CD13/aminopeptidase N mRNA expression and enzyme activity in Systemic Lupus Erythematosus

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ABSTRACT

Aims: To determine the significance of CD13/aminopeptidase N (APN) in systemic Lupus Erythromatus (SLE), we examined its catalytic activity and mRNA expression level in sera and peripheral whole blood cells of patients with SLE.

Methods: In this study, 47 SLE patients and 44 age, sex matched healthy controls were included. The SLE disease activity index score and clinical finding including renal involvement and blood pressure were recorded. Catalytic activities of CD13/APN were measured in serum samples. In addition, CD13 mRNA level in peripheral whole blood cells was analyzed by quantitative real-time polymerase chain reaction (PCR).

Results: A significant higher aminopeptidase activity was observed in serum from patients with SLE than serum from controls. In addition, CD13/APN mRNA expression was 6.12 times higher in SLE patients than in healthy controls. However, CD13/APN mRNA level, or its activity in serum, did not correlate with the score determined according to SLE disease activity index. Additionally, there was not any significant correlation between the complication in organs, including, kidney, and CD13/APN gene expression level or CD13/APN enzyme activity.

Conclusion: CD13/APN enzyme activity and mRNA expression level were higher in SLE patients regardless of their disease activity. More studies are needed to better clarify the role of CD13/APN in the pathogenesis of SLE.

Keyword: Aminopeptidases; Lupus; Nephritis.

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INTRODUCTION

Systemic Lupus Erythromatus (SLE) is a complicated autoimmune disease that affects many organs[1]. The main pathological feature of SLE is aberrant immune responses to auto-antigens, which result in autoantibody production and subsequent tissue injury, due to inflammation. Many inflammatory cells and molecules are known which are involved in the immunopathology of SLE.

CD13/aminopeptidase N is a membrane bound ectoenzyme that widely expressed by variety of mammalian cells, such as monocytes/macrophages, activated T- lymphocyte fibroblasts, neutrophils, endothelial cells, and epithelial cells[2–5]. Although, CD13/APN is a membrane-bound protein, human plasma contains significant amounts of an active soluble form of CD13 (sCD13) [6]. This suggests that certain cells may secrete sCD13, or that sCD13 is released from the plasma membrane by shedding or by specific cleavage[7]. The physiological role of APN is removing the amino acid from the N-terminal of various biologically active peptides, such as enkephalins, angiotensin, neurokinins, and neuropeptids[8,9].

As a membranous protein, CD13/APN has many costimulatory functions, and thus participates in signal transduction and inflammatory or immunological responses[10,11]. It is observed that crosslinking of CD13/APN on monocytes, by monoclonal antibody induces cytokine secretion[12].

Additionally, synthetic inhibitors for APN enzymatic activity induce the production of immunosuppressive cytokine including TGF-beta1, and subsequently, suppress DNA synthesis, and Th1 cytokine production in activated human T cells[13]. Therefore, CD13/APN can be considered as a potential target for the treatment of inflammatory disorders. Conversely, CD13/APN has many anti-inflammatory properties. For example, this enzyme has extensive substrate specificity, enabling it

to digest several important proteins in plasma, including chemokines and pro-inflammatory cytokines[14].

Unexpectedly, an abnormal concentration of this molecule is observed in various pathological disorders, including cancers and autoimmune diseases. Abnormal concentration and activity of APN is associated with diseases such as rheumatoid arthritis, collagen vascular diseases, chronic pain, nephrotoxicity, and renal failure[15–18].

Although, the roles of CD13/APN have been studied in many autoimmune and inflammatory diseases, a few studies have focused on the role of CD13/APN in SLE. Dan H *et al.* showed that the mean activity of CD13/APN in sera, from patients with collagen vascular diseases (CVD) including SLE, were significantly higher compared with that in controls[17]. Nonetheless, correlation between sCD13/APN enzymatic activity and SLE disease activity and organ failure in SLE patients is not evaluated. Additionally, CD13/APN mRNA expression in peripheral whole blood cells from SLE patients has not been determined either. Therefore, the aim of the present study was to investigate the value of the serum APN activity and gene expression in peripheral whole blood cells in patients with SLE.

