Heart Vessels (2003) 18:183–187
DOI 10.1007/s00380-003-0719-7


ORIGINAL ARTICLE

**Minako Yamaoka-Tojo · Taiki Tojo · Takashi Masuda
Yoji Machida · Yoshikazu Kitano · Toshirou Kurosawa
Tohru Izumi**

# C-reactive protein-induced production of interleukin-18 in human endothelial cells: a mechanism of orchestrating cytokine cascade in acute coronary syndrome

Received: December 19, 2002 / Accepted: May 17, 2003

**Abstract** The circulating interleukin (IL)-18 level is a strong predictor of death from cardiovascular causes in patients with coronary artery disease. However, the mechanisms of IL-18 in orchestrating the cytokine cascade and the accelerator of IL-18 production in atherosclerosis are still unknown. In the present study, we measured the serum concentration of IL-18 and other markers of inflammation in 35 patients with acute coronary syndrome. To determine the mechanism of accelerating IL-18 production, we examined the release of IL-18 in human endothelial cells using human recombinant (hr) C-reactive protein (CRP) as a stimulator of IL-18. Furthermore, we investigated the inhibitory effects of hr IL-10 on IL-18 production by hr CRP in human endothelial cells. Circulating levels of IL-18 were significantly higher in patients with acute myocardial infarction than in patients with unstable angina. Incubation with hr CRP, which was equivalent to the serum concentration in patients with acute coronary syndrome, induced IL-18 release. Treatment with hr IL-10 inhibited IL-18 release in the cells stimulated with hr CRP. The serum level of IL-18 was identified as a marker of severity in acute coronary syndrome. Our findings reveal the possibility that circulating CRP by itself could cause a deterioration of the inflammatory cascade in endothelial cells associated with the upregulation of IL-18. This suggests that CRP may contribute to the mechanism of coronary artery disease in addition to being an incidental product of various types of systemic inflammation.

**Key words** Interleukin-18 · C-reactive protein · Acute coronary syndrome

M. Yamaoka-Tojo (✉)[1] · T. Tojo · T. Masuda · Y. Machida · Y. Kitano · T. Kurosawa · T. Izumi
Department of Internal Medicine and Cardiology, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan

*Present address*:
[1] Division of Cardiology, Emory University School of Medicine, 1639 Pierce Drive, WMB Room 325, Atlanta, GA 30322, USA
Tel. +1-404-727-3126; Fax +1-404-727-3330
e-mail: myamaoka-circ@umin.ac.jp

## Introduction

Chronic inflammation causes atherosclerosis[1] and is also involved in atherosclerotic plaque disruption and thrombosis, and may greatly influence the occurrence of acute ischemic syndrome. Current research has mostly focused on C-reactive protein (CRP) as a marker for coronary heart disease and acute coronary syndrome, but CRP itself might also directly provide a proinflammatory stimulus.[2] Interleukin (IL)-18, originally termed interferon (IFN)-γ-inducing factor, is a newly discovered cytokine with pleiotropic activities extending from Th1 polarization of the immune response to a proinflammatory activity.[3,4] The multifunctional properties of IL-18 production in numerous diseases, such as infections, several types of cancer, and in-inflammatory and autoimmune diseases, reflect an inappropriate immune response.[5–7] A recent study showed significant expression of IL-18 in human carotid atherosclerotic plaques.[8] Increasing plasma levels of IL-18 in patients with acute coronary syndrome were reported to the associated with increased mortality.[9] Moreover, the serum IL-18 level was identified as a strong independent predictor of death from cardiovascular causes in patients with coronary artery disease.[10] However, the effects of IL-18 on the production of other cytokines in coronary artery disease are still unknown. We hypothesized that IL-18 could upregulate excessive expression of inflammatory properties, and might play an important role in atherosclerosis development and stability in patients with acute coronary syndrome. In the present study, we evaluated the serum concentration of IL-18 in patients with acute coronary syndrome. Furthermore, we tested the hypothesis that CRP can be a trigger of IL-18 expression in human endothelial cells and that this mechanism is inhibited by IL-10 in vivo. Thus, CRP might provide a trigger for elevated IL-18 levels in acute coronary syndrome.

## Patients and methods

### Study population

The study population consisted of 35 patients [30 men and 5 women; age range 40–87 (mean 66.2) years] with acute coronary syndrome (ST-segment elevation, non-ST-segment elevation myocardial infarction, or unstable angina). They underwent coronary angiography for clinical indications at the Cardiovascular Center of the Kitasato University Hospital, with at least one affected vessel with >75% stenosis in a major coronary artery. The exclusion criteria of this study were evidence of hemodynamically significant valvular heart disease, surgery or trauma within the prior month, known cardiomyopathy, known malignant disease, febrile conditions, or renal failure. Patients were eligible if they had either or both of the following sets of findings: one or more episodes of angina while at rest that lasted at least 5 min and new ST-segment changes; or an abnormal result on a quantitative cardiac troponin-T level above 0.01 ng/ml. Informed consent was obtained from all patients before blood sampling for this study.

