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Research Report

Calprotectin mRNA (MRP8/MRP14) expression in neutrophils of periodontitis patients with type 2 diabetes mellitus

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ABSTRACT

Background: *Calprotectin, a major cytosolic protein of leukocytes, is detected in neutrophils and monocytes/machrophages. This protein is known to be a marker for several inflammatory diseases including periodontitis. In type 2 diabetes mellitus patients, the severity of periodontitis was strongly thought to be caused by decreasing of leukocytes function such as neutrophils. Previous research found that the calprotectin level in serum of periodontitis patients with type 2 DM is higher than periodontits patients non DM.* ***Purpose:*** *The aim of this study was to determine calprotectin mRNA (MRP8/MRP14) expression in human neutrophils of periodontitis patients with type 2 diabetes mellitus.* ***Methods:*** *Neutrophils were isolated from the peripheral blood of periodontitis patients with uncontrolled type 2 DM, controlled type 2 DM, and non DM. The expression of calprotectin mRNA (MRP8 and MRP14) were detected by RT-PCR.* ***Result:*** *The result showed that the value of mRNA calprotectin expression in DM patients were higher than non DM, and the highest expression was on the uncontrolled type 2 DM.* ***Conclusion:*** *The basal level of calprotectin mRNA MRP8/MRP14 expression increased in neutrophil of periodontitis patient with type 2 DM compared non diabetic subjects. It was suggested that high basal level of calprotectin mRNA has a role in the regulation of periodontitis severity with diabetes mellitus patients.*

Key words*: calprotectin mRNA, periodontitis, type2 diabetes mellitus*

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INTRODUCTION

Calprotectin is a calcium binding protein which has a molecular mass of 36.5 kDa, belongs to the S-100 protein family which can be detected in neutrophils, monocytes, and epithelial cells, being composed of two subunits macrophage migration inhibitory factor-related protetin 8 and 14 (MRP8 and MRP14).[1,2] It is known that calprotectin plays an important role in the innate immunity, and its level is markedly increased in plasma, feces, and synovial fluid from patients with infections and inflammatory diseases.[3] Calprotectin level in gingival crevicular fluid (GCF) of periodontitis patients was significantly higher than healthy subjects and it was detected in gingival tissue only from periodontitis patients.[4,5]

Diabetes mellitus (DM) is one of health problems found in the world and about 90 percent were type 2 non insulin dependent diabetes mellitus (NIDDM). In Indonesia, the incidence rate among those who are above 15 years old in Indonesia was 1.2–2.3% and tends to increase.[4] Among the late complications associated to the diabetes mellitus, periodontal disease has been highlighted, and it can be more severe and refractory to treatment than in healthy subjects.[5] The incidence of periodontitis increases, more frequent and severe in diabetic patients with more advanced systemic complications, and the increased susceptibility does not correlate with increased levels of dental plaque or calculus.[6]

The severity of periodontitis in diabetic patient was strongly thought caused by decreasing of leukocytes function such as neutrophils. Periodontitis, as one of very frequent complication of diabetes mellitus, was known as caused by immune response disturbaces such as; decrease of chemotactic, adherence, and phagocytosis of neutrophils.[7] It is known that calprotectin (MRP8 and MRP14), forms about 60% protein in neutrophil, plays a role for neutrophils

function. However, the exact role of calprotectin in periodontitis patient with diabetes mellitus is unclear. In previous research, it was found that the calprotectin level in serum of periodontitis patients with type 2 DM was higher than periodontitis patients non DM.[8] The aim of this study was to determine calprotectin expression in human neutrophils of periodontitis patients with type 2 diabetes mellitus.

MATERIALS AND METHODS

Peripheral venous blood from 10 periodontitis patients with type 2 diabetes mellitus (consisted of 5 uncontrolled DM, 5 controlled DM), and 5 subjects non DM were collected into heparinized tubes to avoid blood aglutination before next procedure. All patients who visited Dr. Sardjito Teaching Hospital Yogyakarta gave their informed consent to participate in this research. Neutrophiles were separated from heparinized blood by density gradient centrifugation using Histopaque®-1077 (Sigma-Aldrich), and this cells were collected in eppendorf tube as samples for RNA determination

Neutrophils from uncontrolled DM patients, controlled DM, and non DM were isolated from its RNA using Trizol® Reagent (Invitrogen) according to the manufacturer's protocol. Trizol Reagent 1 ml was added into pellet cell, suspensed with injection spuit and incubated in room temperature for 5 minutes. Chloroform 20 μl was added and mixed by hands, then centrifugated 12.000 g for 15 minutes in 4° C temperature. The aqueous phase was transfered by mixing with fresh tube and precipitated the RNA from the aqueous phase by mixing with 500 μl isoprophyl alcohol. RNA samples were incubated at room temperature for 10 minutes and then centrifuged at 12.000 × g for 10 minutes at 4° C. RNA pellet was washed with 1 ml ethanol 75% and mixed by vortex and centrifuged at 7.500 × g for 5 minutes at 4° C. Then RNA pellet was dried with vacuum dry for 3 minutes and redisolved in 30 μl RNAse free water.