PATIENTS, MATERIALS, AND METHODS

STUDY GROUPS

In the present study, 47 SLE patients, who had referred to Loghman Hakim educational hospital, were randomly selected. All the patients fulfilled the 1982 revised criteria for classification of SLE[18]. The SLEDAI score was calculated for each patient, according to the clinical and para-clinical findings based on the American Rheumatology Society criteria[19]. Patients were divided into two subgroups: nephritis patients (n=17) and non-nephritis patients (n=30). Additionally, a second subgroup was formed, consisting of patients with either active SLE (n=24) or inactive SLE (n=23), according to SLEDAI score. The entire patients received the dosage of 200-400 mg hydroxychloroquine per day. A total of 44 age- and sex-matched subjects were included in the healthy control group after completing the questionnaire. Exclusion criteria for the control group were: autoimmune disease, cancer, BMI above 30, smoking, and recent infection. The institutional ethics board of Shahid Beheshti University of Medical Sciences, Tehran, approved the present experiment. All the subjects were fully informed and participated voluntarily in the study after signing the consent form.

CD13/APN ENZYME ACTIVITY

The CD13/APN activity was measured using a procedure modified by Ryan *et al.*[17]. Briefly, a total of 90 µl of substrate mixture [1 mM L-Leucine p-Nitroanilide (Sigma) in methanol] and 10 µL of serum were used. The release of p-nitroaniline from L-Leucine p-Nitroanilide was kinetically measured at 405 nm over 60 minutes using a Microplate Reader (Anthos 2020 UK – Biochrom). The millimolar extinction coefficient of p-Nitroaniline at A405nm is 10.8, so enzyme activity of APN was calculated based on the following formula:

$$\text{Unit/ml} = \frac{\Delta A405nm/\text{min } test - \Delta A405nm/\text{min } Blank)}{(dilution\ factor)} \Big/ (10.8)(0.1)$$

CD13/APN MRNA EXPRESSION ANALYSIS

A total of 2 ml venous peripheral blood was taken from each subject. The entire RNA was extracted using QIAamp RNA Blood Mini Kit (Qiagen, Germany). RNAs were converted into complementary DNA (cDNA) using RevertAid First Strand cDNA Synthesis Kit (Thermo, Lithuania). The complete process was carried out according to the manufacturer's instruction in RNase free environment.

CD13/APN mRNA expression was evaluated using quantitative Taqman probe RT-PCR. Exon-exon spanning primers and Taqman probes for CD13/APN and GAPDH genes, as a normalizer, were designed using primer design software (Beacon Designer 7, USA) (Table I). Each well contained reagent for the PCR reaction, as follows: 12.5µL of DFS master mix (Bioron, Germany), 1µL of forward and 1 µL of reverse primer (10 pMol), 0.5 µL of 20 pMol Taqman probe, 3.5 µl DEPC water, and 1.5 µL of single strand cDNA (12.5 ng/µL) in 20 µL final volume. PCR reaction was carried out on a Rotor-gene Q real-time PCR (QIAgen, Germany) using the following process: initially, 2 minutes at 94º C, followed by 40 cycles, which comprised of denaturation for 15 seconds at 95º C and annealing for 60 seconds at 58º C. All the samples were analyzed in duplicates. The relative gene expression was calculated using REST mathematical model, which includes the efficiency (E) of every PCR reaction, as follows:

$\text{Ratio} = (E_{target})^{\Delta Ct\ target\ (control-sample)} / (E_{ref})^{\Delta Ct\ ref\ (control-\ sample)}$[19]

For each gene, cDNA dilution curves were generated and used to calculate the real-time PCR efficiencies [$E=10^{(-1/slope\ of\ dilution\ curve)}$.

STATISTICAL ANALYSIS

Due to the small sample size and deviation of the data from the normal distribution, non-parametric statistical tests (Mann-Whitney rank sum test and Spearman's correlation test) were run for data analysis. Statistical analyses were performed using SPSS (version 21). P-values less than 0.05 were considered as significant.

RESULTS

ENZYME ACTIVITY OF SCD13 IN SERUM

In SLE patients, the enzyme activity of sCD13 was found to be significantly higher than that in healthy subjects (SLE: 3.086 unit/ml vs. control: 2.388 unit/ml]; p=0.028, Figure 1A). Nevertheless, no significant difference was observed in CD13/APN enzyme activity between patients with active and inactive SLE (p> 0.05, Figure 1B). Additionally, lupus nephritis and non-nephritis patients were not significantly different in sCD13 activity level (nephritis: 2.994 unit/ml vs. non-nephritis: 3.136 unit/ml; p>0.05, Figure 1C).

