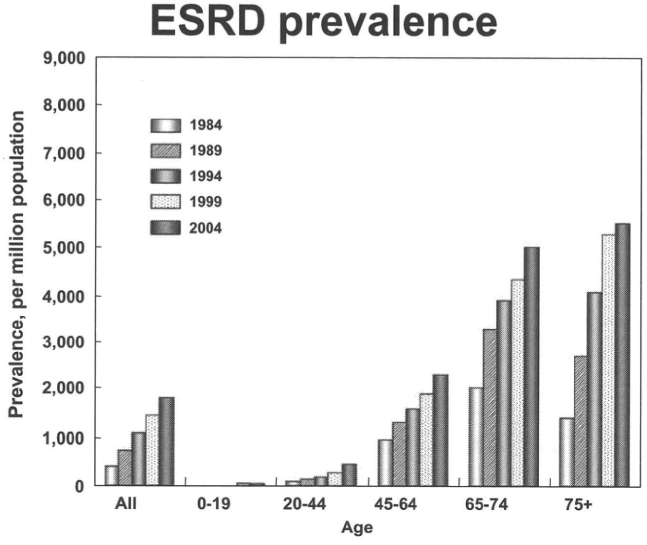

Fig. 1 Prevalence of dialysis patients in Japan. Data are cited from the Japanese Society for Dialysis Society. ESRD, end-stage renal disease.

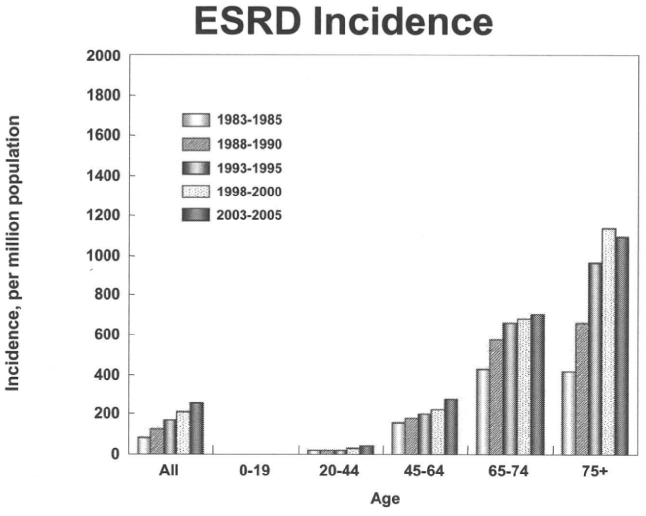

Fig. 2 Incidence of dialysis patients in Japan. Data are cited from the Japanese Society for Dialysis Society. ESRD, end-stage renal disease.

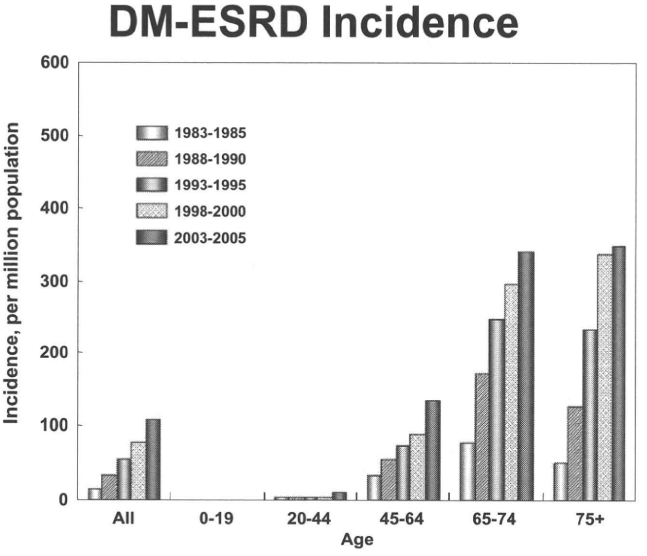

Fig. 3 Incidence of diabetes mellitus (DM) dialysis patients in Japan. Data are cited from the Japanese Society for Dialysis Society. ESRD, end-stage renal disease.

Table 1 Important predictors of end-stage renal disease

1. Proteinuria
2. Hypertension
3. Hyperglycaemia
4. Hyperuricaemia
5. Anaemia
6. Obesity
7. Metabolic syndrome
8. Low glomerular filtration rate

proteinuria and hypertension.[8–13] Effects of obesity on CKD and ESRD were complex and we observed that the decrease in body mass index was a risk factor for developing CKD[14] and ESRD.[15] Low glomerular filtration rate (GFR) per se was not significant, unless otherwise associated with proteinuria.[16] The annual incidence of ESRD was approximately 1% in those with dip-stick 3+ and over and renal biopsy recipients.

The Japanese Society of Nephrology (JSN) has estimated the prevalence of CKD stage 3 to be 10.4%, 7.6% within the range of 50–59 mL/min per 1.73 m^2, in the screened population. The annual GFR decline rate was approximately 0.36 mL/min per 1.73 m^2.[17] Among those who visited twice in 10 years, GFR declined only in the aged group, 60 years and over.[18] Other than high blood pressure and proteinuria, factors related to this age-related GFR decline were not certain. Prevalence of proteinuria, hypertension, DM, anaemia, and metabolic syndrome increased with the decline in estimated GFR (eGFR).

UNIVERSAL OR TARGETED SCREENING FOR CKD?

In April 2008, the Ministry of Health, Labour and Welfare started Tokutei-Kenshin for all residents aged 40–74 years. This strategy is to implement lifestyle modification for those diagnosed with metabolic syndrome. Initially, the urine test was set as optional, not mandatory for this program. This screening program was not originally planned to detect CKD. The cost for measuring microalbuminuria is only covered for DM patients without obvious nephropathy and the test can be repeated every 3 months. The cost is ¥1150 (>$US 10). A

cost–benefit analysis examining the frequency and extent of screening including microalbuminuria is currently under survey in Japan.

ONGOING STUDIES

Both the JSN and JSDT are working together to educate people and collecting evidence for preventing ESRD and related cardiovascular disease (CVD). The JSN has published the GFR estimation equation based on inulin clearance.[19] Using the nationwide registry, Japan Kidney Disease Registry (J-KDR), several cohort studies are underway.

Late referral to nephrologists, which is defined as dialysis started within 1 year after referral is common.[20,21] According to the 2007 annual report of the JSDT, the late referral rate was 69.3%, and that of less than 1 month was 37.7%. Such 'late referral' has a negative impact on survival after starting dialysis. Preliminary result of the JSDT supports the notion that the longer the duration of pre-haemodialysis (HD) treatment, the better the survival. The explanations of such an observation remained speculative. Differences in the control of hypertension, nutritional status and comorbid conditions identified by different nephrologists might play a role.[22] The Japan Incident Dialysis Cohort Study (J-IDCS) has been started to examine the current status of the incidence of Japanese HD patients and how they progress into ESRD.

There are two other ongoing projects in Japan. The Japanese Government (Ministry of Health and Labour) assigned CKD as a national target disease for the strategic medical research in 2007. The Japan Kidney Foundation was asked to launch the investigation: project leader, Professor K Yamagata; Frontier of Renal Outcome Modifications in Japan (FROM-J). The main objective of this research is to observe the CKD progression between two treatment strategies such as intervention A and B, and the target number of total patients is 2500. In both groups, CKD patients are treated by a general physician (Kakarituke doctor) based on the CKD practice guide of the JSN. In intervention B, patients are also followed by a registered dietician and monitored by outside personnel every month. The primary outcomes are: (i) the dropout rate; (ii) the referral rate to registered nephrologists; and (iii) progression rate of CKD to ESRD. The expected difference in the incidence in ESRD is 15% in 5 years between the two groups. This target was set using the following reports. The 2002 DM survey conducted by the Ministry of Heath, Labour and Welfare of Japan stated that only 33.3% of patients had been controlled their HbA1c less than 6.5%; that hypertension is not adequately controlled because less than 50% of subjects with hypertension are taking medications for hypertension in Ibaraki, Japan;[23] and renin angiotensin inhibitors have been used less in the area where the incidence of ESRD is high.[24] Sorensen *et al.* reported that significant decrease (15%) in DM nephropathy was achieved with aggressive management of blood pressure and glucose.[25] In this study, GFR change will also be followed using the JSN original equation.[19] The second is the chronic kidney disease-Japan cohort (CKD-JAC).[26] The natural course of CKD has not been studied in a large cohort of patients. Risk factors of CKD progression with respect to the development of CVD are not known in Japan. The study will enrol 3000 CKD patients, eGFR 10–59 mL/min per 1.73 m^2, in 18 clinical centres around Japan. Each clinical centre will enrol approximately 200 patients over 12 months and monitoring the incidence of ESRD, CVD and all-cause mortality will be determined in 4 years. The study will also examine the relationship between eGFR and quality of life. The enrolment was started in September 2007.

