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ScienceDirect
Metabolism Clinical and Experimental 60 (2011) 453–459
Metabolism
Clinical and Experimental
www.metabolismjournal.com

Advanced glycation end products, carotid atherosclerosis, and circulating endothelial progenitor cells in patients with end-stage renal disease

Hiroki Ueno[a], Hidenori Koyama[a,*], Shinya Fukumoto[a], Shinji Tanaka[a], Takuhito Shoji[a], Tetsuo Shoji[a], Masanori Emoto[a], Hideki Tahara[a], Masaaki Inaba[a], Ryusuke Kakiya[b], Tsutomu Tabata[b], Toshio Miyata[c], Yoshiki Nishizawa[a]

[a]*Department of Metabolism, Endocrinology and Molecular Medicine, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan*
[b]*Division of Internal Medicine, Inoue Hospital, Suita 564-0053, Japan*
[c]*Center for Translational and Advanced Research, Tohoku University Graduate School of Medicine, Sendai, Miyagi 980-8575, Japan*

Received 18 December 2009; accepted 6 April 2010

Abstract

Numbers of endothelial progenitor cells (EPCs) have been shown to be decreased in subjects with end-stage renal disease (ESRD), the mechanism of which remained poorly understood. In this study, mutual association among circulating EPC levels, carotid atherosclerosis, serum pentosidine, and skin autofluorescence, a recently established noninvasive measure of advanced glycation end products accumulation, was examined in 212 ESRD subjects undergoing hemodialysis. Numbers of circulating EPCs were measured as $CD34^+$ $CD133^+$ $CD45^{low}$ $VEGFR2^+$ cells and progenitor cells as $CD34^+$ $CD133^+$ $CD45^{low}$ fraction by flow cytometry. Skin autofluorescence was assessed by the autofluorescence reader; and serum pentosidine, by enzyme-linked immunosorbent assay. Carotid atherosclerosis was determined as intimal-medial thickness (IMT) measured by ultrasound. Circulating EPCs were significantly and inversely correlated with skin autofluorescence in ESRD subjects ($R = -0.216$, $P = .002$), but not with serum pentosidine ($R = -0.079$, $P = .25$). Circulating EPCs tended to be inversely associated with IMT ($R = -0.125$, $P = .069$). Intimal-medial thickness was also tended to be correlated positively with skin autofluorescence ($R = 0.133$, $P = .054$) and significantly with serum pentosidine ($R = 0.159$, $P = .019$). Stepwise multiple regression analyses reveal that skin autofluorescence, but not serum pentosidine and IMT, was independently associated with low circulating EPCs. Of note, skin autofluorescence was also inversely and independently associated with circulating progenitor cells. Thus, tissue accumulated, but not circulating, advanced glycation end products may be a determinant of a decrease in circulating EPCs in ESRD subjects.

1. Introduction

Endothelial progenitor cells (EPCs) play a key role in the maintenance and repair of vascular integrity in response to endothelial injury [1-3]. Upon cytokine stimulation and ischemic injury, EPCs can be mobilized from bone marrow, home to ischemic tissue, and contribute to neovascularization and angiogenesis [4-6]. Decreased number of circulating EPCs is associated with risk of coronary artery disease and cardiovascular mortality and morbidity [7-9].

Patients with diabetes mellitus and end-stage renal disease (ESRD) are known to have increased risks for cardiovascular mortality and morbidity [10,11]. Most, but not all, of the studies have shown that the numbers of circulating EPCs are decreased in patients with ESRD [12-16] and diabetes [17,18], the underlying mechanisms of which remained poorly characterized. Age-dependent depression in circulating EPCs is also reported and is implicated in the risk for cardiovascular diseases [19,20].

A common feature of aging, diabetes, and ESRD is the accumulation of advanced glycation end products (AGEs) [21], which is implicated in the pathogenesis of chronic vascular complications [22-25]. We therefore reasoned that decrease in number of circulating EPCs might be associated with AGE accumulation in patients with ESRD. In the present study, we examined the association between the number of circulating EPCs, serum pentosidine, and skin autofluorescence, a recently developed noninvasive measure

* Corresponding author. Tel.: +81 6 6645 3806; fax: +81 6 6645 3808.
E-mail address: hidekoyama@med.osaka-cu.ac.jp (H. Koyama).

0026-0495/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2010.04.001

for tissue AGE accumulation [26], in 212 ESRD patients. We also measured carotid atherosclerosis, determined as intimal-medial thickness (IMT) by ultrasound, to examine if the association between AGEs and EPCs is independent of the levels of atherosclerosis.