CD13/APN MRNA EXPRESSION

The statistical analyses revealed that CD13/APN mRNA expression was 6.12 times higher in SLE patients than in healthy controls (p≤ 0.001, Figure 2A). Despite this difference in the expression between patients and control groups, subsequent analyses revealed that there was no significant difference in the CD13/APN mRNA level between active or non-active SLE patients (p> 0.05, Figure 2B). Additionally, no significant correlation was observed between CD13/APN mRNA expression and SLEDAI, either (p> 0.05). Meanwhile, CD13/APN mRNA expression in lupus nephritis patients was 1.35 times higher than that in non-nephri-

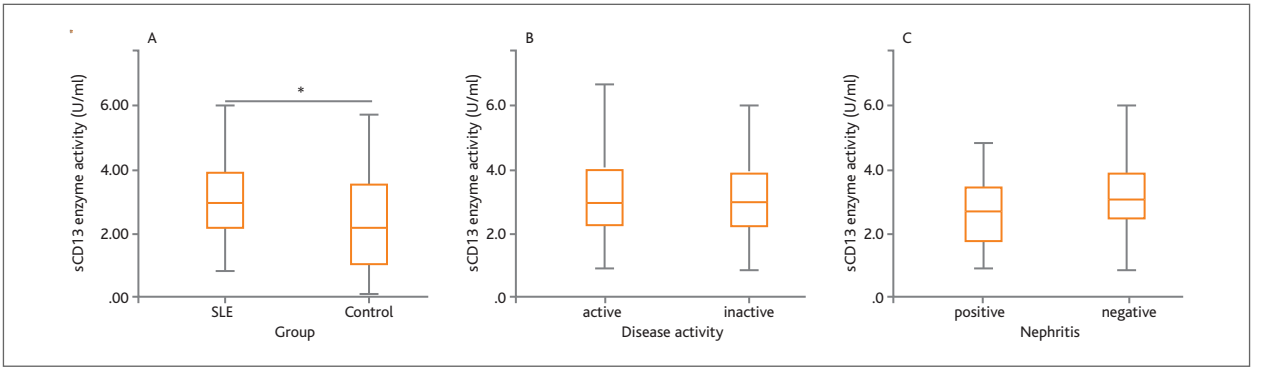

FIGURE 1. Enzyme activity of sCD13 was quantified in serum using spectrophotometry. A. sCD13 activity was significantly higher in SLE patients than in healthy control participants (p<0.05). B. The catalytic activity level of sCD13 in the serum of patients with active SLE was not significantly higher than that in non-active SLE patients. C. sCD26 level was not significantly different between nephritis and non-nephritis patients (p>0.05). p=0.05 is shown by*.

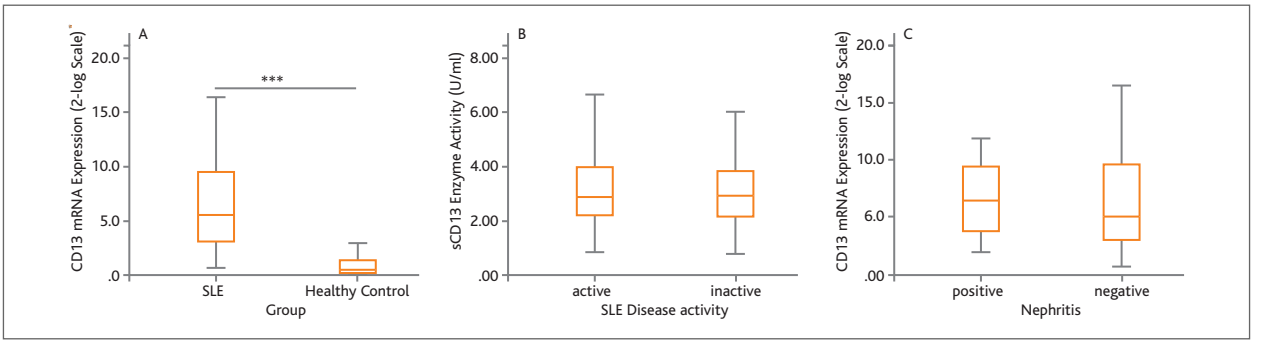

FIGURE 2. CD13/APN mRNA expression was evaluated using quantitative RT-PCR. A. The mRNA level of CD13/APN was significantly higher in SLE patients as compared to that in healthy controls (p<0.001). CD13/APN mRNA level was not statistically different between patients with active and inactive SLE. C. There was no statistically significant difference between the two subgroups of nephritis patients and non-nephritis patients (p>0.05). p=0.001 is shown by***.

tis patients, however the difference was not statistically significant (p>0.05, Figure 2C).

CORRELATION BETWEEN ENZYME ACTIVITY OF SCD13 AND CD13 MRNA EXPRESSION

Statistical analysis revealed that there is not any significant correlation between CD13/APN mRNA expression and sCD13 enzyme activities (p>0.05). There was no significant correlation between neither blood pressure and serum APN activity nor mRNA expression in SLE patients. Additionally, there was not any significant correlation between the complication in other organs, such as, skin rash, vasculitis, pericarditis, seizure, and arthritis and CD13/APN gene expression level (p>0.05). Moreover, the results did not reveal any significant correlation between gene expression and the prescribed prednisolone dose (p>0.05).