### Blood processing

Peripheral venous blood was taken and immediately centrifuged for 15 min at 4°C to measure plasma brain natriuretic peptide (BNP), serum IL-18, IL-10, and high-sensitivity (hs)-CRP. All samples were stored at −80°C before analysis.

### Measurement of BNP and hs-CRP

Circulating levels of plasma BNP were measured by radioimmunoassay (Shionoria BNP kit, Osaka, Japan; sensitivity, 18.4 pg/ml). The concentration of hs-CRP was determined from serum samples in patients with CHF (LPIA-CRP, Iatron Laboratories, Tokyo, Japan; sensitivity, 20 μg/dl).

### Materials

Human umbilical vein endothelial cells (HUVECs) from Clonetics (Walkersville, MD, USA) were grown in endothelial basal medium (EBM-2; Clonetics) supplemented with hydrocortisone, human fibroblast growth factor-B, human recombinant vascular endothelial growth factor, long R insulin-like growth factor-1, ascorbic acid, heparin, fetal bovine serum, human recombinant epidermal growth factor, gentamicin, and amphotericin-B in a humidified incubator containing 5% $CO_2$ in air at 37°C. Cells were used at passages 3–4. Human recombinant (hr) C-reactive protein (CRP) was purchased from Wako Pure Chemical Industries (Osaka, Japan). The confluent monolayers of HUVECs were stimulated with several doses of hr CRP in fresh medium. For inhibitory experiments, the cells were pretreated with hr IL-10 (Strathmann Biotech, Hannover, Germany). After 1 h, the cells were incubated with hr CRP for 24 h.

### Immunoactivities of cytokines

The levels of IL-18 in the culture supernatant or serum levels of IL-18 were measured using a commercially available immunoassay kit (MBL, Nagoya, Japan) according to the manufacturer's instructions. The assay uses two monoclonal antibodies against two different epitopes of human IL-18. Briefly, diluted serum samples were incubated on microtiter plate wells precoated with antihuman IL-18 monoclonal antibody (125-2H) for 1 h at room temperature. After washing, the peroxidase-conjugated antihuman monoclonal antibody (159-12B) was added to the microwell and incubated for 1 h at room temperature. After another washing, the peroxidase substrate was mixed with chromogen and allowed to incubate for 30 min at room temperature. An acid solution was then added to each well to terminate the enzyme reaction and to stabilize the developed color. The optical density (OD) of each well was then measured at 450 nm using a microplate reader. In each case duplicate readings were converted into pg/ml using the standard curves generated from each plate. The mean (±SD) serum concentration of IL-18 from 46 healthy blood donors measured by the assay was 126.0 ± 44.5 pg/ml. The sensitivity of the assay was 12.5 pg/ml. Serum levels of IL-10 were measured using a highly sensitive human IL-10 kit (Human IL-10 US ELISA; BioSource International, Camarillo, CA, USA). A monoclonal antibody specific for human IL-18 was coated onto the microtiter wells. During the first incubation, the IL-10 antigen bound to the immobilized antibody on one site. After washing, a biotinylated monoclonal antibody specific for IL-10 was added. During the second incubation, this antibody bound to the immobilized IL-10 captured during the first incubation. After removal of the excess second antibody, streptavidin-peroxidase was added. After the third incubation and washing to remove all unbound enzyme, a substrate solution was added, which was acted upon by the bound enzyme to produce color. The intensity of this colored product was measured at 450 nm using a microplate reader. The minimum detectable dose of human IL-10 was <0.2 pg/ml. The mean level of human IL-10 from 26 sera evaluated in this assay was 3.6 pg/ml (ranging from 1.4 to 8.2 pg/ml).

### Statistical analyses

The results are presented as the mean ± standard error for variables. If blood test results were below the limit of detectability of a test, the lower limit of detection was recorded. Univariate analysis was performed using Student's *t*-test. Categorical data were compared against a chi-squared distribution. Comparisons between multiple groups were determined by one-way analysis of variance (ANOVA), and were followed by Scheffe's *F*-test for normal variables. The levels of IL-18, hs-CRP, troponin-T, IL-10, and BNP among the patient subgroups were compared with a Kruskall-Wallis one-way analysis of variance on ranks. Linear regression analysis was employed to determine the relationship between continuous variables. Differ-

ences were considered significant at $P < 0.05$. Statistical analyses were performed with StatView 4.58 software (Abacus Concepts, Calabasa, CA, USA).