CONCLUSION

Japan is an emerging 'elderly' society. CKD is common in Japan and is expected to increase, particularly in the elderly population. Proteinuria and hypertension are common denominators of CVD, DM, obesity and metabolic syndrome. Further studies are necessary to determine the benefits of proteinuria screening and automatic reporting of eGFR on the incidence of ESRD. More research is needed to determine the natural course of CKD progression, particularly in the elderly population.

CONFLICT OF INTEREST

The Authors state that there is no conflict of interest regarding the material discussed in the manuscript.

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C-Reactive Protein Is a Predictor for Developing Proteinuria in a Screened Cohort

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Key Words

C-reactive protein · Proteinuria · Estimated glomerular filtration rate · Chronic kidney disease

Abstract

Background: The relationship between C-reactive protein (CRP) and proteinuria is not known. ***Methods:*** We examined 20,077 screenees (men: 52.4%) of the Okinawa General Health Maintenance Association (OGHMA) registry who were examined between 2004 and 2006. Cross-sectional and longitudinal relationships between CRP and dipstick proteinuria were examined. The OGHMA central laboratory measured creatinine and CRP levels using an autoanalyzer (normal: <0.30 mg/dl). The glomerular filtration rate was estimated using the Japanese formula. ***Results:*** The prevalence of dipstick-positive proteinuria increased from 5.2% in screenees with a low CRP level of <0.10 mg/dl to 12.3% in those with high CRP levels (0.30–0.90 mg/dl). The CRP values did not affect the mean (SD) estimated glomerular filtration rate: 76.9 (13.7) with low CRP and 76.4 (15.1) with high CRP levels. We examined the relationship between baseline CRP and the development of proteinuria among subjects screened in 2004. Of 8,315 subjects without proteinuria examined again by 2006, 370 (4.4%) had developed proteinuria. The odds ratio (95% CI) for high CRP levels (0.30–0.90 mg/dl; reference CRP: <0.10 mg/dl) was 1.433 (1.013–2.028; p = 0.0422) after adjusting for multivariate variables, suggesting that CRP is closely associated with the prevalence and incidence of proteinuria. ***Conclusion:*** A prospective study on the development of proteinuria among those with high CRP levels is warranted. Screenees with high CRP levels may need to be followed up carefully despite the absence of traditional risk factors for proteinuria.

Introduction

C-reactive protein (CRP) is a nonspecific acute-phase reactant associated with inflammation. Individuals with chronic kidney disease (CKD) are exposed to several potential inflammatory stimuli as they often have traditional risk factors for atherosclerosis. In both the general population and dialysis patients, CRP is independently associated with cardiovascular outcome [1, 2]. Inflammation may be the unifying concept between kidney disease and the enhanced risk for cardiovascular disease in patients with CKD.

The prevalence of end-stage renal disease (ESRD) is high in Japan [3, 4], and may be due to the high incidence of CKD or the more pronounced progression of CKD in Japanese. In asymptomatic subjects of the general population, the prevalence of microalbuminuria and proteinuria is higher in the Japanese than in other populations [5–9]. A cross-sectional study revealed that microalbuminuria is associated with CRP. However, microalbuminuria is not usually measured in a mass screening setting. The inter-

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Table 1. Demographics of the screened subjects (n = 20,077)

Variables	All (n = 20,077)	Low CRP level (n = 12,336)	High CRP level (n = 1,596)
Age at screening, years	49.4 (11.3)	48.7 (11.2)	50.4 (11.8)**
Men, n	10,523 (52.4%)	5,968 (48.4%)	940 (58.9%)**
Body mass index	24.1 (3.5)	23.2 (3.1)	26.0 (4.4)**
Systolic blood pressure, mm Hg	122.1 (16.2)	120.4 (16.0)	125.6 (16.7)**
Diastolic blood pressure, mm Hg	75.2 (10.6)	74.1 (10.4)	77.6 (11.0)**
Blood urea nitrogen, mg/dl	13.6 (3.6)	13.6 (3.6)	13.5 (3.7)
Serum creatinine, mg/dl	0.77 (0.19)	0.76 (0.18)	0.79 (0.18)**
eGFR, ml/min/1.73 m^2	76.5 (13.9)	76.9 (13.7)	76.4 (15.1)**
Total protein, g/dl	7.2 (0.4)	7.1 (0.4)	7.2 (0.4)**
Serum albumin, g/dl	4.3 (0.2)	4.4 (0.2)	4.3 (0.2)**
Total cholesterol, mg/dl	201.9 (33.8)	199.3 (32.7)	204.0 (35.8)*
Triglyceride, mg/dl	129.6 (109.7)	115.3 (86.4)	150.1 (113.5)**
Uric acid, mg/dl	5.7 (1.5)	5.4 (1.4)	6.1 (1.5)**
Fasting blood glucose, mg/dl	101.6 (21.4)	99.2 (17.7)	107.6 (28.6)**
Hemoglobin A1c, %	5.2 (0.7)	5.1 (0.6)	5.5 (1.0)**
Hematocrit, %	42.7 (4.1)	42.3 (4.1)	43.3 (4.0)**
Proteinuria, %	6.8	5.2	12.3**
CRP, mg/dl	0.11 (0.13)	0.05 (0.03)	0.47 (0.16)**

Data are means with SD in parentheses unless otherwise specified. Low CRP level: CRP <0.10 mg/dl. High CRP level: 0.30–0.90 mg/dl. Abnormally high levels of CRP (>0.90 mg/dl) were excluded from the study (n = 413; 2.0% of the total of 20,490). Proteinuria: dipstick 1+ or over. Screening was performed during 2004 to 2006 in Okinawa, Japan. * $p < 0.05$, ** $p < 0.01$.

action between CRP and CKD has not been investigated in a general population study. We have been studying the relationship between dipstick proteinuria and renal outcomes in a large community-based screening cohort. In the present study, we examined the effect of CRP on the development of dipstick-positive proteinuria.

Materials and Methods

Study Design

This study was an observational study in a community-based, longitudinal, limited-access data set designed to examine the relationship between CRP and CKD, and also the effect of CRP on the development of dipstick-positive proteinuria.

Between 2004 and 2006, the study population, a total of 20,610 participants aged 20–89 years, was examined at the Okinawa General Health Maintenance Association (OGHMA) central clinic. The OGHMA, a nonprofit organization founded in 1972 and currently under the direction of Drs. Ikemiya and Kinjo, conducts a large annual community-based health examination [8, 9]. Once each year, the staff, doctors and nurses visit residences and workplaces throughout the prefecture to perform health examinations or to examine those who visited the central clinic. All examinations were performed at the OGHMA laboratory, and participants visited voluntarily. The OGHMA personnel provided mass screening, informed the participants of their results and, when necessary, recommended further evaluation or treatment. This process included an interview concerning health status, a physical examination, and urine and blood tests. A nurse or doctor measured blood pressure using a standard mercury sphygmomanometer with the subject in the sitting position. Dipstick testing for proteinuria, hematuria and glucosuria (Ames Dipstick, Tokyo, Japan) was performed in spontaneously voided fresh urine. Proteinuria was defined as a dipstick urinalysis score of 1+ or more. The body mass index was calculated as weight (kilograms) divided by the square of the height (meters). Hypertension was defined as systolic blood pressure of ≥140 mm Hg and/or diastolic blood pressure of ≥90 mm Hg. Diabetes mellitus was defined as fasting blood glucose of ≥126 mg/dl or hemoglobin A1c of ≥6.0%.

Participants studied again during 2005 to 2006 were identified by their ID number. The annual numbers of participants were 11,887 in 2004, 12,815 in 2005 and 13,627 in 2006. The ethics committee of the OGHMA approved the study protocol. Only coded data were used for the study.

Laboratory Tests

Serum creatinine levels were measured using the automated enzymatic method. The glomerular filtration rate (GFR) was estimated using the equation developed by the Japanese Society of Nephrology: estimated GFR (eGFR) = 194 × serum creatinine$^{-1.094}$ × age$^{-0.287}$ (× 0.739, if female). We defined CKD as an eGFR of less than 60 ml/min/1.73 m^2 [10]. High-sensitivity serum

Table 2. Mean (SD) levels of CRP based on eGFR and/or proteinuria (n = 20,077)

	CRP, mg/dl	
	proteinuria (–)	proteinuria (+)[1]
eGFR		
<60 ml/min/1.73 m^2	0.12 (0.13)	0.18 (0.18)
	n = 1,714	n = 252
60–89 ml/min/1.73 m^2	0.11 (0.13)	0.15 (0.16)
	n = 14,137	n = 908
≥90 ml/min/1.73 m^2	0.11 (0.14)	0.17 (0.17)
	n = 2,856	n = 210
Dipstick for proteinuria		
(–) (n = 16,428)	0.11 (0.12)	
(+/–) (n = 2,279)	0.13 (0.14)	
1+ (n = 1,016)	0.15 (0.16)	
2+ (n = 304)	0.16 (0.16)	
3+ and over (n = 50)	0.18 (0.18)	

Data are means with SD in parentheses. Abnormally high levels of CRP (>0.90 mg/dl) were excluded from the study (n = 413; 2.0% of the total of 20,490). Screening was performed during 2004 to 2006 in Okinawa, Japan.