DISCUSSION

The results of the current study showed that CD13/APN activity in sera as well as mRNA expression in peripheral whole blood cells obtained from patients with SLE was significantly higher than those in normal subjects. The results reported in the previous studies, as well, suggested that serum CD13/APN activity was significantly lower in normal groups than in patients with CVD[17]. Membranous and soluble forms of CD13/APN play many roles in inflammation. An increase in the CD13/APN serum concentration enhances cell migration to the site of inflammation. Soluble CD13/APN is a chemoattractant for activated T lymphocytes[20,21]. Additionally, CD13/APN is expressed on monocytes and endothelial cells, and it is demonstrated that membranous form of CD13/APN has also a role in the migration of leukocytes to the inflammatory site[8,22]. Among the various organs, kidney is the main target for the migration of leukocytes during many autoimmune diseases, such as SLE. It seems that local increase in the production of CD13/APN is correlated with the migration of inflammatory cells to the kidney. The previous studies have reported that in the initial phase of clinical acute renal failure, CD13/APN's urinary activity is considerably raised[23]. Also, increased aminopeptidase activity in urine obtained from patients with diabetic nephropathy was reported[24]. In the current study, although serum CD13/APN activity and relative mRNA expression were higher in patients with nephritis than in non-nephritis lupus patients, the difference was not significant. The lack of significant difference may be due to the fact that we measured enzyme activity in the serum rather than in renal tissue or urine sample. Previously, Dan H *et al.* showed that the activity of CD13/APN, locally produced in the disease site, is a useful marker for CVD[17].

The result from the current study revealed that the mRNA expression level of APN/CD13 was 6.12 times higher in peripheral whole blood cells obtained from SLE patients than that obtained from healthy controls (p<0.001). However, CD13/APN mRNA level, or its activity in serum, did not correlate with SLEDAI. In other word, CD13/APN activity and mRNA expression were higher in SLE patients regardless of their disease activity. To explain this seemingly contradictory finding, other activities of this molecule should be considered, as well. CD13/APN cleaves several pro-inflammatory cytokines and chemokines, including CXCL11, CXCR4, and stromal cell-derived factor-1, and thus decreases inflammation[14,25]. In addition, high concentrations of circulating sCD13 raise the possibility that the soluble form acts as an endogenous modulator of CD13-mediated adhesion. Moreover, new studies put further emphasis on the inhibitory role of CD13/APN. CD13/APN negatively regulates Tool-like reseptor 4 signaling[26]. In addition, CD13/APN negatively modulates receptor-mediated antigen uptake in dendritic cells to control T-cell activation in adaptive immunity[27]. Recently, Zotz JS *et al.* showed that CD13/APN is a novel negative regulator of mast cell activation in vitro and in vivo[28]. Thus, the contrasting functions of CD13/APN suggest that increased CD13/APN activity or mRNA level in lupus patients could be considered as an effort by the immune system to modulate autoimmune responses and inflammation. Chemotactic activity and contribution of CD13/APN to the migration of cells may also be in agreement with its anti-inflammatory function. In agreement with this hypothesis, Rahman *et al.* reported that CD13/APN regulates migration of mesenchymal stem cells to inflamed tissues. They also showed that tissue repair was impaired in CD13 knockout animals[29].

Beside nephropathy, the prevalence of hypertension in patients with SLE is markedly high. There are several lines of evidence to suggest that renal CD13/APN regulates tubule salt handling, influences blood pressure, and plays a pathogenic role in hypertension[30]. Yet, the findings of the present study did not show any significant correlation between blood pressure and neither serum APN activity nor mRNA expression in SLE patients.

The mechanism of elevated soluble CD13 in SLE remains unclear. Up regulation of CD13/APN mRNA expression in peripheral whole blood cells indicate that secretion/shedding of CD13/APN from peripheral whole blood cells may be responsible for the aminopeptidase activity detected in the serum obtained from SLE patients. However, membrane bound CD13/APN is extensively expressed on multiple tissue types, including those affected by SLE. CD13/APN is also reported to be expressed in fibroblasts, neutrophils, and endothelial cells[2–5]. Further studies are necessary to determine the cells contributing to aminopeptidase activity in serum.

CONCLUSION

CD13/APN gene expression and enzymatic activity in blood obtained from SLE patients was found to be higher compared with those in the control group's blood. Yet, the high expression and enzymatic activity of APN was not correlated with disease activity. A greater understanding of mechanisms that regulate the production and action of this multifunctional enzyme may lead to new insights to control and treat SLE disease.

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