2. Methods

2.1. Subjects

This study was approved by the Ethics Committee at Osaka City University Graduate School of Medicine (approval no. 835). This study adheres to the Declaration of Helsinki, and informed consent was obtained from all subjects who participated in the study. The present study included ESRD patients who were treated by regular hemodialysis 3 times a week at Inoue Hospital, Suita, Japan. Among 312 patients who agreed to participate, 212 patients (nondiabetic, 177; diabetic, 35) completed all measurements including circulating EPCs, skin autofluorescence, serum pentosidine, and carotid atherosclerosis. Table 1 summarizes the characteristics of the ESRD subjects. *Diabetic subjects* were defined as those meeting the following criteria: fasting plasma glucose greater than 126 mg/dL, casual plasma glucose greater than 200 mg/dL, or history of treatment of diabetes. Presence of vascular complications was diagnosed as described previously [27].

Table 1
Characteristics of the subjects

No. of subjects	212
Age (y)	59.9 ± 10.1
Sex (% male)	59.9
Etiology of ESRD (%)	
Glomerulonephritis	63.7
Diabetic nephropathy	15.5
Polycystic kidney disease	6.6
Nephrosclerosis	1.4
Others	12.8
Diabetes (%)	16.5
Vascular complications (%)	27.8
Coronary heart diseases (%)	16.5
Cerebrovascular diseases (%)	10.2
Peripheral artery diseases (%)	7.5
Hemodialysis vintage (years)	13.0 (1.9-30.9)
Current smoking (%)	21.2
Body mass index (kg/m^2)	21.4 ± 2.8
Systolic blood pressure (mm Hg)	149 ± 17
Hemoglobin (g/dL)	9.80 ± 1.04
Non-HDL cholesterol (mg/dL)	103.2 ± 32.5
HDL cholesterol (mg/dL)	47.6 ± 12.4
Calcium (mg/dL)	9.58 ± 0.81
Phosphate (mg/dL)	5.80 ± 1.35
Intact parathyroid hormone (pg/mL)	135 (10-1656)
Carotid IMT (mm)	0.762 ± 0.163
Serum pentosidine (μg/mL)	0.51 ± 0.18
Skin autofluorescence	0.018 ± 0.007

Continuous variables are summarized as mean ± SD, whereas median values (limits of observed values) are shown for variables with skewed distribution.

2.2. Quantification of EPCs

Numbers of EPCs were measured by flow cytometry essentially as described previously [16]. In brief, 4 mL of peripheral blood was drawn; and mononuclear cells were isolated by Ficoll density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare, Buckinghamshire, UK). Mononuclear cells were stained with fluorescein isothiocyanate–conjugated anti-CD34 monoclonal antibody (Beckman Coulter, Fullerton, CA), PC5-conjugated anti-CD45 mAb (Beckman Coulter), phycoerythrin-conjugated monoclonal anti-CD133 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany), and allophycocyanin (activated protein C)-conjugated anti-VEGF R2 (R&D Systems, Minneapolis, MN). Samples were subjected to a 2-dimensional side scatter fluorescence dot plot analysis (FACS CANTO; Becton-Dickinson, Franklin Lakes, New Jersey). After appropriate gating with low cytoplasmic granularity, cells were serially gated with $CD34^+$, low expression of CD45, $CD133^+$, and $VEGFR2^+$ fraction, enabling quantification of $CD34^+$ $CD45^{low}$ $CD133^+$ $VEGFR2^+$ cells (EPCs) per 10^6 mononuclear cells. In 14 healthy volunteers, numbers of EPCs and endothelial colony-forming units determined by Endothelial Progenitor Culture Assay (StemCell Technology, Vancouver, BC, Canada) exhibited a linear relationship ($r^2 = 0.866$). The total numbers of $CD34^+$ $CD45^{low}$ $CD133^+$ cells were also counted as progenitor cells (PCs). The intraassay coefficient of variation of EPCs and PCs based on multiple measurements of a sample was 8.6% and 3.4%, respectively.

2.3. Skin autofluorescence

Skin autofluorescence was assessed by the autofluorescence reader (AGE Reader; Diagnoptics, Groningen, the Netherlands) as previously described in detail [26]. The measure of autofluorescence used was the average light intensity per nanometer in the range 420 to 600 nm divided by the average light intensity per nanometer in the range 300 to 420 nm. The intraassay coefficient of variation based on repeated measurements on the same day was 2.8% (n = 5). For a nonwhite population, skin autofluorescence in Japanese healthy volunteer was 0.013 ± 0.005 (n = 110) and was found to be a predictor of arterial stiffness [28].

