Elevated Plasma Osteopontin Levels Were Associated With Osteopontin Expression of CD4+ T Cells in Patients With Unstable Angina

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Background  Plaque instability in patients with unstable angina (UA) is associated with stimulated CD4+ T cells, so the present study investigated whether there is a relationship among plaque instability, osteopontin and CD4+ T cells.

Methods and Results  Peripheral blood mononuclear cells were collected from 51 consecutive patients with UA, 60 patients with stable angina (SA), and 39 patients with chest pain syndrome (CPS). Osteopontin-producing CD4+ T cells were quantified by flow cytometry. Plasma osteopontin levels (ng/ml) were measured by ELISA and were higher in patients with UA (792.0±316.7) than in those with SA (626.0±195.0, p<0.005) or CPS (594.7±239.4, p<0.005). The frequency (%) of osteopontin-producing CD4+ T cells was higher in patients with UA (26.7±13.3) than in those with SA (19.5±11.1, p<0.05) or CPS (16.6±9.0, p<0.005). Furthermore, the plasma osteopontin level correlated with the frequency of osteopontin-producing CD4+ T cells (r=0.327, p=0.0004), as did the high-sensitivity C-reactive protein level (r=0.360, p=0.0002).

Conclusions  The plasma osteopontin levels are elevated in patients with UA, accompanied by an increase in the number of osteopontin-production of circulating CD4+ T cells. Circulating CD4+ T cells may play a role through osteopontin in the pathophysiology of UA. (Circ J 2006; 70: 851–856)

Key Words:  Osteopontin; T cell; Unstable angina

Osteopontin is abundantly produced in the early stage of T-cell and macrophage activation and is expressed by both cell types.1–4 Functional studies have shown that osteopontin supports T-cell and macrophage chemotaxis and can costimulate T-cell proliferation.5–7 Recently, it has been demonstrated that osteopontin is associated with both atherosclerosis8 and coronary artery disease9. Activated inflammatory cells, mainly T cells and macrophages, are known to be associated with plaque rupture in atherosclerotic plaques.10–12 Constitutive stimulation of T cells and macrophages in unstable angina (UA) is not limited to the vascular lesion but also involves peripheral immune cells.13–15 Because it is unclear if there is a relationship between plaque instability and plasma osteopontin levels, we examined whether plasma osteopontin levels were higher in patients with UA and whether osteopontin-production of circulating CD4+ T cells affected plasma osteopontin levels.

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Study Population

Some reports show an association between osteopontin and calcification16–18 so we excluded patients with coronary calcification detected by coronary arteriography. We studied 150 patients who underwent diagnostic catheterization (106 men, 44 women; mean age ±SD. 67±11 years) between October 2003 and February 2005. Of them 51 consecutive patients (39 men, 12 women; mean age 66±11 years) had UA, which was defined as chest pain at rest with documented transient ST-segment depression or elevation of 0.1 mV in at least 2 continuous electrocardiographic (ECG) leads. The last spontaneous attack was required to have occurred within a period of 24 h before entry into the study. Patients with new Q-wave development or with an increase in the creatine kinase level of more than twice the normal upper limit were excluded. We confirmed by coronary arteriography that all patients with UA had significant coronary artery stenosis but no coronary spasm. The remaining patients were 60 with stable angina (SA) (43 men, 17 women; mean age 69±10 years) who had typical exertional chest discomfort associated with horizontal or down-sloping ST-segment depression >1.0 mm on exercise test, ≥90% narrowing of the major coronary arteries, and no acetylcholine-induced coronary spasm. Moreover, these patients had not experienced any acute events, worsening of symptoms during the previous 6 months, or any anginal episodes within the week preceding enrolment. There were 39 patients with chest pain syndrome (CPS)
(24 men, 15 women; mean age 66±13 years) whose chest pain was not accompanied by ECG changes, coronary organic stenosis, or coronary spasm when an intracoronary injection of acetylcholine was given during coronary arteriography.

None of the patients was treated with steroids and none had collagen disease, advanced liver disease, renal failure, malignant disease, septicemia, or other inflammatory disease. Written informed consent was given by each patient. The study conformed to the guidelines approved by the institutional ethics committee.

Blood Samples
Blood samples from an antecubital vein were obtained with a 21-gauge needle from all patients while recumbent. The first 3 ml of blood was used for biochemical assessment and a subsequent 3-ml sample was collected into 2 evacuated tubes containing 0.3 ml of EDTA for flow cytometric analysis (FCA) of osteopontin production and plasma osteopontin levels.