Calprotectin mRNA (MRP8 and MRP14) expression was determined by reverse transcriptase polymerase chain reaction (RT-PCR) according to manufacturer's procedure. We used two steps RT-PCR procedure, the first step was cDNA synthesis from RNA samples and continued with PCR procedure as the second step. Master mix for cDNA syntesis were ; 10 x Reaction Buffer, 25mM $MgCl_2$, Deoxy Nucleotid Mix, Primer pd $(T)_6$, RNAse Inhibitor, and AMV RT. To determined mRNA calprotectin, cDNA samples and PCR primers for calprotectin (Table 1) were amplified by polymerase chain reaction (PCR). PCR products then were checked by electrophoresis to measured band intesity of the MRP8 and MRP14 Calprotectin mRNA. The intensity of each band was normal if compared to the GAPDH band. The expression of calprotectin (MRP18 and MRP14) mRNA was represented as the intensity of bands that were checked by thin layer chromatography.

Table 1. PCR Primers

Oligonucleotide	Sequence	Product
MRP8 sense	5'-GCTGGAGAAAGCCTTGAACTC-3'	232 bp
MRP8 antisense	5'-CCACGCCCATCTTTATCACCA-3'	
MRP14 sense	5'-TCGCAGCTGGAACGCAACATA-3'	213 bp
MRP14 antisense	5'-AGCTCAGCTGC TTGTCTGCAT-3'	
GADPH sense	5'-TCCACACC CTGTTGCTGTA-3'	558 bp
GAPDH antisense	5'-ACCACAGTCCATGCCATCAC-3'	

RESULT

Polymerase chain reaction (PCR) product of calprotectin mRNA (MRP8 and MRP14) and also GAPDH were checked by electrophoresis to know each band position. In this study, band position of all oligonucleotides were suitable with base pairs (bp) values of MRP8, MRP14, and GAPDH as mentioned by the manufacturer's protocol. Compared with the 100 bp DNA ladder, these bands were in the correct position as described on Figure 1.

To investigate whether Diabetes Mellitus affects the MRP8 and MRP14 expression in human neutrophils, the expression of MRP8/14 mRNA was examined. When neutrophils were isolated from diabetic and non diabetic subjects, the expression of MRP8 mRNA significantly higher than MRP14 mRNA. The intensity of bands markedly increased in uncontrolled DM patient (Figure 2).

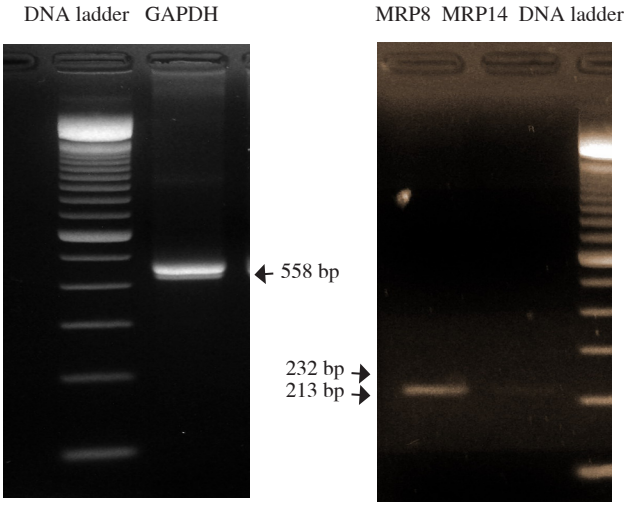

Figure 1. Band position of MRP8, MRP14, GAPDH primers comparing with 100bp DNA ladder. All primers were in the correct positions where the values of base pairs (bp) were 558 bp for GAPDH, 213 bp for MRP14, and 232 bp for MRP8.

Both MRP8 and MPR14 mRNA expression in periodontitis patients with type 2 DM were higher than non DM patients, while the highest expression of mRNA MRP8/MRP14 was in uncontrolled DM (Figure 3); suggesting that MRP8/MRP14 mRNA has important role on severity of periodontitis in diabetic patient.