2.4. Ultrasonography

Ultrasonographic scanning of the carotid artery was performed by an ultrasonic phase-locked echotracking system that was equipped with a high-resolution real-time scanner. The site of the most advanced atherosclerotic lesion was examined in the longitudinal and transverse projections of common carotid artery to record the maximum IMT as previously described [29,30].

2.5. Biochemical analyses

Serum pentosidine levels were measured as previously described [27]. Serum levels of total cholesterol and high-

density lipoprotein (HDL) cholesterol were measured by enzymatic methods adapted to an autoanalyzer (Hitachi 7470; Hitachi, Tokyo, Japan). Non-HDL cholesterol was calculated by subtracting HDL cholesterol from total cholesterol.

2.6. Statistical analyses

Statistical analyses were performed with StatView V software (SAS Institute, Cary, NC). To evaluate relationship between factors, simple regression analyses and stepwise multiple regression analyses were performed. Findings of $P < .05$ or F value> 4.0 were considered significant.

3. Results

As shown in Fig. 1A, numbers of circulating EPCs were significantly and inversely correlated with skin autofluorescence, but not with serum pentosidine, in ESRD subjects. Circulating EPCs also tended to be inversely associated with carotid atherosclerosis (Fig. 1B). Female, presence of diabetes, low blood hemoglobin, and non-HDL cholesterol were also associated with low circulating EPCs in ESRD patients (Table 2). Carotid IMT tended to correlate with skin autofluorescence (r = 0.133), whereas it was significantly associated with serum pentosidine (r = 0.159) (Fig. 2A). As previous study demonstrated [31], the relation between skin autofluorescence and AGE plasma levels was completely absent in our subjects (Fig. 2B). To further determine the significance of skin autofluorescence, serum pentosidine, and carotid atherosclerosis on circulating EPCs, stepwise multiple regression analyses were performed (Table 3). In model 1, variables included classic and nonclassic risk factors for cardiovascular diseases in ESRD, but excluded carotid IMT, serum pentosidine, and skin autofluorescence. In this model, female, presence of diabetes, and low blood hemoglobin were identified to be independently associated with low circulating EPCs. In model 2, adding carotid IMT to model 1, significant relationships were not affected. Although model 3, adding serum pentosidine to model 2, did not affect the results, inclusion of skin autofluorescence to model 2 (model 4) resulted in skin autofluorescence being independently associated with low circulating EPCs together with low blood hemoglobin. In this model, sex and diabetes were not significantly associated with EPCs any more. To further examine whether relation between skin autofluorescence and circulating EPCs was independent of blood hemoglobin, multiple regression analysis was performed using age, diabetes, hemoglobin, carotid IMT, and skin autofluorescence as variables (R^2 = 0.109, $P <$ .001). Even in this model, skin autofluorescence (β =