[1] Dipstick 1+ or over.

Table 3. Demographics of screened subjects examined in 2004 (baseline) and followed up by 2006 (reexamined) versus those not reexamined in Okinawa, Japan (n = 11,626)

	Reexamined (n = 8,926)	Not reexamined (n = 2,700)
Age at screening, years	49.6 (10.1)	49.3 (12.7)
Men, n	5,216 (58.4%)	1,353 (50.1%)**
Body mass index	24.2 (3.4)	24.0 (3.5)**
Systolic blood pressure, mm Hg	122.4 (15.8)	122.5 (16.4)
Diastolic blood pressure, mm Hg	75.0 (10.5)	74.3 (10.7)**
Blood urea nitrogen, mg/dl	13.9 (3.6)	14.1 (3.8)*
Serum creatinine, mg/dl	0.79 (0.18)	0.77 (0.24)**
eGFR, ml/min/1.73 m^2	75.6 (13.5)	76.9 (14.5)**
Total protein, g/dl	7.1 (0.4)	7.2 (0.4)**
Serum albumin, g/dl	4.3 (0.2)	4.3 (0.2)
Total cholesterol, mg/dl	201.7 (32.9)	201.2 (34.9)
Triglyceride, mg/dl	132.4 (114.5)	128.6 (102.9)
Uric acid, mg/dl	5.8 (1.5)	5.6 (1.5)**
Fasting blood glucose, mg/dl	102.1 (20.9)	102.5 (23.3)
Hemoglobin A1c, %	5.2 (0.7)	5.2 (0.8)
Hematocrit, %	42.9 (4.0)	42.5 (4.1)**
Proteinuria, %	6.9	7.5
CRP, mg/dl	0.12 (0.13)	0.12 (0.13)

Data are means with SD in parentheses unless otherwise specified. Abnormally high levels of CRP (>0.90 mg/dl) at baseline were excluded from the study. Proteinuria: dipstick 1+ or over.

* $p < 0.05$, ** $p < 0.01$.

CRP levels were measured using the latex-enhanced immunonephelometric method (Daiichi-Chemical, Tokyo, Japan). Both within-variance and the coefficient of variance of the method were less than 5% of the normal range of less than 0.30 mg/dl.

Statistics

Data were analyzed by SAS/STAT software (version 6.03; SAS Institute, Tokyo, Japan). Student's t test and the χ^2 test were performed to compare the significance of discrete variables. The odds ratio (95% CI) was calculated by a logistic regression procedure using multivariate models. $p < 0.05$ was considered significant.

Results

A total of 20,610 subjects visited the OGHMA and were examined during 2004 to 2006. CRP and dipstick proteinuria data were available for the majority of the subjects (20,490; 99.4%). Abnormally high levels of CRP (>0.90 mg/dl) were excluded from the study (n = 413; 2.0% of the total), and therefore we studied a total of 20,077 subjects (97.4%). The demographics of the subjects are summarized in table 1. The prevalence of dipstick-positive proteinuria increased from 5.2% in screenees with a CRP level of <0.10 mg/dl to 12.3% in those with CRP levels of 0.30–0.90 mg/dl. The CRP values did not affect the mean (SD) eGFR: 76.9 (13.7) for CRP levels of <0.10 mg/dl and 76.4 (15.1) for CRP levels of 0.30–0.90 mg/dl. Several variables were significantly different between subjects with low CRP (<0.10 mg/dl) and high CRP levels (0.30–0.90 mg/dl), such as body mass index, proteinuria, serum albumin, uric acid, blood pressure, total cholesterol, triglyceride and diabetes mellitus at baseline.

The relationship between baseline CRP, eGFR and dipstick proteinuria is shown in table 2. CRP levels were associated with degree of proteinuria, but not with GFR.

In 2004, a total of 11,626 screenees participated, 8,926 (76.8%) of whom had data on urine dipstick tests for proteinuria and CRP and were examined again by 2006. The baseline demographics were slightly different between those who were examined again and those who were not reexamined by 2006 (table 3). Nevertheless, the baseline levels of CRP were similar.

The incidence of proteinuria was related to the baseline serum CRP levels (fig. 1, 2). Among screenees without proteinuria (n = 8,315), the incidence was 4.4% of the

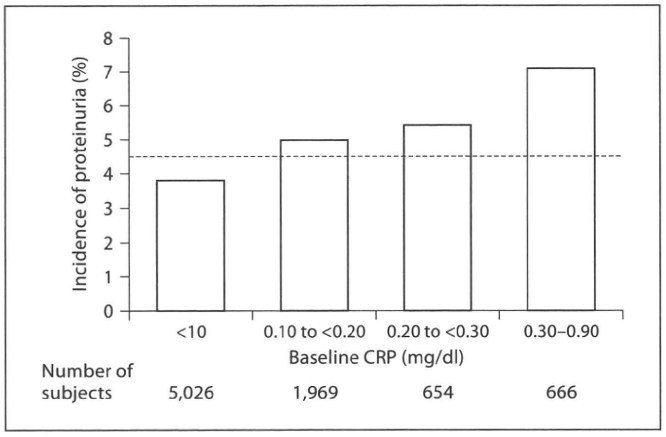

Fig. 1. Incidence of proteinuria by baseline CRP. Baseline screening was performed in 2004, and the follow-up study was performed by 2006. All screenees were negative for dipstick proteinuria in 2004 (n = 8,315).

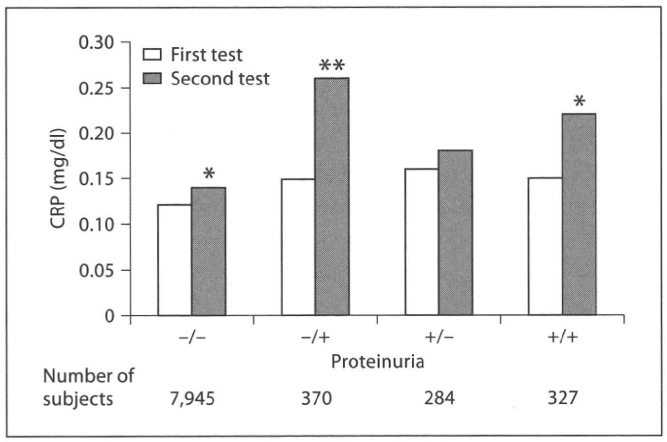

Fig. 2. Relationship between CRP and proteinuria. Mean levels of CRP by combining the levels of proteinuria, either absence or presence, at the first and second test. Baseline screening was performed in 2004, and the follow-up study was performed by 2006 (n = 8,926). * $p < 0.05$, ** $p < 0.01$, paired t test.

total: 3.8% in subjects with a CRP level of <0.10 mg/dl, 5.0% in those with CRP levels of 0.10 to <0.20 mg/dl, 5.4% in those with CRP levels of 0.20 to <0.30 mg/dl, and 7.1% in those with CRP levels of 0.30–0.90 mg/dl. The GFR-adjusted odds ratio (95% CI) of CRP was 1.433 (1.013–2.028; p = 0.0422) for developing proteinuria (table 4). Mean CRP levels are shown by the combination of the presence or absence of proteinuria at baseline and follow-up (fig. 2). Those who developed proteinuria had significantly increased levels of CRP at the follow-up visit (paired t test: p = 0.0008).

Table 4. Risk of developing proteinuria by baseline levels of CRP

CRP levels	Odds ratio	95% CI	p
Model 1			
<0.10 mg/dl	1.000		
<0.20 mg/dl	1.333	1.039–1.711	0.0239
<0.30 mg/dl	1.439	0.994–2.084	NS
0.30–0.90 mg/dl	1.933	1.389–2.688	<0.0001
Model 2			
<0.10 mg/dl	1.000		
<0.20 mg/dl	1.299	1.011–1.668	0.0408
<0.30 mg/dl	1.391	0.960–2.016	NS
0.30–0.90 mg/dl	1.888	1.356–2.628	0.0002
Model 3			
<0.10 mg/dl	1.000		
<0.20 mg/dl	1.081	0.835–1.401	NS
<0.30 mg/dl	1.112	0.760–1.627	NS
0.30–0.90 mg/dl	1.433	1.013–2.028	0.0422

Baseline CRP level was categorized into 4 groups: <0.10, <0.20, <0.30, and 0.30–0.90 mg/dl. Multivariate analysis was performed using the variables presented in the table. Subjects were those who were screened in 2004 and visited again by 2006 in Okinawa, Japan (n = 8,315). Variables used for analysis: none for model 1; age and sex for model 2; age, sex, body mass index, systolic blood pressure, diastolic blood pressure, serum creatinine, hematocrit, total cholesterol, triglyceride, serum albumin, uric acid and fasting blood glucose for model 3. NS = Not significant.

