-negative Rac suppressed Ras-induced activation of Raf-MEK-ERK pathway, indicating that Ras requires either Rho or Rac function in activation of Raf-MEK-ERK pathway. Based on these findings, it is speculated that the inability of farnesylpyrophosphate to rescue the cells from the inhibition of OPN expression by Pitavastatin might be caused by suppression of Rho family function in Pitavastatin-treated cells. Further study is necessary to prove this possibility.

Acknowledgments

This work was supported by grants-in-aid 12770633, 13216018, 13204010, and 14571086; grants from the Ministry of Health, Labor, and Welfare; grants from Japan Heart Foundation; and grants from Mitsui Sumitomo Welfare Foundation, which were provided to Koutaro Yokote. Seijiro Mori received grants from the Ministry of Health, Labor, and Welfare, Comprehensive Research on Aging and Health, and Research on Specific Diseases, and from Yamanouchi Pharmaceutical Co, Ltd.

References

1. Sodek J, Ganss B, McKee MD. Osteopontin. *Crit Rev Oral Biol Med.* 2000;11:279–303.
2. Xie Y, Sakatsume M, Nishi S, Narita I, Arakawa M, Gejyo F. Expression, roles, receptors, and regulation of osteopontin in the kidney. *Kidney Int.* 2001;60:1645–1657.
3. Cantor H. The role of Eta-1/osteopontin in the pathogenesis of immunological disorders. *Ann N Y Acad Sci.* 1995;760:143–150.
4. Senger DR, Perruzzi CA, Papadopoulos A. Elevated expression of secreted phosphoprotein I (osteopontin, 2ar) as a consequence of neoplastic transformation. *Anticancer Res.* 1989;9:1291–1300.
5. Craig AM, Bowden GT, Chambers AF, Spearman MA, Greenberg AH, Wright JA, McLeod M, Denhardt DT. Secreted phosphoprotein mRNA is induced during multi-stage carcinogenesis in mouse skin and correlates