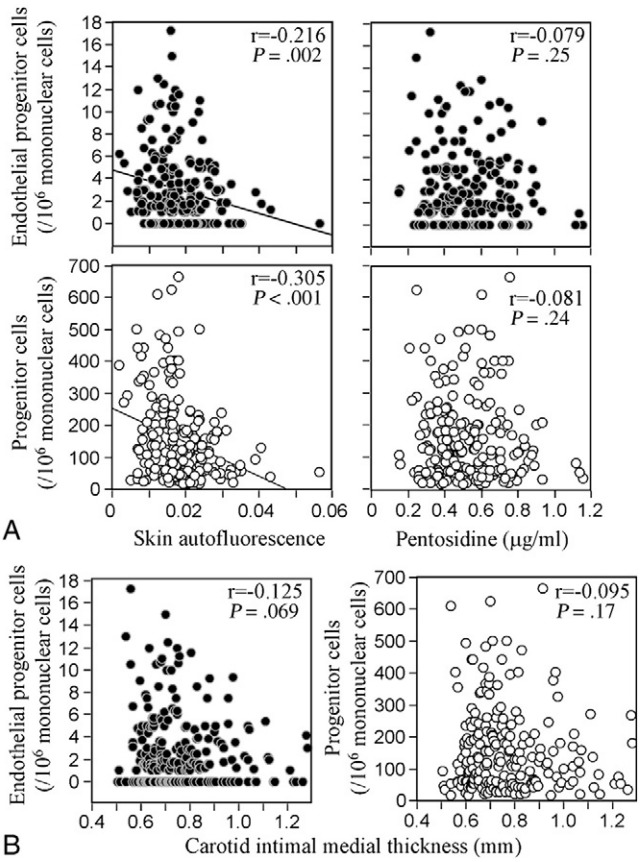

Fig. 1. A, The numbers of both circulating PCs and EPCs were inversely and significantly correlated with skin autofluorescence, but not with serum pentosidine, in 212 ESRD subjects. Endothelial progenitor cells were quantified as $CD34^+$ $CD45^{low}$ $CD133^+$ $VEGFR2^+$ cells per 10^6 mononuclear cells by flow cytometry. Progenitor cells were defined as $CD34^+$ $CD45^{low}$ $CD133^+$ cells. B, Intimal-medial thickness in carotid artery measured by ultrasound tended to be inversely associated with circulating EPCs.

Table 2
Simple regression analyses of factors associated with numbers of circulating EPCs and PCs

	EPC	PC
Age	−0.075	−0.142*
Sex (male = 0, female = 1)	−0.236†	−0.243†
Diabetes (no = 0, yes = 1)	−0.147*	−0.160*
Vascular complications (no = 0, yes = 1)	−0.081	−0.133
Hemodialysis vintage (short = 0, long = 1)	−0.113	−0.165*
Current smoking (no = 0, yes = 1)	0.099	0.076
Body mass index	0.052	0.109
Systolic blood pressure	−0.046	−0.028
Hemoglobin	0.255†	0.235†
Non-HDL cholesterol	−0.145*	−0.119
HDL cholesterol	0.053	0.064
Calcium	0.075	0.003
Phosphate	0.053	0.099
Intact parathyroid hormone (logarithm transformed)	0.034	0.034
Carotid IMT	−0.125	−0.095
Serum pentosidine	−0.079	−0.081
Skin autofluorescence	−0.216†	−0.305†

Hemodialysis vintages are classified into long (>13.0 years) and short (≤13.0 years) according to the median. R values (coefficients of correlation) are shown.

* $P < .05$.

† $P < .01$.

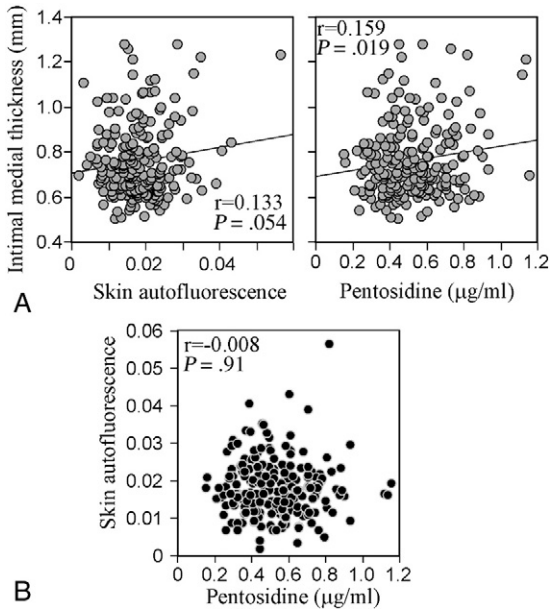

Fig. 2. A, Skin autofluorescence and serum pentosidine show weak correlation with carotid IMT. B, Lack of association between skin autofluorescence and serum pentosidine.

−0.142, P = .040) was significantly and inversely associated with circulating EPCs, with the relation independent of age (β = 0.030, P = .67), diabetes (β = −0.105, P = .12), blood hemoglobin (β = 0.229, P < .001), and carotid IMT (β = −0.075, P = .28).

We also examined the determinants of circulating PCs, potential precursors of EPCs. Circulating PCs and EPCs showed moderate correlation (R = 0.608, P < .001). As shown in Fig. 1A, circulating PCs were significantly and inversely associated with skin autofluorescence, whereas serum pentosidine failed to be significantly correlated. The relation between carotid IMT and circulating PCs was not significant (Fig. 1B). Besides skin autofluorescence, higher age, female, presence of diabetes, long hemodialysis duration, and low blood hemoglobin levels were associated with low circulating PCs (Table 2). Stepwise multiple regression analyses revealed that female, presence of diabetes, long hemodialysis vintage, and low hemoglobin were independently associated with low circulating PCs (model 1). As in the case of EPCs, addition of skin autofluorescence to variables resulted in identification of skin autofluorescence being independently associated with low circulating PCs, with sex, diabetes, and dialysis duration being expelled from significant variables correlated (Model 4). Neither serum pentosidine nor carotid atherosclerosis level was significantly associated with circulating PCs (model 2 or model 3). Similar to EPCs, in multiple regression analysis using age, diabetes, hemoglobin, carotid IMT, and skin autofluorescence as variables (R^2 = 0.143, P < .001), skin autofluorescence (β = −0.249, P < .001) was significantly and inversely associated with circulating PCs, with the relation independent of age (β = −0.040, P = .56), diabetes (β = −0.105, P = .12), blood hemoglobin (β = 0.180, P = .006), and carotid IMT (β = −0.021, P = .76).