Discussion

Dipstick proteinuria is the strongest predictor of ESRD among the commonly measured variables in a mass screening setting [11, 12]. We further investigated the role of CRP in the prevalence and incidence of proteinuria. CRP is not usually measured for screening purposes unless otherwise indicated. It is now included among the standard laboratory tests among our screening tools, however, because CRP is a known marker of atherosclerosis and a predictor of cardiovascular disease and microalbuminuria. CRP is a useful laboratory test for predicting the risk of developing proteinuria independently of the baseline GFR level. Therefore, CRP might be another marker for those at high risk of developing CKD and eventually ESRD, in addition to other traditional risk factors for proteinuria.

The higher the levels of proteinuria, the more rapidly CKD progresses. Renin-angiotensin system (RAS) inhibitors are beneficial in the treatment of proteinuric renal disease [13]. Except for the hypotensive action of RAS inhibitors, their effects are not well described. RAS inhibitors also effectively suppress chronic inflammation and therefore lower CRP [14]. The findings of the present study support the notion that chronic inflammation has a causative role in the development of proteinuria and cardiovascular disease.

Several studies have evaluated inflammatory markers in CKD and cardiovascular disease risk. CKD has both traditional and nontraditional risk factors [15, 16]. The relationship between GFR and proteinuria, however, may not be simple. CRP is often associated with an increased risk for cardiovascular disease even without CKD. The effects of proteinuria may be independent of the GFR level. Although Weiner et al. [17] recently reported that both CKD and inflammation are associated with an increased risk for cardiovascular disease and mortality, their study did not consider proteinuria. Proteinuria per se is a cause of inflammation. Higher proteinuria levels are associated with increased tubulointerstitial damage [18]. Lowering proteinuria levels effectively prevents the progression of GFR decline independently of lowering blood pressure [13].

The prevalence of stage 3 CKD is relatively high and is expected to increase with the growing elderly population, particularly in Japan [19, 20]. The incidence and prevalence of ESRD are also quite high in Japan and are generally expected to increase in Asian countries [7]. Therefore, it is important to define those at risk of developing ESRD among subjects with stage 3 CKD. Metabolic syndrome is a predictor of CKD and proteinuria [21, 22]. Obesity causes glomerulopathy [23], as confirmed by meta-analysis [24]. The results of the present study demonstrate a significant association between CRP and body mass index. Several mechanisms may underlie this entity; visceral adipocytes are often associated with hypercytokinemia, leading to inflammation [25].

The strength of this paper is that the registry used in this study is a well-described, community-based screening registry in Okinawa, Japan [11, 12], and that the number of subjects is sufficiently large. Renal outcomes were investigated using several variables including dipstick proteinuria. The subjects participated voluntarily and most were ambulatory. Both cross-sectional and follow-up studies supported the role of CRP in the development of proteinuria. The present study confirms that atherosclerotic processes are related to chronic inflammation.

This study has several limitations. First, it is a retrospective observational study, and pertinent information such as lifestyle-related factors, comorbid conditions and treatments was not available. Compared to the effects of body mass index and blood pressure, the effects of smoking and alcohol intake were small on the development of proteinuria [26]. Low intake of alcohol was rather inhibitory of proteinuria development. Subjects with proteinuria and high levels of CRP might have been treated with RAS inhibitors, which influence CRP levels and proteinuria. Since the participants were largely normotensive, the effect of RAS inhibitors would have been very small. Second, the causes of high CRP levels, 0.30–0.90 mg/dl and higher, are not clear. In the majority of subjects who were examined twice or more, CRP levels remained in the range of 0.15 mg/dl and proteinuria was not detected (fig. 2). Subjects with symptomatic urinary tract infection, other signs of infection, or a history of cardiovascular disease or malignancies were not completely excluded from the screening. We have excluded those with abnormally high levels of CRP (>0.9 mg/dl), therefore, such a possibility would be very small, if existent. Third, dipstick proteinuria is a semiquantitative method and is not so accurate. But a positive result indicates that the proteinuria (or albuminuria) level is higher than that of the range of microalbuminuria. Microalbuminuria is relatively common in screened Japanese subjects [5, 6]. A significant relationship between CRP and microalbuminuria has been reported in apparently healthy individuals in a cross-sectional cohort [6]. Quantification of microalbuminuria is more sensitive than that of albuminuria, but it would require a longer follow-up to track the development of ESRD. The predictive power of proteinuria for the development of ESRD is significant.

In conclusion, CRP is a useful predictor of dipstick-positive proteinuria in a screening setting. In the present study, the risk of developing proteinuria was associated with increased CRP levels, even those with an increase within the normal range. Subjects with high CRP levels (0.30–0.90 mg/dl) may require further follow-up, despite the absence of traditional risk factors for CKD. The benefits of a comeasurement of CRP remains to be shown among the screened subjects with dipstick-negative proteinuria.

Acknowledgments

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Methylglyoxal augments intracellular oxidative stress in human aortic endothelial cells

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Abstract

Methylglyoxal (MGO) is a non-enzymatic metabolite in the glycolytic pathway and its concentration in blood and tissues is elevated in diabetes and renal failure. MGO induces tissue injuries via ROS; however, the mechanism remains to be clarified. The present study examined the harmful actions of MGO. Human aortic endothelial cells were assessed under real-time fluorescent microscopy with continuous superfusion. Increases in intracellular ROS were measured with fluorescent indicator, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (DCFH-DA). The addition of MGO rapidly increased the ROS in a dose-dependent manner. The increment of DCF was entirely abolished by pre-treatment with superoxide anion scavenger and membrane-permeable catalase, indicating that MGO induces superoxide production. The increment was completely inhibited by 2-thenoyltrifluoroacetone or carbonyl cyanide 3-chlorophenylhydrazone and partially inhibited by N-methyl-L-arginine. These data suggest that MGO stimulates superoxide production from mitochondria and partially stimulates nitric oxide synthase in human endothelial cells.

Keywords: *Methylglyoxal, oxidative stress, endothelial cell, carbonyl stress*

Introduction

Endothelial dysfunction is the initial step in the development of atherosclerosis. Diabetes mellitus and end-stage renal disease (ESRD) cause atherosclerosis and arteriosclerosis through endothelial damage [1].

Methylglyoxal (MGO) is a highly reactive dicarbonyl compound produced mainly by the degradation of glucose, via fructose in the glycolytic pathway. MGO reacts with the peptide forming MGO-derived lysine dimer, one of the advanced glycation end-products (AGEs) [2]. MGO is also known as a protein-bound uremic toxin [3] and the free form of MGO also exists stably in plasma. The plasma level of MGO is elevated in diabetic patients [4], patients with end-stage renal disease [5] and the corresponding animal models [6–8]. Several studies have suggested that MGO induces peritoneal tissue injury via oxidative stress [9]. MGO stimulates the transcription of angiopoietin-2 expression, which causes endothelial cell death and acellular capillary formation in the retina [10] and kidney [11]. In addition, a substantial portion of glucose-induced tissue injuries are caused by MGO [12]. Based on such findings, increased attention has been focused on the role of MGO in various pathological conditions such as diabetes mellitus, renal failure and hypertension [12–14]. However, the biological actions of MGO have not been well studied.

Reactive oxygen species (ROS), such as superoxide anion ($O^{\cdot}_2^-$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) are crucial risk factors in the progression of endothelial damage [15–17]. We have recently reported that MGO itself is not an ROS, as

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determined by a chemiluminescence method; however, we detected free radicals by an electron spin resonance spectrum method when MGO was present with H_2O_2 [18]. MGO induces ROS production in neutrophils [19], platelets [20] and vascular smooth muscle cells [21]. However, the pathway of ROS production induced by MGO has not been examined enough in aortic endothelial cells, especially in the acute period after stimulation.

In the present study, we determined if MGO at concentrations detected in physiological or pathological conditions induced ROS in human aortic endothelial cells (HAECs) *in vitro*. We also examined the pathways of MGO-induced ROS production.

Materials and methods

Cell culture and reagents

Human aortic endothelial cells (HAECs) were obtained from Cambrex (Charles City, IA) and cultured according to the supplier's instructions. Methylglyoxal, elastase, polyethylene-glycol binding catalase (PEG-Cat), 2-thenoyltrifluoroacetone (TTFA), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), apocynin and N-methyl-L-arginine (L-NAME) were purchased from Sigma-Aldrich (St. Louis, MO) and 5- (and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCFH-DA) was purchased from Invitrogen (Carlsbad, CA). Tiron and H_2O_2 were purchased from Dojindo (Kumamoto, Japan).