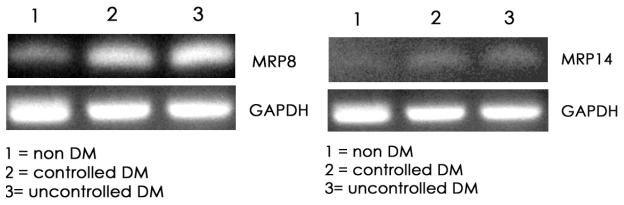

Figure 2. Calprotectin MRP8/MRP14 mRNA expression of neutrophils from diabetic and non diabetic subjects. The expression of mRNA was determined in 1ug RNA from resting neutrophils by RT-PCR using MRP8, MRP14, and GAPDH primers.

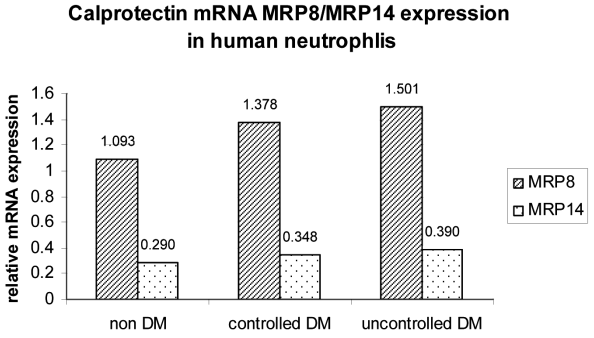

Figure 3. Expression of MRP8 and MRP14 mRNA in neutrophils of uncontrolled type 2 DM, controlled DM, and non DM subjects.

DISCUSSION

This study identified that calprotectin mRNA (MRP8/MRP14) expression in neutrophils of periodontitis with type 2 DM was different from periodontitis patients without DM, and the highest calprotectin expression was on uncontrolled type 2 DM patients compared with controlled DM and non DM (Figure 3). The difference expression of calprotectin from neutrophils on diabetic and non diabetic patients were strongly suspected to be correlated with impairment of immune cell function, especially innate immunity cells such as neutrophils and monocytes. Some authors mentioned that this impairment of function including chemotaxis, diapedesis, and phagocytosis of neutrophils,[9] but which one of those functions that was very dominant to cause the severity of diabetic periodontitis is unclear. Our result showed that calprotectin MRP8/14 mRNA expression in periodontitis patients with diabetes mellitus was different from non diabetic subjects, while calprotectin has been well known as a chemotactic factor.[10,11] The previous study demonstrated that diabetic patients with severe periodontitis had depressed PMN (neutrophils) chemotaxis compared to those with periodontitis on non diabetic subjects with severe or mild periodontitis.[6]

The result also identified that the highest calprotectin MRP8/14 mRNA expression were in periodontitis patients with uncontrolled type 2 diabetes mellitus. It maybe caused by pro inflammatory cytokines that markedly increased in blood of uncontrolled diabetes mellitus patient. Pro inflammatory cytokines such as TNF-alfa and IL1-beta were present in large amount in blood circulating diabetic patients, and it was reported that the level was higher in uncontrolled DM than controlled DM.[12,13] These cytokines, both TNF-alpha and IL-1beta, are found in the circulating peripheral blood and their level were increased in several inflammatory diseases, including periodontitis. In previous study, it was reported that expression of MRP8/MRP14 was increased in monocyte by several factors and compounds including TNF-alpha, and it was also known that this cytokine caned stimulate calprotectin expression in human neutrophils.[14,15]

MRP8/MRP14 is found predominantly in a cytosolic location in both neutrophils and monocytes, it represents about 45–60% of the total neutrophils cytosolic protein.[1,16] Large amounts of MRP8/MRP14 is necessary in cell such as the neutrophil which must make quick responses to environmental signals.[10] After activations of neutrophils, MRP8 and MRP14 are released into the compartment extracellular via tubulin dependent pathway, where they are known to promote the adhesion of neutrophlis on endothelium.[16] Previously, high basal level concentration of calprotectin intracellular in monocyte and neutrophils that was determined by ELISA kit was identified (unpublished data). Increasing basal level of calprotectin in serum of periodontitis patients with type 2 diabetes mellitus was also found.[8] In this study, the same pattern of increasing calprotectin mRNA expression in uncontrolled type 2 diabetic patients was found. It can be understood, because uncontrolled diabetic patients have persistently high concentration of TNF-alpha in their blood circulation, whereas TNF-alpha potentially stimulate neutrophils to increase MRP8/MRP14 mRNA expression and calprotectin production. In conclusion, the calprotectin mRNA MRP8/MRP14 expression was increased in neutrophil of periodontitis patient with type 2 DM compared to non diabetic subjects. It was suggested that high expression of calprotectin mRNA has a role in the regulation of periodontitis with severity in diabetes mellitus.

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