Table 3
Stepwise multiple regression analyses of factors associated with numbers of circulating EPCs and PCs

EPCs

Variables	Model 1	Model 2	Model 3	Model 4
Age	NS	NS	NS	NS
Sex (male = 0, female = 1)	−0.170*	−0.170*	−0.170*	NS
Diabetes (no = 0, yes = 1)	−0.148*	−0.148*	−0.148*	NS
Vascular complications (no = 0, yes = 1)	NS	NS	NS	NS
Hemodialysis vintage (short = 0, long = 1)	NS	NS	NS	NS
Current smoking (no = 0, yes = 1)	NS	NS	NS	NS
Body mass index	NS	NS	NS	NS
Systolic blood pressure	NS	NS	NS	NS
Hemoglobin	0.178*	0.178*	0.178*	0.225*
Non-HDL cholesterol	NS	NS	NS	NS
HDL cholesterol	NS	NS	NS	NS
Calcium	NS	NS	NS	NS
Phosphate	NS	NS	NS	NS
Intact parathyroid hormone (logarithm transformed)	NS	NS	NS	NS
Carotid IMT	–	NS	NS	NS
Serum pentosidine	–	–	NS	–
Skin autofluorescence	–	–	–	−0.179*
R^2	0.107*	0.107*	0.107*	0.096*

PCs

Variables	Model 1	Model 2	Model 3	Model 4
Age	NS	NS	NS	NS
Sex (male = 0, female = 1)	−0.159*	−0.159*	−0.159*	NS
Diabetes (no = 0, yes = 1)	−0.197*	−0.197*	−0.197*	NS
Vascular complications (no = 0, yes = 1)	NS	NS	NS	NS
Hemodialysis vintage (short = 0, long = 1)	−0.180*	−0.180*	−0.180*	NS
Current smoking (no = 0, yes = 1)	NS	NS	NS	NS
Body mass index	NS	NS	NS	NS
Systolic blood pressure	NS	NS	NS	NS
Hemoglobin	0.160*	0.160*	0.160*	0.190*
Non-HDL cholesterol	NS	NS	NS	NS
HDL cholesterol	NS	NS	NS	NS
Calcium	NS	NS	NS	NS
Phosphate	NS	NS	NS	NS
Intact parathyroid hormone (logarithm transformed)	NS	NS	NS	NS
Carotid IMT	–	NS	NS	NS
Serum pentosidine	–	–	NS	–
Skin autofluorescence	–	–	–	−0.273*
R^2	0.137*	0.137*	0.137*	0.128*

Hemodialysis duration (long; >13.0 years according to the median).
NS = not significant. "–" indicates not included in variables.
* F value >4.0.

4. Discussion

This study is the first to demonstrate the potential involvement of tissue AGE accumulation, but not

circulating AGEs, in decrease in number of circulating EPCs in humans. Moreover, the relation between tissue AGEs and circulating EPCs was independent of the level of subclinical atherosclerosis.

Recent flow cytometric technique enabled us to measure samples large enough to make a solid conclusion [16,32,33]. As previously described [16], we defined $CD34^+$ $CD133^+$ $CD45^{low}$ $VEGFR2^+$ cells as EPCs and used this fraction of cells to examine regulation of EPCs in subjects with ESRD. We also analyze PCs, defined as $CD34^+$ $CD133^+$ $CD45^{low}$ cells, as previously reported [33]. Consistent with the previous finding that EPCs are mainly derived from $CD34^+$ $CD133^+$ $CD45^{low}$ PCs [34,35], number of PCs exhibited a significant and positive correlation with that of EPCs.