Assessment of direct reaction between DCFH-DA and MGO

To confirm that DCFH-DA does not chemically react with MGO in a cell-free condition, 1, 10 and 100 μmol/L MGO or various concentrations of H_2O_2 were added to DCFH-DA in Falcon 96-well plates. Elastase was added to the well for conversion of DCFH-DA to DCFH 3 min before the addition of H_2O_2 or MGO. DCFH reacted with H_2O_2 or $ONOO^-$, but not $O^{\cdot}{}_2^-$, and was converted to the fluorescent form of the probe oxidation product (2',7'-dichlorofluorescein, DCF). The fluorescent signals of DCF were captured with excitation at 480 nm and emission at 535 nm by using a Fluoroscan Ascent plate reader (Thermo Labsystems, Waltham, MA). Data were analysed with Ascent software (Thermo Labsystems, Waltham, MA).

Measurement of DCF under real-time fluorescent microscopy with a continuous superfusion system

HAECs at the 5–7th passage were cultured on 15 mm cover slips and grown to 80–90% confluence under 95% O_2 and 5% CO_2. The cover slip was then placed on the stage of a real-time fluorescent microscope IX71 (Olympus, Tokyo, Japan) and the chamber was set for continuous superfusion.

The temperature of the chamber was maintained at 37°C by warming the superfusate with a TC-344 and TA-29 thermo-warmer (Warner Instruments, Hamden, CT). The superfusion rate of the buffer was maintained at 1 ml/min with a PHD2000 syringe pump (Harvard Apparatus, Holliston, MA) and the chamber volume was kept at 5 mL by continuous aspiration. Signals of DCF were detected through an Ixon cool CCD video camera (Andor Co., Tokyo, Japan) equipped with an emission filter of 535 nm after excitation at 480 nm by a DG-4 and 175-W Xenon-Arc lamp (Sutter Instruments, Navato, CA). The signals were captured every 10 s and quantified with MetaFluor imaging software (Universal Imaging Co., Ypsilanti, MI).

Protocol

Cells were loaded with a membrane-permeable, non-fluorescent probe, DCFH-DA (5 μmol/L) for 15 min at 37°C in Hanks' Balanced Salt Solution (HBSS) under the light-shielding condition [22]. After washing the dye with superfusion buffer for 5 min, the vehicle buffer was exchanged with buffer that contained 0, 1, 10 or 100 μmol/L MGO and cells were observed for 20 min. Then the fluorescence of DCF was recorded. For the inhibition study, cells were pre-treated with inhibitors such as Tiron, TTFA, CCCP, apocynin and L-NAME for 6 h prior to the study. In the case of PEG-catalase, the cells were treated for 15 min before loading DCFH-DA. To compare ROS productivity after each treatment, mean rates of oxidation (units/s) were calculated for 800 s immediately after starting MGO superfusion during every experiment. These values are rough but illustrative estimates of the rate of oxidation of the probe which is not constant in time (especially for higher rates).

Statistical analysis

All data from at least five independent experiments were expressed as means ± SEM. The unpaired Student's *t*-test was used to analyse the data of mean oxidation rates in each independent experimental group.

Results

MGO did not chemically produce DCF in the cell-free assay

DCFH-DA is a fluorescent indicator used extensively to detect intracellular H_2O_2, but it may have potential artifacts. We assessed the direct interaction between MGO and DCFH-DA by using microplate readers to confirm that DCFH-DA is useful as a fluorescent

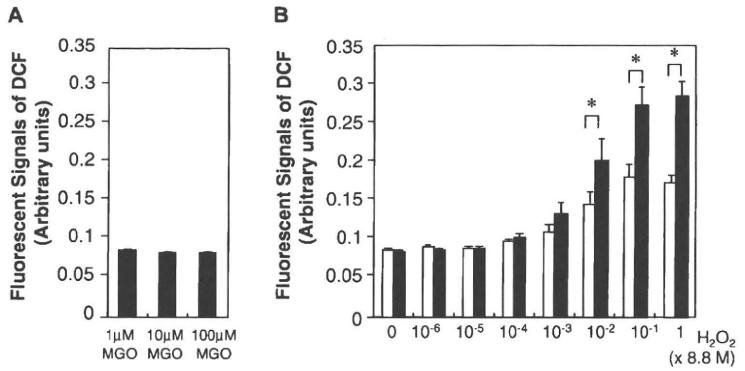

Figure 1. DCFH-DA did not react directly with MGO. (A) MGO did not form DCF from DCF. (B) DCFH-DA formed DCF from DCFH in the presence of H_2O_2 at concentrations higher than 8.8 mmol/L. Elastase was added to form DCFH from DCFH-DA. Open bars, absence of elastase; closed bars, presence of elastase. $^*p < 0.05$ absence of elastase vs the presence of elastase; $\#p < 0.05$ vs H_2O_2 $8.8*10^{-4}$ mol/L.

marker to measure intracellular H_2O_2 and $ONOO^-$. The amount of DCF in the presence of elastase was increased in a H_2O_2-concentration dependent manner and significantly greater than the amount in the absence of elastase at concentrations of H_2O_2 greater than 8.8 mM (Figure 1B). However, MGO did not directly convert DCFH to DCF (Figure 1A). These results suggested that MGO is not a ROS molecule that chemically reacts with DCFH, so DCFH-DA is available in the next protocol with HAECs.

MGO induced DCF formation dose-dependently

MGO increased the amount of intracellular DCF in HAECs. In order to evaluate the rate of increase, the intensities of the DCF were plotted against the elapsed time (Figure 2) and the mean rates of oxidation were calculated (Figure 3).

MGO increased the DCF signals in a dose- and time-dependent manner. Superfusion of MGO at the concentration of 10 and 100 μmol/L significantly increased the production of ROS. The rate for 0 (control), 1, 10 and 100 μmol/L MGO was 0.51 ± 0.18, 0.42 ± 0.09, 1.90 ± 0.12* and 2.64 ± 0.23 units/s*, respectively ($^*p = 0.0029$, 10 μmol/L MGO vs control, $p = 0.0014$, 100 μmol/L MGO vs control).

Inhibition study of MGO-induced ROS formation

To determine the ROS production by MGO in HAECs (Figure 3), the cells were pre-treated with a membrane-permeable type of catalase (50 U/mL of PEG-Cat) or $O^{\bullet}_2{}^-$ scavenger [23] (1 mmol/L of Tiron). Pre-treatment with either PEG-Cat (0.26 ± 0.25 units/s, $p = 0.00\,071$ vs 100 μmol/L MGO) or Tiron (0.29 ± 0.07 units/s, $p = 0.00\,005$ vs 100 μmol/L MGO) completely abolished the MGO-induced DCF to the vehicle level. These data suggest that MGO promotes the production of $O^{\bullet}_2{}^-$ and that the $O^{\bullet}_2{}^-$ is converted to H_2O_2, which increases the intracellular levels of DCF.

Next, we investigated which pathway was involved in the MGO-induced $O^{\bullet}_2{}^-$ production. Cells were pre-treated with an uncoupler of oxidative phosphorylation

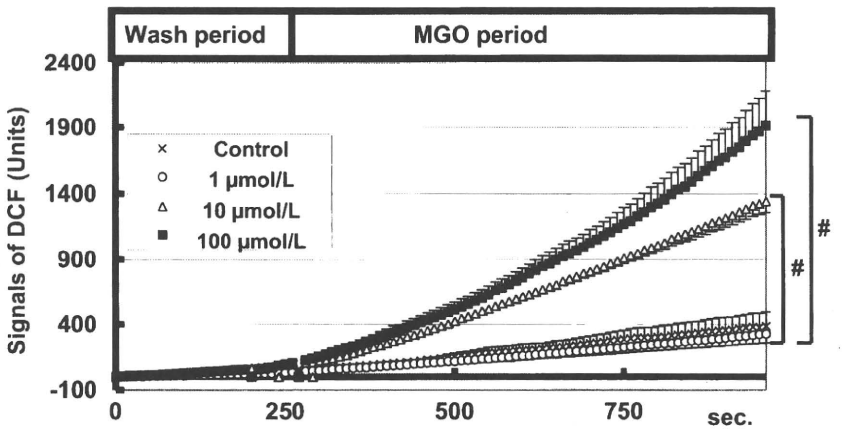

Figure 2. Intracellular ROS production by MGO. Increases in the amounts of DCF indicate the production of MGO-induced ROS. Acute infusion of MGO induced the production of intracellular ROS in a dose- and time-dependent manner in HAECs. The wash period indicates the vehicle in the each experiment. $\#p < 0.05$, control vs 10 μmol/L or 100 μmol/L.

