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# Association between oxidative DNA damage and telomere shortening in circulating endothelial progenitor cells obtained from metabolic syndrome patients with coronary artery disease

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**Abstract**

Metabolic syndrome (MS) induces an increase in oxidative stress and may be an important contributory factor for coronary artery disease (CAD). Telomere shortening of endothelial progenitor cells (EPCs) may be the key factor in endothelial cell senescence. The rate of telomere shortening is highly dependent on cellular oxidative damage. This study analyzed the relationship between telomere shortening and oxidative DNA damage in EPCs obtained from CAD patients with MS and without MS. We analyzed circulating EPCs in peripheral blood obtained from 57 patients with CAD (acute myocardial infarction [AMI], $n=26$; stable angina pectoris [AP], $n=31$) and 21 age-matched healthy subjects (control). Telomere length and telomerase activity were significantly lower in CAD patients than in controls, and were lower in AMI patients than in AP patients. Oxidative DNA damage was higher in CAD patients compared with controls, and oxidative DNA damage in AMI patients was also higher than in AP patients. There was a negative correlation between telomere length and oxidative DNA damage. Telomere length and telomerase activity were lower in CAD patients with MS than in those without MS. Oxidative DNA damage in CAD patients with MS was higher than in those without MS. In our *in vitro* study, oxidative treatments induced telomere shortening and decrease in telomerase activity of EPCs. These results suggest that EPC telomere shortening *via* increased oxidative DNA damage may play an important role in the pathogenesis of CAD. In addition, MS may be related to increased oxidative DNA damage and EPC telomere shortening.


*Keywords:* Acute myocardial infarction; Flow cytometry; Flow-FISH; Stable angina pectoris; Telomerase

## 1. Introduction

Metabolic syndrome (MS) is a cluster of insulin resistance, impaired glucose tolerance, dyslipidemia, obesity, and elevated blood pressure that has reached epidemic proportions in industrialized countries [1]. In recent clinical trials, MS was associated with increased risk of coronary artery disease (CAD) [2]. Oxidative stress plays a critical role in the pathogenesis of CAD in patients with MS [3]. Oxidative stress has been implicated in the development of atherosclerosis through a variety of mechanisms, especially those leading to endothelial dysfunction [4,5]. Oxidative stress also induces damage or apoptosis of endothelial cells [6].

Recent studies have identified that normal adults have a small number of circulating endothelial progenitor cells (EPCs) in the peripheral blood [7]. It has also been reported that patients at risk for CAD have a decreased number of circulating EPCs with impaired activity [8]. EPCs are regarded as having a key role in the maintenance of vascular integrity and the replacement of apoptotic or damaged endothelial cells in response to various cardiovascular risk factors, such as MS [9,10]. Cell division is associated with telomere shortening,

*Abbreviations:* AMI, acute myocardial infarction; AP, angina pectoris; CAD, coronary artery disease; PBMCs, peripheral blood mononuclear cells; TG, triglycerides.

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leading to senescence once telomere length reaches a critical threshold [11]. Telomeres are composed of noncoding double-stranded repeats of G-rich tandem DNA sequence TTAGGG extending over 6–15 kb at the end of eukaryotic chromosomes and are necessary for both successful DNA replication and chromosomal integrity [12]. Telomere shortening is modulated by the rate of cell turnover and oxidative stress [13]. In this study our aim was to determine whether oxidative stress is related to EPC telomere shortening in CAD patients with MS.