FCA of Cytokine Production (Osteopontin)
One of the 1.5 ml blood samples containing EDTA was immediately mixed with 1.5 ml of medium [10% fetal calf serum-supplemented RPMI1640 with 40 mg/ml Brefeldin A (Sigma, St Louis, MO, USA)] and then incubated for 24 h at 37°C and 5% CO₂. After washing with ice-cold phosphate-buffered saline, cells were recovered by centrifugation and adjusted to 5×10⁵ white blood cells per test. The fixation and permeabilization of cells were both performed using IntraPrep™ reagent (Beckman Coulter, High Wycombe, UK). The cells were successively stained with anti-human osteopontin monoclonal antibody (Immuno-Biological Laboratories, Fujioka, Japan), phycoerythrin-labeled anti-mouse IgG antibody (BD Pharmingen, San Diego, CA, USA), and fluorescein isothiocyanate labeled anti-human CD4 antibody (BD Pharmingen). The FCA was performed to evaluate cytokine production using a FACScan™ instrument (Becton Dickinson, San Jose, CA, USA). Nonspecific staining with the isotype-matched control monoclonal antibody was <1%.

Plasma Osteopontin Measurement
The other 1.5 ml blood sample containing EDTA was used for analyzing plasma osteopontin levels by ELISA (Immuno-Biological Laboratories). Plasma samples were immediately stored at –80°C until analysis. The recently
developed ELISA kit was based on the method reported by Kon et al.\textsuperscript{19} and it measures the total concentration of phosphorylated and nonphosphorylated forms of osteopontin in plasma. The intra- and inter-assay coefficients of variation for the kit are 6.8% and 13.3%, respectively. The normal (mean±SD) value for plasma osteopontin level measured in our laboratory (n=20) is 556±173 ng/ml.

**Statistical Analysis**

All data are given as mean±SD. The comparisons of continuous data among the 3 patient groups were performed with 1-way ANOVA followed by Scheffé’s test. Comparisons of osteopontin data among the 3 patient groups were performed using the Kruskal-Wallis test followed by the Dunnett procedure. Frequency data among the 3 patient groups were compared using the $\chi^2$ test. Linear regression analysis was used to determine the correlation between the plasma osteopontin levels and the frequency of osteopontin-producing CD4$^+$ T cells. P-values <0.05 were considered to be statistically significant.

**Results**

**Characteristics of the Patient Groups**

The clinical characteristics of the UA, SA, and CPS groups are shown in Table 1. They were matched for age, gender, frequency of coronary risk factors, and lipid levels.

**Assessment of Plasma Osteopontin Levels**

The levels (ng/ml) were 792.0±316.7, 626.0±195.0 and
594.7±239.4 in the UA, SA and CPS groups, respectively. The plasma level of osteopontin was statistically significantly higher in the UA group than in the SA and CPS groups at p<0.005 (Fig 1). Differences in frequency between the SA and CPS groups were not significant.

Assessment of the Frequency of Osteopontin-Producing CD4+ T Cells

There was no difference in the absolute lymphocyte numbers among the 3 patient groups. First, we performed a preliminary measurement of only the plasma osteopontin levels in 11 patients with UA, 16 with SA and 9 with CPS, so there are no data for the osteopontin-producing CD4+ T cells in these 36 patients. The frequency of peripheral CD4+ T cells staining for osteopontin was 26.7±13.3%, 19.5±11.1% and 16.6±9.0% in the UA, SA and CPS groups, respectively. Representative dot plots of the FACScans are shown in Fig 2. The frequency of osteopontin-producing CD4+ T cells was found to be statistically significantly higher in the UA group than in the SA and CPS groups at p<0.05 and p<0.005 (Fig 3). Differences in the frequencies between the SA and CPS groups were not significant.

Assessment of the Relationship Between Plasma Osteopontin Level and the Frequency of Osteopontin-Producing CD4+ T Cells

The plasma osteopontin levels were significantly and positively correlated with the frequency of osteopontin-producing CD4+ T cells (r=0.327, p<0.0004, Fig 4).

Assessment of High-Sensitivity C-Reactive Protein (CRP) Level

The plasma high-sensitivity CRP level (mg/L) was 6.35±9.27, 2.37±4.66 and 2.26±3.75 in the UA, SA and CPS groups, respectively. The level was statistically significantly higher in the UA group than in the SA and CPS groups at p<0.05 (Fig 5). Differences in the frequencies between the SA and CPS groups were not significant. The high-sensitivity CRP level correlated with the frequency of osteopontin-producing CD4+ T cells (r=0.360, p<0.0002, Fig 6).