Both numbers and function of circulating EPCs have been shown to be decreased in patients with ESRD [12-16] and diabetes [12,13]. Because accumulation of AGEs is a promising pathogenetic mechanism for complications in both diabetes and ESRD, we reasoned that tissue or circulating AGE levels might be associated with circulating EPCs in subjects with ESRD. Our results clearly showed that tissue AGE accumulation, but not circulating AGEs, may be a determinant of low circulating EPCs. Importantly, this association was independent of the potential confounders, including aging, sex, presence of diabetes, history for cardiovascular diseases, and other classic risk factors for atherosclerosis. Moreover, relation between circulating EPCs and AGE accumulation was independent of the level of carotid atherosclerosis, which is an important surrogate marker for cardiovascular diseases, and could be interrelated both with AGEs and circulating EPCs. Serum pentosidine levels were shown to be associated with carotid atherosclerosis in ESRD patients with peritoneal dialysis [36] and diabetic patients [37], which is in agreement with our current observations in hemodialysis patients. Although we did not observe significant correlation between IMT and circulating EPCs as reported in type 1 diabetes mellitus patients [38], previous reports did find significant correlation between EPCs and carotid IMT in healthy [39] or with plaque burden in patients with coronary disease [40].

In the current study, we have used carotid IMT as a surrogate marker for subclinical atherosclerosis. However, measurement of arterial stiffness by pulse wave velocity (PWV), which is significantly and positively associated with skin autofluorescence in ESRD subjects, could be a better marker of subclinical atherosclerosis. In subjects (n = 101) who are overlapped with previous report and have data for PWV [28], multiple regression analysis (R^2 = 0.124, P = .026) revealed that skin autofluorescence was still significantly and inversely associated with circulating PCs (β = 0.238, P = .020), with the relation independent of age, diabetes, hemoglobin, and PWV. However, in these small numbers of subjects, significant independent relation between skin autofluorescence and EPCs was not observed (data not shown), which may be the result of lack of enough power due to small cohort size or of close relationship between skin autofluorescence and PWV. Altogether, in these observations, although subclinical atherosclerosis, circulating or tissue AGEs, and circulating EPCs are mutually interrelated in ESRD patients, tissue accumulated, but not circulating, AGEs could be a determinant for a decrease in circulating EPCs.

It is unclear at present how tissue AGEs, but not circulating AGEs, affect circulating EPCs. Because skin autofluorescence has been shown to correlate with tissue levels of AGEs [26], one intriguing possibility is that skin autofluorescence may reflect AGEs in bone marrow, which could directly influence mobilization of PCs. Indeed, skin autofluorescence, but not serum pentosidine, is also inversely and independently associated with circulating PCs in this study. It is reported that bone marrow mononuclear cells obtained from diabetic mice have abrogated angiogenic potential in postischemic revascularization reaction [41], suggesting impairment of function or decrease in number of PCs or EPCs at the level of bone marrow. Because receptor for AGEs (RAGE) has recently been shown to be expressed in PCs [42], EPC generation and mobilization may be modulated by AGEs [43]. In rat uremic model, we have preliminary observed that PC mobilization induced by hind limb ischemia is inversely correlated with carboxymethyllysine levels in bone marrow [44]. Although these observations are in support of our hypothesis, this should be directly proved by measuring bone marrow AGEs in humans to examine its impact on PC mobilization in a future study.

Skin autofluorescence has been found to be increased in patients with diabetes or ESRD [45,46] and has been shown to correlate with development of long-term complications in diabetic patients [46,47]. In addition, skin autofluorescence is a predictor of cardiovascular mortality in diabetes and ESRD [48,49]. It is still not entirely clear, however, how accumulation of AGEs could contribute to increase in cardiovascular risk. We have recently shown that AGE accumulation is an independent predictor of arterial stiffness [28], a well-established risk factor for cardiovascular mortality [50,51]. The present findings suggest an alternative possibility that a decrease in circulating EPCs, a well-defined risk predictor for cardiovascular diseases [7-9], may mediate the adverse effects of tissue AGE accumulation in patients with ESRD. Because AGE accumulation and RAGE expression could be interrelated [52], the present findings may also be an underlying mechanism for low circulating endogenous secretory RAGE as a risk predictor for cardiovascular events in ESRD subjects [27], which is an intriguing question to be explored in a future study.

This study has one principal limitation. It is unclear whether the skin autofluorescence measured to estimate AGE accumulation represents AGE modification in the bone marrow. Nevertheless, the present findings reveal

important aspects of the pathogenesis and pathophysiology of cardiovascular diseases in ESRD and provide insights into therapeutic target for the prevention of these disorders.

Acknowledgment

This study was supported by a Grant-in-Aid for Scientific Research (20591067 to HK) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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