with the metastatic potential of murine fibroblasts. *Int J Cancer* 1990;46: 133–137.
6. Kohri K, Nomura S, Kitamura Y, Nagata T, Yoshioka K, Iguchi M, Yamate T, Umekawa T, Suzuki Y, Sinohara H, Kurita T. Structure and expression of the mRNA encoding urinary stone protein (osteopontin). *J Biol Chem.* 1993;268:15180–15184.
7. Liaw L, Birk DE, Ballas CB, Whitsitt JS, Davidson JM, Hogan BL. Altered wound healing in mice lacking a functional osteopontin gene (spp1). *J Clin Invest.* 1998;101:1468–1478.
8. Shanahan CM, Cary NR, Metcalfe JC, Weissberg PL. High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. *J Clin Invest.* 1994;93:2393–2402.
9. Liaw L, Lombardi DM, Almeida MM, Schwartz SM, deBlois D, Giachelli CM. Neutralizing antibodies directed against osteopontin inhibit rat carotid neointimal thickening after endothelial denudation. *Arterioscler Thromb Vasc Biol.* 1997;17:188–193.
10. Takemoto M, Yokote K, Nishimura M, Shigematsu T, Hasegawa T, Kon S, Uede T, Matsumoto T, Saito Y, Mori S. Enhanced expression of osteopontin in human diabetic artery and analysis of its functional role in accelerated atherogenesis. *Arterioscler Thromb Vasc Biol.* 2000;20: 624–628.
11. Takemoto M, Yokote K, Yamazaki M, Ridall AL, Butler WT, Matsumoto T, Tamura K, Saito Y, Mori S. Enhanced expression of osteopontin by high glucose in cultured rat aortic smooth muscle cells. *Biochem Biophys Res Commun.* 1999;258:722–726.
12. Morisaki N, Takahashi K, Shiina R, Zenibayashi M, Otabe M, Yoshida S, Saito Y. Platelet-derived growth factor is a potent stimulator of expression of intercellular adhesion molecule-1 in human arterial smooth muscle cells. *Biochem Biophys Res Commun.* 1994;200:612–618.
13. Takemoto M, Kitahara M, Yokote K, Asaumi S, Take A, Saito Y, Mori S. NK-104, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, reduces osteopontin expression by rat aortic smooth muscle cells. *Br J Pharmacol.* 2001;133:83–88.
14. Chaulet H, Desgranges C, Renault MA, Dupuch F, Ezan G, Peiretti F, Loirand G, Pacaud P, Gadeau AP. Extracellular nucleotides induce arterial smooth muscle cell migration via osteopontin. *Circ Res.* 2001;89: 772–778.
15. Sawada N, Itoh H, Ueyama K, Yamashita J, Doi K, Chun TH, Inoue M, Masatsugu K, Saito T, Fukunaga Y, Sakaguchi S, Arai H, Ohno N, Komeda M, Nakao K. Inhibition of Rho-associated kinase results in suppression of neointimal formation of balloon-injured arteries. *Circulation.* 2000;101:2030–2033.
16. Kandabashi T, Shimokawa H, Mukai Y, Matoba T, Kunihiro I, Morikawa K, Ito M, Takahashi S, Kaibuchi K, Takeshita A. Involvement of rho-kinase in agonists-induced contractions of arteriosclerotic human arteries. *Arterioscler Thromb Vasc Biol.* 2002;22:243–248.
17. Shimokawa H, Morishige K, Miyata K, Kandabashi T, Eto Y, Ikegaki I, Asano T, Kaibuchi K, Takeshita A. Long-term inhibition of Rho-kinase induces a regression of arteriosclerotic coronary lesions in a porcine model in vivo. *Cardiovasc Res.* 2001;51:169–177.
18. Laufs U, Marra D, Node K, Liao JK. 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors attenuate vascular smooth muscle proliferation by preventing rho GTPase-induced down-regulation of p27(Kip1). *J Biol Chem.* 1999;274:21926–21931.
19. Seasholtz TM, Majumdar M, Kaplan DD, Brown JH. Rho and Rho kinase mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration. *Circ Res.* 1999;84:1186–1193.
20. Shibata R, Kai H, Seki Y, Kato S, Morimatsu M, Kaibuchi K, Imaizumi T. Role of Rho-Associated Kinase in Neointima Formation After Vascular Injury. *Circulation.* 2001;103:284–289.
21. Guijarro C, Blanco-Colio LM, Ortego M, Alonso C, Ortiz A, Plaza JJ, Diaz C, Hernandez G, Egido J. *Circ Res.* 1998;83:490–500.
22. Kataoka C, Egashira K, Inoue S, Takemoto M, Ni W, Koyanagi M, Kitamoto S, Usui M, Kaibuchi K, Shimokawa H, Takeshita A. Important role of Rho-kinase in the pathogenesis of cardiovascular inflammation and remodeling induced by long-term blockade of nitric oxide synthesis in rats. *Hypertension.* 2002;39:245–250.
23. Chen H, Ikeda U, Shimpo M, Ikeda M, Minota S, Shimada K. Fluvastatin upregulates inducible nitric oxide synthase expression in cytokine-stimulated vascular smooth muscle cells. *Hypertension.* 2000;36: 923–928.
24. Danesh F, Sadechi MM, Amro N, Phillips C, Zeng L, Lin S, Sahai A, Kanwar YS. 3-Hydroxy-3-methylglutaryl CoA reductase inhibitors prevent high glucose-induced proliferation of mesangial cells via modulation of Rho GTPase/p21 signaling pathway: implications for diabetic nephropathy. *Proc Natl Acad Sci U S A.* 2002;99:8301–8305.
25. Miao L, Calvert JW, Tang J, Zhang JH. Upregulation of small GTPase RhoA in the basilar artery from diabetic (mellitus) rats. *Life Sci.* 2002; 71:1175–1185.
26. Parrish AR, Weber TJ, Ramos KS. Osteopontin overexpression in vascular smooth muscle cells transfected with the c-Ha-rasEJ oncogene. *In Vitro Cell Dev Biol Anim.* 1997;33:584–587.
27. Li W, Chong H, Guan KL. Function of the Rho family GTPases in Ras-stimulated Raf activation. *J. Biol Chem.* 2001;276:34728–34737.
28. Mainiero F, Murgia C, Wary KK, Curatola AM, Pepe A, Blumemberg M, Westwick JK, Der CJ, Giancotti FG. The coupling of $\alpha 6\beta 4$ integrin to Ras-MAP kinase pathways mediated by Shc controls keratinocyte proliferation. *EMBO J.* 1997;16:2365–2375.