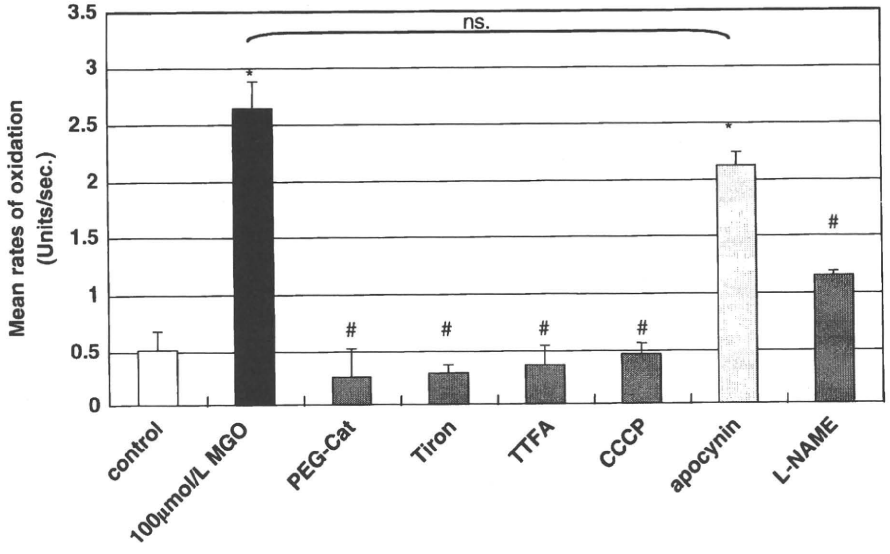

Figure 3. Effects of inhibitors on the MGO-induced ROS production. MGO (100 μmol/L) significantly increased the intracellular ROS production (*$p < 0.05$). Pre-treatment with PEG-Cat, Tiron, TTFA or CCCP completely inhibited the 100 μmol/L MGO-induced ROS (#$p < 0.05$). Pre-treatment with apocynin did not significantly inhibit the ROS formation vs 100 μmol/L MGO (ns), but significantly increased the formation of ROS vs control (*$p < 0.05$). Pre-treatment with L-NAME partially and significantly inhibited the formation of ROS (#$p < 0.05$).

that abolishes the mitochondrial membrane proton gradient (5 μmol/L of CCCP), an inhibitor of the mitochondrial electron transport chain complex II (10 μmol/L of TTFA), nitric oxide synthase (NOS) inhibitor (100 μmol/L of L-NAME) or an inhibitor of NADPH oxidase (NOX) (1 mmol/L of apocynin) for 6 h. Increases in the level of DCF were completely inhibited to the level of vehicle by pre-treatment with CCCP (0.46 ± 0.09 units/s, $p = 0.0001$ vs 100 μmol/L MGO) or TTFA (0.36 ± 0.18 units/s, $p = 0.0002$ vs 100 μmol/L MGO). Pre-treatment with L-NAME (1.14 ± 0.04 units/s, $p = 0.0012$ vs 100 μmol/L MGO) significantly suppressed the response to 54% of the response induced by 100 μmol/L MGO. These results suggest that pathological concentrations of MGO increase the intracellular production of $O^{\bullet}{}_2^-$ in HAECs and that the production is mainly mediated by the mitochondrial electron-transport pathway and partially mediated by the NOS pathway. However, pre-treatment with apocynin also partially suppressed the production of DCF, although not significantly (2.12 ± 0.11 units/s, $p = 0.09$ vs 100 μmol/L MGO, $p = 0.0016$ vs control).

Discussion

MGO is one of the uremic toxins found in patients with diabetic nephropathy and ESRD, but its contribution to tissue toxicity has remained controversial. In the present study, we demonstrate that MGO increased the production of ROS in HAECs by using real-time fluorescent microscopy. For the first time, we demonstrated that the increase occurred immediately after exchanging the vehicle buffer with MGO in the chamber. DCFH is useful as an indicator of $ONOO^-$ as well as H_2O_2 [24]. Superoxide anion is converted into H_2O_2 or $ONOO^-$, so DCF is able to indirectly measure the total amounts of superoxide. Tiron inhibited DCF after MGO stimulation; thus, probably MGO primarily stimulates the production of $O^{\bullet}{}_2^-$. Tiron has been reported to be a Ca^+ chelator in addition to a scavenger of $O^{\bullet}{}_2^-$ like a SOD mimetic [25]. Therefore, it is possible that tiron diminishes ROS production via another unknown pathway.

Certain levels of MGO exist in normal tissues throughout the whole body and the plasma level of MGO is increased in hyperglycaemic conditions (1–4 μmol/L) [26], in end-stage renal disease (9.7–11.5 μmol/L) [5] or by ageing [27,28]. In the present study we used 1, 10 or 100 μmol/L of MGO, which are the concentrations found in the plasma of healthy persons, diabetic patients and ESRD patients, respectively. MGO is a key component in pathological conditions such as DM and ESRD. We measured the plasma concentration of MGO in healthy individuals and found that it was less than 1 μmol/L. Even after healthy individuals consumed beverages, MGO concentrations were not greater than 1 μmol/L (unpublished data). Thus, the daily intake of food does not appear to contribute to endothelial toxicity in healthy individuals, because our results show that a low level of MGO does not increase the production of intracellular ROS. A previous study showed that cigarette smoke contains 0.19–0.83 μmol of MGO per cigarette [29]. Chronic oral administration of MGO at 0.69 mmol per kg of body weight led to

kidney collagen accumulation in an animal study [30]. Thus, the toxicity of MGO may be involved not only in some disease conditions, but also in activates such as smoking and the large intake of foods containing high levels of MGO.

The present study demonstrated that pathological concentrations of MGO rapidly elicit ROS production in human endothelial cells. Previous studies have used rather high concentrations of MGO to demonstrate its biological actions [19–21]. The higher sensitivity of our system may be due to our superfusion system and real time measurements of ROS. When cells are treated with MGO for a prolonged period under culture conditions, secondary changes may occur, and these changes may obscure the detection of subtle changes. Since our system allowed real-time measurements in the absence of the influences of secondary changes, we were able to detect significant effects of MGO at low concentrations.

Davidson and Duchen [31] reported that mitochondria play an important role in production of $O^{\cdot}_2{}^-$ in the endothelial cells. Superoxide is generated from two main sites in the inner mitochondrial membrane, NADH dehydrogenase at complex I and at the interface between ubiquinone and complex III. High glucose loads stimulate mitochondrial $O^{\cdot}_2{}^-$ production from the latter site in aortic endothelial cells [32] and retinal endothelial cells [33]. Rosca et al. [34] showed that the MGO-induced modification of mitochondrial complex III was associated with the excessive formation of $O^{\cdot}_2{}^-$ in streptozotocin-derived diabetic rats. Long-standing hyperglycaemia alters the mitochondrial function and increases the formation of $O^{\cdot}_2{}^-$ due to alterations in mitochondrial metabolism [35]. Shangari et al. [36] reported that a monocarbonyl compound metabolized from glucose, glyoxal markedly increased ROS-induced cytotoxicity in rat hepatocytes. In the present study, two types of mitochondrial inhibitors (TTFA and CCCP) completely suppressed the MGO-induced ROS. TTFA inhibits mitochondrial electron transport chain complex II and CCCP uncouples oxidative phosphorylation, which abolishes the mitochondrial membrane proton gradient. Our results suggest that the mitochondrial pathway is crucial for MGO-induced ROS production, which probably originates from $O^{\cdot}_2$.

Superoxide reacts with nitric oxide (NO) very quickly and forms $ONOO^-$ [37]. Zou et al. [38] demonstrated that increases in the level of $ONOO^-$ reduced the amount of BH_4 and increased the uncoupling form of eNOS. Cai [39] suggested that H_2O_2 originating from vascular NAD(P)H oxidases propagates its own production via the enhancement of several pathways of ROS production including uncoupling eNOS. The uncoupling eNOS increases the production of $O^{\cdot}_2{}^-$. However, our protocols of measuring DCF could not distinguish the production of H_2O_2 from $ONOO^-$. Thus, we could not distinguish uncoupling

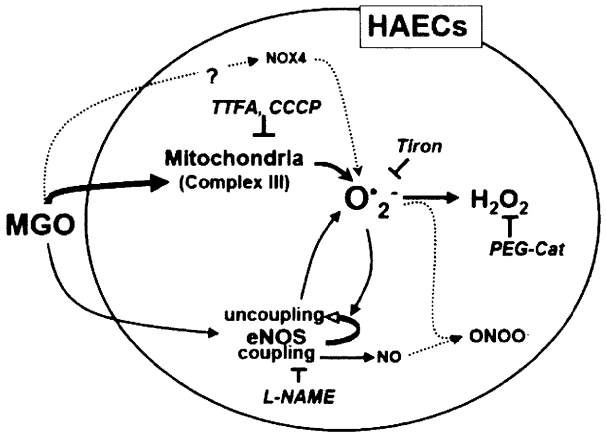

Figure 4. Putative pathways by which MGO induces ROS production in HAECs. To increase $O^{\cdot}_2{}^-$ production, MGO primarily affects the mitochondria and partially affects eNOS. MGO might also potentially affect Nox. The increment of intracellular $O^{\cdot}_2{}^-$ increases the uncoupling of eNOS and probably trigs a vicious cycle on the oxidative stress.

eNOS from coupling eNOS to consider their individual contributions to the ROS production. $ONOO^-$ is also a harmful ROS molecule and L-NAME inhibits uncoupling eNOS as well as coupling eNOS. Based on our results, eNOS is associated with the MGO-mediated ROS production. These findings suggest that MGO triggers a vicious cycle involved in formation of uncoupling eNOS (Figure 4). This pathway may augment the MGO toxicity in the cells.