Discussion

In the present study, we found that the plasma osteopontin level was elevated in patients with UA and that it was associated with the production of osteopontin of circulating CD4+ T cells. Osteopontin is associated with T-cell and macrophage activation and is expressed by both cell types. Monocyte chemoattractant protein-1 is a potent specific chemoattractant for monocytes and is reported to be elevated in patients with coronary artery disease and UA.16,17 Recently, high levels of osteopontin mRNA and protein were reported in human atherosclerotic plaque from the aorta, carotid and coronary arteries.18-20 Osteopontin facilitates the migration of endothelial cells and inhibits nitric oxide synthase expression in endothelial cells.21 An in vitro study has shown that osteopontin also promotes the proliferation of smooth muscle cells.22 Some groups have demonstrated that osteopontin transgenic mice develop significantly larger atherosclerotic lesions than nontransgenic mice;23,24 In contrast, osteopontin-deficient mice develop smaller atherosclerotic lesions than non-transgenic mice.25 These lines of evidence suggest that osteopontin plays an important role in the development of atherosclerotic plaques.

Recently, it was reported that the infiltration of CD45+ leukocytes into neointimal lesions was reduced in atherosclerotic plaques of osteopontin-deficient mice.28 Coronary lesions that are infiltrated with immune cells, including macrophages, T lymphocytes and mast cells, are suspected to be unstable.29-31 In this regard, we reported that in directional coronary atherectomy specimens, the area of macrophage infiltration was larger in patients with UA than it was in those with SA, and that tissue factor expression on...
macrophages was also more frequently observed in patients with UA.\(^\text{32}\) Liuzzo et al have demonstrated that plaque instability in patients with UA is associated with monocytes activated by interferon-\(\gamma\) derived from stimulated CD4\(^+\) T cells.\(^\text{3,4,5}\) We also demonstrated that the frequency of interferon-\(\gamma\)-production of CD4\(^+\) T cells is higher in patients with UA than in patients with SA.\(^\text{33}\) In the present study, the frequency of osteopontin-production of CD4\(^+\) T cell was higher in patients with UA than in those with SA or CPS. It has been also reported that T-cell-mediated endothelial cell injury is a novel pathway of tissue damage that contributes to plaque destabilization.\(^\text{34}\) All these findings suggest that aberrations in the global immune system are of functional importance to plaque inflammation in patients with UA. In the present study, plasma osteopontin levels were associated with production of osteopontin of circulating CD4\(^+\) T cells and furthermore, plasma osteopontin levels were positively correlated with the frequency of osteopontin-production of circulating CD4\(^+\) T cells. The increase in the plasma osteopontin level may reflect CD4\(^+\) T cell activation in patients with UA.

Yasue et al reported that in patients who underwent coronary angiography on suspicion of coronary artery disease those who were smokers had elevated blood levels of inflammatory markers, such as the leukocyte count, CRP and thrombogenic markers, without apparent inflammation.\(^\text{35}\) CRP has recently been found in human atherosclerotic plaque, where the immunoreaction of CRP was localized proximal and distal to the unstable plaque,\(^\text{36}\) CRP has recently been found in human atherosclerotic plaque, where the immunoreaction of CRP was localized proximal and distal to the unstable plaque.\(^\text{37}\) In the present study, high-sensitivity CRP levels were increased in patients with UA and the level positively correlated with the frequency of osteopontin-production of circulating CD4\(^+\) T cells. These findings suggest that increased CRP affects the osteopontin production of circulating CD4\(^+\) T cells in patients with UA. Previous studies have demonstrated a reduction in CRP with a variety of statins\(^\text{38}\) and there is a possibility that osteopontin levels and the production of osteopontin of T cells were affected by statin therapy in the present study.

Further study is needed to elucidate the relationships among serum CRP level, plasma osteopontin level and the frequency of osteopontin-producing CD4\(^+\) T cells and to assess whether these variables reflect the status of coronary artery plaque. In addition, the osteopontin production of other cell types should be investigated in patients with UA.

In conclusion, we have demonstrated for the first time that an increase in the osteopontin production of circulating CD4\(^+\) T cells is associated with elevated plasma osteopontin levels in patients with UA. Osteopontin, as well as CRP, may play an important role in UA in its association with activation of CD4\(^+\) T cells.

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