Available online at www.sciencedirect.com
ScienceDirect
Diabetes Research and Clinical Practice 75 (2007) 27–29
DIABETES RESEARCH AND CLINICAL PRACTICE
www.elsevier.com/locate/diabres

A patient with Werner syndrome and adiponectin gene mutation

Naotake Hashimoto [a,*], Sachiko Hatanaka [d], Koutaro Yokote [c], Hiroko Kurosawa [a], Tomohiko Yoshida [d], Rie Iwai [b], Hidenori Takahashi [b], Katsuya Yoshida [a], Atsuya Horie [a], Kenichi Sakurai [c], Kazuo Yagui [c], Yasushi Saito [c], Shouji Yoshida [a]

[a] *Department of Internal Medicine, Asahi General Hospital, I-1326, Asahi, Chiba 289-2511, Japan*
[b] *Department of Clinical Laboratory, Asahi General Hospital, I-1326, Asahi, Chiba 289-2511, Japan*
[c] *Department of Clinical Cell Biology, Graduate School of Medicine, Chiba University, Japan*
[d] *Department of Internal Medicine, Naruto Hospital, Japan*

Received 6 October 2005; received in revised form 20 April 2006; accepted 9 May 2006
Available online 27 June 2006

Abstract

Werner syndrome is a premature aging disease characterized by genomic instability and increased cancer risk. Here, we report a 45-year-old diabetic man as the first Werner syndrome patient found to have an adiponectin gene mutation. Showing graying and loss of hair, skin atrophy, and juvenile cataract, he was diagnosed with Werner syndrome type 4 by molecular analysis. His serum adiponectin concentration was low. In the globular domain of the adiponectin gene, I164T in exon 3 was detected. When we examined effects of pioglitazone (15 mg/day) on serum adiponectin multimer and monomer concentrations using selective assays, the patient's relative percentage increased in adiponectin concentration was almost same as that in the 18 diabetic patients without an adiponectin mutation, but the absolute adiponectin concentration was half of those seen in diabetic patients treated with the same pioglitazone dose who had no adiponectin mutation. The response suggested that pioglitazone treatment might help to prevent future Werner syndrome-related acceleration of atherosclerosis. Present and further clinical relevant to atherosclerosis in this patient should be imformative concerning the pathogenesis and treatment of atherosclerosis in the presence of hypoadiponectinemia and insulin resistance.

Keywords: Werner syndrome; Adiponectin mutation; Diabetes mellitus; Hypoadiponectinemia; Thiazolidine therapy

1. Introduction

Werner syndrome is an autosomal recessive hereditary disease characterized by premature aging, genomic instability, and accelerated atherosclerosis, and increased cancer risk [1,2]. The defective gene product in Werner syndrome belongs to the ReqQ family of DNA helicases [3]. Here, we report the first patient with Werner syndrome found to have an adiponectin gene mutation as well. We examined changes in adiponectin secretion in response to pioglitazone therapy.

2. Case presentation

A 45-year-old man was diagnosed with diabetes when cataract developed at the age of 25 years. He did not seek further treatment until he was 39 years old, when he was admitted to another hospital. There he was given insulin and was noted to have abdominal fat accumulation. He was referred to our hospital in April 2004.

* Corresponding author. Tel.: +81 479 63 8111; fax: +81 479 60 1210.
E-mail address: naohasi@hospital.asahi.chiba.jp (N. Hashimoto).

0168-8227/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.diabres.2006.05.010

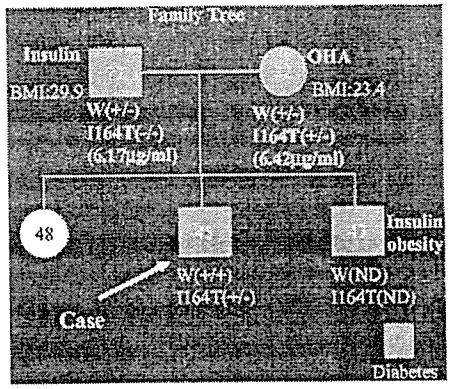

Fig. 1. The patient's father, mother, and uncle had diabetes; the father and uncle were treated with insulin, and the mother with oral hypoglycemic agents. Both parents were heterozygous for Werner syndrome type 4, and heterozygosity for the adiponectin gene mutation I164T was identified in the mother. Values shown are serum adiponectin concentrations (μg/ml).