In vascular endothelial cells, the Nox family is an important source of $O^{\cdot}_2{}^-$. Nox2 and Nox4 are abundantly expressed in human endothelial cells [40]. A previous study suggested that Nox was responsible for the MGO-induced $O^{\cdot}_2{}^-$ formation in mesangial cells [41]. However, we did not find a significant contribution of Nox to the acute ROS production induced by MGO in HAECs when we used apocynin to inhibit Nox. Apocynin is activated in the presence of H_2O_2 and myeloperoxidase (MPO) [42] and it inhibits the activation of Nox2 by inhibition of the translocation of p47phox. A recent report found that apocynin is an antioxidant and is not used as an NADPH oxidase inhibitor [43]. HAEC has a rare expression pattern of MPO that is different from neutrophils. Furthermore, the pre-treated apocynin was washed out before the experimental period, so apocynin could not exert antioxidant effects in our protocol. Nox4 requires p22phox for its activity but does not require other regulatory sub-units [44]. It is possible that Nox4 produced superoxide in our study, but we could not address this possibility in the present study.

Recently we reported that MGO reacted with H_2O_2, forming a novel carboxy-methyl radical [18]. The radical was formed higher under MGO with H_2O_2 than under glyoxal (GO) with H_2O_2. This observation is consistent with our supplemental data (online version only) showing that the ROS production rate by MGO

(4.11 ± 0.79 units/s) is greater than that by GO (0.82 ± 0.05 units/s, $p = 0.02$). As MGO reacts with glutathione and forms S-D-lactoylglutathione, it is possible that MGO decreases the intracellular capacity of antioxidants [45], but GO does not [46]. On the other hand, H_2O_2 is known as an endothelial-derived hyperpolarization factor [47]. Thus, MGO may reduce H_2O_2 localized around endothelial cells and damage the artery.

MGO is a radical modulator that forms advanced glycation end-products (AGEs). Aminoguanidine (AG) inhibits AGE formation from glucose and methylglyoxal [48]. AGE is also an inducer of $O^{\bullet}_2{}^-$. We examined the ability of AG and arginine (L-Arg) to inhibit AGE formation from MGO. As shown in the supplemental figure (online version only), both L-Arg and AG entirely inhibited the ROS production (mean oxidation rates (units/s): MGO alone, 4.11 ± 0.79; MGO with L-Arg, 0.014 ± 0.01; MGO with AG, −0.09 ± 0.06; control, 0.31 ± 0.12). MGO reacts with guanidine residue [49] and produces superoxide, which is inhibited by AG [50]. Arginine is a kind of guanidino compound. It is possible that the extracellular $O^{\bullet}_2{}^-$ generated form L-Arg and MGO was continuously drained from the chamber because of our continuous superfusion system. As DCF is an accumulative indicator, it is probable that AG almost completely inhibited ROS by the basal production and that we observed a photo-bleaching phenomenon of DCF. Because the guanidine residue is a constituent of cyclic GMP, which is an intracellular signal of NO to dilate vessels, MGO may interfere with NO functions. It has been reported that MGO activated caspase-3 and induced the cell death of endothelial cells [51]. The expression and activity of caspase-3 was stimulated by superoxide [51]. This study supports our finding that MGO stimulates the production of superoxide in endothelial cells. Here, we show the mechanism by which MGO produces ROS in endothelial cells. Further studies are needed to conclusively determine if the produced ROS is derived from $O^{\bullet}_2{}^-$.

In conclusion, we found that high concentrations of MGO rapidly induce the production of ROS in HAECs. The putative source of the ROS is the mitochondrial pathway and also partially the eNOS pathway (Figure 4). The increased level of MGO in patients with renal dysfunction and/or diabetes probably plays an important role in the endothelial dysfunction and is a putative risk for developing atherosclerosis.

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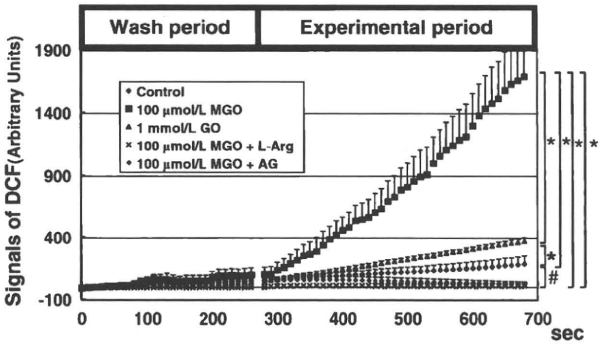
Supplementary Material



Supplementary Figure 1. Vehicle buffer was continuously superfused during the wash period and then switched to buffer that contained MGO (100 μmol/L) or glyoxal (GO, 1 mmol/L) during the experimental period. Signals of DCF were detected and captured by a 535-nm emission filter with 480-nm excitation wavelength every 10 s. The responses by MGO were measured in the presence or absence of 10 mmol/L Aminoguanidine (AG) or 100 μmol/L L-arginine (L-Arg). $^{*}p < 0.05$, $\#p = 0.016$ control vs 100 μM MGO + AG.

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Biological Effects of Electrolyzed Water in Hemodialysis

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Key Words
Hemodialysis · Electrolyzed water · Oxidative stress · Methylglyoxal · Neutrophils

Abstract
Background/Aims: The application of electrolyzed water (EW) at the cathode side to manufacture reverse osmosis (RO) water and hemodialysis (HD) solution can actually lead to less oxidative capacity in chemical terms. The present study examined the biological actions of this water on human polymorphonuclear leukocytes (PMNs), and the clinical feasibility of applying this technology to HD treatment. ***Methods:*** RO water using EW (e-RO) exhibited less chemiluminescence in luminol-hydrogen peroxide and higher dissolved hydrogen levels (~99.0 ppb) compared with control RO water. The effects of e-RO on PMN viability were tested. HD using e-RO was performed for 12 consecutive sessions in 8 patients for the feasibility test. ***Results:*** Basal cellular viability and function to generate superoxide radicals of PMNs were better preserved by e-RO application. In the clinical trial, reductions of blood pressure were noted, but no adverse events were observed. There were no changes in the blood dialysis parameters, although methylguanidine levels were significantly decreased at the end of study. ***Conclusion:*** The present study demonstrated the capacity of e-RO to preserve the viability of PMNs, and the clinical feasibility of applying this water for HD treatment. The clinical application of this technology may improve the bio-compatibility of HD treatment.

Introduction

Evidence suggests that enhanced oxidative stress plays a crucial role in poor outcomes of patients on maintenance hemodialysis (HD) [1, 2]. In addition to a uremic milieu [1], several factors in the HD system have been found to be involved in the pathological mechanism of these poor outcomes, including bio-incompatibility of the dialysis membrane, contamination of the HD solution and loss of antioxidants during HD [3–7].

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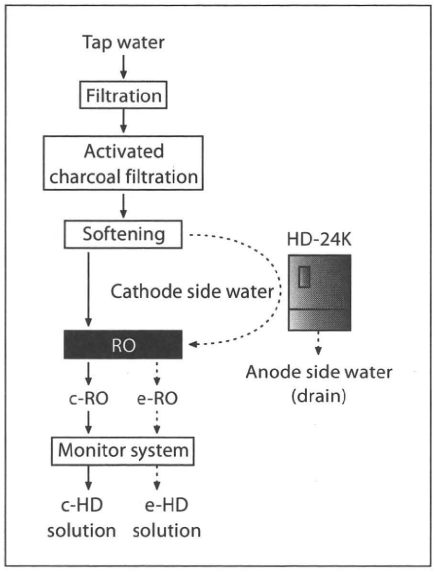

Fig. 1. Manufacturing process for HD solution using reverse osmosis water from the cathode-side of electrolyzed water. c-RO = control reverse osmosis water; e-RO = reverse osmosis water using electrolyzed water at the cathode side.