## 2. Materials and methods

### 2.1. Study population

Peripheral blood samples were obtained from 57 consecutive patients with CAD (26 patients with acute myocardial infarction [AMI] and 31 patients with stable angina pectoris [AP]). All patients with AMI were admitted within 12 h of onset of AMI. The diagnosis of AMI was made on the basis of the presence of prolonged chest pain, typical electrographic changes, and increased concentrations of serum cardiac enzymes. AP according to the exclusion parameters described for AMI and meeting the following criteria: history of typical chest pain on effort, lasting unchanged for more than 3 months and not associated with rest angina; documented exercise-induced myocardial ischemia; and angiographically proven CAD. Patients were excluded from the study if they had clinical signs of acute infection, severe renal failure or rheumatoid disease, or if they were suspected of having a malignant or primary wasting disorder. Peripheral blood samples were immediately taken from AMI patients at the time of admission and before percutaneous coronary intervention (PCI) procedure. Peripheral blood samples were also taken from AP patients before PCI procedure. As a control, peripheral blood samples were obtained from 21 age- and sex-healthy subjects without any evidence of CAD by history and physical examination. Metabolic syndrome was defined as the presence of at least three out of five risk determinants according to the modified NCEP ATP-III report (a waist circumference $\geq$85/90 cm in men/women, fasting triglycerides [TG] >150 mg/dL, low HDL-cholesterol [HDL-C] [men <40 mg/dL, women <50 mg/dL], systolic blood pressure >130 mm Hg and/or diastolic blood pressure >85 mm Hg, and fasting blood glucose level >100 mg/dL) [14,15]. Approval was obtained from the ethical committee of the Iwate Medical University School of Medicine (H17-73), and written informed consent was obtained from all subjects.

### 2.2. Quantification of circulating EPCs

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples obtained from all subjects by Ficoll–Paque density gradient centrifugation and lymphocyte separation solution (Nacalai Tesque Inc.). PBMCs were resuspended at a final concentration of $1 \times 10^6$ cells/mL in RPMI1640 (Sigma). PBMCs were incubated with 10 μL of FITC-conjugated anti-human CD34 m monoclonal antibody (mAb) (Becton Dickinson) and 10 μL of PE-conjugated anti-human KDR mAb (R&D system), followed by incubation at 4 °C for 30 min. After incubation, cells were fixed with 1% paraformaldehyde. Isotype immunoglobulin IgG antibody was used as a control (Becton Dickinson). The number of CD34 and KDR-double positive cells among $1 \times 10^6$ cells were counted using a FACScan analyzer (Becton Dickinson).

### 2.3. Cell culture enrichment of EPCs

PBMCs ($8 \times 10^6$) were plated on fibronectin-coated culture dishes (Sigma) and maintained in endothelial basal medium-2 (EBM-2; Clonetics, Guelph, Canada) supplemented with EGM-2-MV-SingleQuots (Clonetics) containing 5% fetal bovine serum, 50 ng/mL human vascular endothelial growth factor (VEGF), 50 ng/mL human insulin-like growth factor 1, and 50 ng/mL human epidermal growth factor. To exclude contamination with mature circulating endothelial cells, we carefully removed the culture supernatant 8 h after initial seeding and placed it into new fibronectin-coated culture dishes. After 4 days of culture, nonadherent cells were removed by washing, new medium was applied, and the culture was maintained through day 4. Adherent cells of endothelial lineage were identified by the concurrent binding of FITC-conjugated *Ulex europeaus* agglutinin I (UEA-1, Sigma) and the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanime (DiI)-labeled acetylated low-density lipoprotein (acLDL, Molecular Probes). Adherent cells were visualized with an inverted fluorescent microscope, and adherent cells that stained for both FITC-UEA-1 and DiI-acLDL were considered to be EPCs. Two independent investigators evaluated the number of EPCs per $mm^2$ by counting dual-staining cells in 15 randomly selected high power fields using an inverted fluorescent microscope (Olympus, Tokyo, Japan).

### 2.4. Determination of human telomere length by flow-FISH

Telomere lengths of freshly isolated PBMCs and cultured EPCs were measured using a Dako Telomere peptide nucleic acid kit/FITC for flow cytometry (Dako Cytomation, Ely, U.K.). Relative telomere length (RTL) was determined by comparing isolated EPCs with a control cell line (1301; subline of the Epstein–Barr virus genome negative T-cell leukemia line CCRF-CEM) [16]. A total of $5 \times 10^5$ cells were resuspended in 300 μL of hybridization solution containing 70% formamide either with no probe (unstained control) or with FITC-conjugated telomere PNA probe. These cells were heated for 10 min at 82 °C for DNA denaturation. Hybridization was performed overnight at room temperature in the dark. After washing, cells were resuspended in 0.5 mL of