The patient's father and uncle had diabetes; recently, his mother also had been diagnosed with diabetes. Fig. 1 shows the patient's family tree. His parents both were found to be heterozygous for the Werner mutation, while his mother was heterozygous for an I164T mutation in the adiponectin gene. No consanguinity was reported.

Height was 151.8 cm and weight was 38 kg. Blood pressure was 158/80 mmHg and the pulse was regular with a rate of 92 min^{-1}. The patient injected insulin before each meal (Penfil R 6U) and before sleep (Penfil N 6U). Hemoglobin (Hb) A1c was 6.7%; total serum cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride concentrations were 208, 59, and 190 mg/dl, respectively. Urinary albumin excretion was 26.5 mg/g creatinine. The serum C-peptide concentration was 6.61 ng/ml with a simultaneous plasma glucose concentration of 156 mg/dl, suggesting that insulin secretory capacity was preserved and implying that insulin resistance was likely. The patient showed graying and loss of hair, skin atrophy, and juvenile cataract. We diagnosed him with Werner syndrome type 4 according to molecular analysis [4].

Yokote et al. [5], previously, reported serum adiponectin concentrations to be decreased in Werner syndrome (mean 3.1 μg/ml); our patient's serum adiponectin concentration was particularly low (2.24 μg/ml; to adiponectin monomer assay kit, Otsuka, Tokyo, Japan). When we sequenced the adiponectin gene, heterozygous mutation representing I164T in exon 3 was seen in the globular domain, as was demonstrated in his mother. This mutation has been reported to be atherogenic and to promote insulin resistance, leading to ischemic heart disease [6]. As the adiponectin and Werner genes are located on chromosome 3 and 8, respectively. We concluded that the two mutations were associated coincidentally.

To evaluate vascular atherosclerosis, carotid intima media thickness (IMT) was examined ultrasonographically. While this was only 0.6 mm, calcified plaques 2 mm in thickness were observed in right and left carotid arteries.

We next examined the effects of pioglitazone (15 mg/day) on adiponectin concentrations in the patient using separate adiponectin assay kits to detect the total monomers (Otsuka) and multimeric forms (Fujirebio, Tokyo, Japan). We compared his response to treatment with those in 18 diabetic patients whose adiponectin exon sequences were normal. Responses of serum adiponectin concentrations in the assay for monomers to 15 mg/day of pioglitazone in the other 18 diabetic patients were as follows: 5.68 ± 0.67 μg/ml before pioglitazone, 11.76 ± 1.85 μg/ml (at 1 month), and 11.81 ± 2.20 μg/ml (at 2 months, mean ± S.E.M.). In the Werner patient, the pretreatment adiponectin monomer concentration was 2.32 μg/ml; the1-month value, 6.07 μg/ml; the 2-month value, 5.02 μg/ml (Fig. 2A). Expressed relative to basal concentrations, responses of adiponectin monomer concentrations in

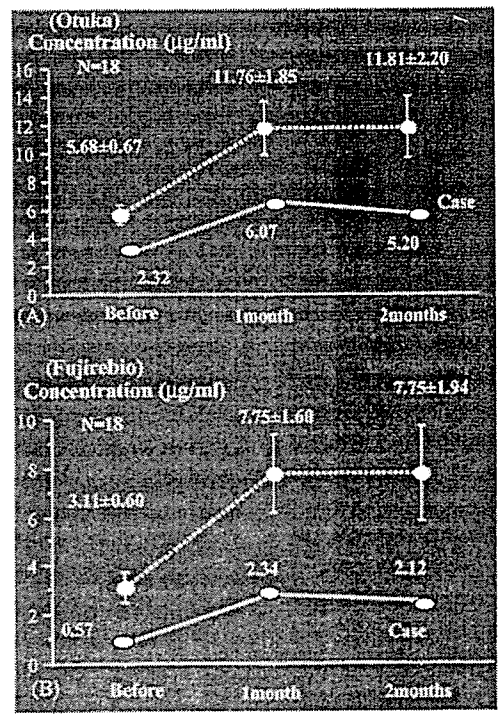

Fig. 2. A: Serum adiponectin concentrations (A, monomer; B, multimer) in response to 15 mg/day of pioglitazone. Data are shown for 18 diabetic patients without an adiponectin gene mutation (means ± S.E.M., broken line) and for the Werner patient (solid line).