Water electrolysis renders 2 types of water: acidic water at the anode side and alkali water at the cathode side. It has been shown that alkali water (called electrolyzed water, EW) at the cathode side exhibits unique chemical properties, such as highly dissolved hydrogen with antioxidative capacity [8, 9]. An in vitro study showed that chemical reactions to generate superoxide anions and hydrogen peroxide (H_2O_2) were suppressed by EW treatment [8]. Based on these findings, therapeutic application of EW or water with highly dissolved hydrogen has been studied in animal models of diabetes [10, 11] and ischemic brain injury [12].

Furthermore, EW has been used to manufacture HD solution to reduce oxidative stress in patients [13–15]. To date, the limited clinical experience using this technology shows that EW suppresses oxidative/inflammatory markers in HD patients [13, 14]; however, most of the biological and clinical effects of EW remain unclear. The present study aimed to: (1) examine the biological action of EW in terms of whether it could ameliorate injury to human polymorphonuclear leukocytes, which may play a central role in excess inflammation or oxidative stress in HD patients [16], and (2) to test the clinical feasibility of applying this technology to HD treatment.

Materials and Methods

Manufacture of Test Solutions

Details of the manufacturing process were reported previously [15]. Briefly, test solutions were manufactured as follows (fig. 1): prefiltered water was processed by activated charcoal filtration and water softening to supply the water electrolysis system HD-24K (Nihon Trim, Osaka, Japan), where water was electrolyzed by direct current supply to the anode and cathode electrode plates. Water at the anode side was drained out, and EW was collected to supply the reverse osmosis equipment (MH500CX, Japan Water System Corp., Tokyo, Japan) at 500 ml/min. The intensity of the electrolysis was adjusted to maintain a pH $\leq$10.0. The reverse osmosis water made by EW (e-RO) was supplied to a personal HD monitoring system (DBB-22B, Nikkiso, Tokyo, Japan) to make the HD solution by mixing with a liquid dialysis solution concentrate.

The pH of the e-RO ranged from 9.0 to 10.0, with mean dissolved hydrogen levels of 99.0 ppb. The dissolved hydrogen level was detected by a gas analyzer (DH-35A hydrogen gas analyzer, Mitsuwa Rikagaku, Osaka, Japan). The hemodialysis solution made by e-RO (e-HD) did not differ from the control HD (c-HD) solution in respect of the electrolyte composition or pH; however, the former solution had a higher level of dissolved hydrogen (80 vs. 0 ppb).

Biological Effects of EW on Polymorphonuclear Leukocytes

Measurement of Cellular Viability

Human polymorphonuclear leukocytes (PMNs) were obtained from healthy volunteers or patients on chronic HD as indicated. Briefly, whole blood was withdrawn from healthy volunteers, and heparinized samples were placed onto the Mono-poly Resolving Medium (Dainippon Pharmaceutical, Osaka, Japan) to collect PMNs. After centrifugation at 1,800 rpm for 30 min at 18°C, the intermediate PMN-enriched layer was recovered, washed, and resuspended in RPMI 1640 culture medium supplemented with 10% fetal calf serum or phosphate buffered saline (PBS). Collected PMNs were adjusted to a concentration of 5×10^5 cells/ml. 50 μl of cell suspension was placed in a 96-well microplate and 50 μl of the test solution containing methylglyoxal (MGO), a toxic dicarbonyl compound elevated in uremic patients, and/or 4-β phorbol 12-β-myristate 13-α-acetate (PMA; a stimulator of the respiratory burst of PMNs) in PBS, was added. The viability of PMNs was examined using a commercially available kit (CellTiter-Glo® luminescent cell viability assay, Promega Corp., Wisc., USA) as previously reported [17]. After each treatment, 100 μl of the sample was placed in a microplate and 100 μl CellTiter-Glo® reagent (Promega), which contains beetle luciferin and luciferase was added immediately. This reacts with adenosine triphosphate (ATP), which was released from lysed cells, to produce oxyluciferin; it also generates chemiluminescence as a function of the increased ATP levels. After incubation, luminescence was measured using a chemiluminescence analyzer (Glo-Max™ 20/20n luminometer, Promega). Each measurement was made 5 times, and the mean values for each sample were calculated for analysis after correction for cell-free levels.

Measurement of Radical Generation from PMN

The rate of superoxide release from human PMNs was determined by measuring the reduction of ferricytochrome C, as reported elsewhere [18]. PMNs obtained by the same procedure described above were adjusted to a concentration of 1×10^6 cells/ml

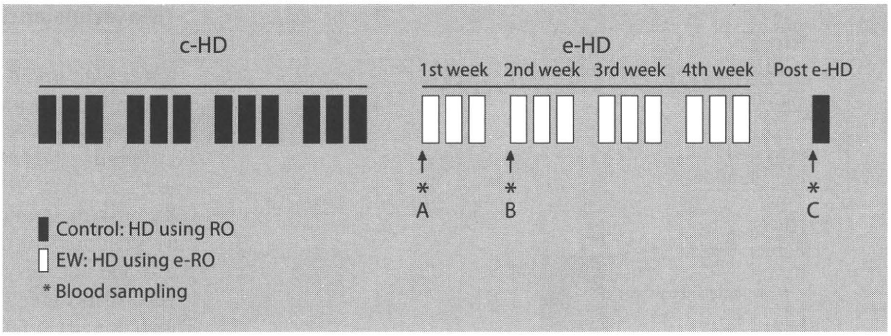

Fig. 2. Feasibility study protocol of HD applying e-RO based HD solution (e-HD). e-RO = reverse osmosis water using electrolyzed water at the cathode side; A = the start of the first e-HD session (first week); B = the start of the fourth e-HD session (second week); C = the start of c-HD after 12 e-HD sessions (post-e-HD).

Table 1. Patient demographics

Case	Sex	Age years	HD vintage months	Underlying renal disease	Dialyser	Prescription	
						AHD	statin
1	female	65	58	NS	BG-1.6	ARB	–
2	male	35	111	CGN	APS-2.1	–	–
3	female	64	216	CGN	BG-1.8	–	–
4	male	70	70	DN	BG-1.8u	ARB	–
5	male	75	77	CGN	AMBC-1.5	ARB	+
6	male	67	335	CGN	APS-185	–	–
7	male	72	116	DN	BG-2.1	CCB+ARB	–
8	male	70	6	NS	PS-1.6	ARB	–

AHD = Anti-hypertensive drug; ARB = angiotensin receptor blockade; CCB = calcium channel blocker; CGN = chronic glomerulonephritis; DN = diabetic nephropathy; NS = nephrosclerosis.

in a solution of 80 μM cytochrome C diluted in PBS. Immediately after adding 10 μl of control or PMA (100 μg) to 700 μl of the sample, 250 μl of the sample was placed on the microplate. Absorbance was measured for 15 min at 550 nm, and was expressed as nanomoles of superoxide production per 0.25×10^6 cells/10 min.

In these 2 experiments, PBS was prepared either with control reverse osmosis water (c-RO) or e-RO. Manufactured e-RO was stocked in the closed flask immediately after being made and then used in the study.

Clinical Feasibility of a HD System Using EW

Patients and Study Design

Eight patients on regular HD treatment at Kashima Hospital Dialysis Center (Iwaki, Japan) were enrolled in the trial (table 1). They consisted of 6 men and 2 women, with a mean age of 67 years (range 35–75 years) and a mean dialysis duration of 85 months (range 6–33.5 months). Their underlying renal diseases were chronic glomerulonephritis in 4 patients, nephrosclerosis in 2 and diabetic nephropathy in 2. All patients had been on regular HD treatment 3 times a week for 4 h (n = 6) or 5 h (n = 2) each session. All patients had been using high-flux membrane dialyzers. Among them, 4 were receiving an angiotensin receptor blocker, 1 was receiving an angiotensin receptor blocker and a calcium channel blocker, and 1 was receiving a statin. Patients who were taking ascorbic acid or tocopherol were excluded.

Patients were treated by regular HD regimen using c-HD solution for 1 month, followed by the use of e-HD solution for another month. Both 1-month regimens comprised 3 sessions per week, for a total of 12 sessions (fig. 2). During the study, no changes in HD modes or concomitant medications were made.

Blood sampling was obtained just before each HD session. Blood was immediately centrifuged and serum was stored at –80°C until measurements were made by commercially available kits or high performance liquid chromatography (HPLC). Interleukin-6 was assessed by the CLEIA method (Human IL-6 CLEIA, Fujirebio C, Tokyo, Japan), highly sensitive C reactive protein by nephrometry (N High Sensitive CRP, Dade Behring, Marburg, Germany), creatol and methylguanidine by HPLC, 8-OHdG by an enzyme-linked immunosorbant assay (ELISA) kit (high sensitive 8-OHdG check, Nikken C, Shizuoka, Japan) and pentosidine by an ELISA kit (FSK pentosidine, Fushimi C, Marugame, Japan).

All patients were monitored regarding subjective symptoms during the study periods. Blood pressure was measured using a sphygmomanometer at the upper arm with the patient in the supine position just before and after each HD session. The mean of the 12 measurements obtained was determined.