## Results

### IL-18 and coronary artery disease

Among the 30 male and 5 female patients, the mean left ventricular ejection fraction was 37.3%. Among these patients, 7 died of cardiovascular causes within a year after acute coronary syndrome. A total of 40% of these patients had had previous myocardial infarction. Four patients had had coronary bypass surgery at least 2 years previously. The circulating level of IL-18 in the patients was 311.2 ± 38.6 pg/ml. IL-18 concentrations did not correlate with BNP, left ventricular ejection fraction, or age. The circulating level of hs-CRP was 2493.8 ± 788.8 μg/dl, troponin-T was 0.42 ± 0.22 ng/ml, IL-10 was 12.3 ± 2.7 pg/ml, and BNP was 638.9 ± 110.1 pg/ml in the 35 patients (mean ± SE). The serum level of IL-18 in patients with triple-vessel disease (389.8 ± 78.6 pg/ml) tended to be higher than in patients with single- or double-vessel disease (251.1 ± 42.7, 281. 1 ± 69.7 pg/ml, respectively). There was no consistent relationship found between the extent of coronary artery disease and the levels of IL-18, hs-CRP, troponin-T, left ventricular ejection fraction, or age.

### IL-18 and other markers

IL-18 concentrations did not correlate with the inflammatory marker, hs-CRP, and the sensitive myocardial necrosis marker, troponin-T, in patients with acute coronary syndrome. Patients were divided into two groups according to the presence or absence of an elevated troponin-T level at the time of blood sampling (Table 1). The median troponin-T level was 0.05 ng/ml in the 35 patients. In those with troponin-T elevation ($n = 18$), the serum levels of IL-18 were significantly greater than in those with low troponin-T levels (466.1 ± 50.9 vs 147.2 ± 18.5 pg/ml; $P < 0.0001$; Fig. 1). However, no difference was found in left ventricular ejection fraction, plasma levels of BNP, and serum levels of hs-CRP between these two groups.

**Table 1.** Demographic characteristics of patients with acute coronary syndromes according to troponin-T levels

| TnT (ng/ml) | ≦0.05 ($n = 17$) | >0.05 ($n = 18$) | *P* value |
|---|---|---|---|
| Age (years) | 64.8 ± 2.2 | 67.5 ± 2.4 | NS |
| Male (%) | 88.2 | 83.3 | NS |
| LVEF (%) | 40.5 ± 4.0 | 34.3 ± 2.3 | NS |
| Previous myocardial infarction (%) | 47.1 | 33.3 | NS |
| Triple-vessel disease (%) | 29.4 | 44.4 | NS |
| BNP (pg/ml) | 480.6 ± 152.4 | 788.4 ± 154.2 | NS |
| hs-CRP (μg/dl) | 1354.2 ± 543.4 | 3570.1 ± 1420.0 | NS |
| IL-18 (pg/ml) | 147.2 ± 18.5 | 466.1 ± 50.9 | <0.0001 |

Data presented are percentage of patients or mean ± SE
TnT, troponin-T; LVEF, left ventricular ejection fraction; BNP, brain natriuretic peptide; hs-CRP, high sensitivity test for C-reactive protein; IL, interleukin; NS, nst significant

### CRP activates the releasing of IL-18 in human endothelial cells

To investigate the direct effect of CRP in HUVECs, the cells were incubated with hr CRP. Forty-eight-hour time course studies are shown in Fig. 2. Stimulation with hr CRP at a concentration of 100 μg/ml induced a significant secretion of IL-18, with the maximum effect at 48 h (a 2.3-fold increase at 48 h; $P < 0.0001$). To confirm the influence of clinical levels of circulating CRP, the cells were incubated with 1, 10, and 100 μg/ml of hr CRP, which was a lower concentration than the serum levels of hs-CRP in the patients (serum levels of hs-CRP in patients with acute coronary syndrome range from 10 to 24073 μg/dl). As shown in Fig. 3, dose-response experiments performed with 24-h incubation showed a significant induction of IL-18 even with 10 μg/ml (from 71.5 ± 5.8 (mean ± SEM of triplicates) pg/ml at baseline to 130.4 ± 16.3 with 10 μg/ml of hr CRP). These results represent three independent experiments.

### hr IL-10 prevents IL-18 releasing by CRP

We also tested the effects of IL-10 on the proinflammatory effects of IL-18 in HUVECs. The inhibitory effect of pretreatment with hr IL-10 on the release of IL-18 in the cells treated with hr CRP was significant (Fig. 3). When the cells were preincubated with anti-inflammatory cytokine, hr IL-10 (10 ng/ml), the maximum inhibitory effects of hr IL-10 on IL-18 release was seen in HUVECs stimulated with 10 μg/ml of hr CRP (130.4 ± 16.3 to 85.5 ± 9.2 pg/ml, P < 0.001).

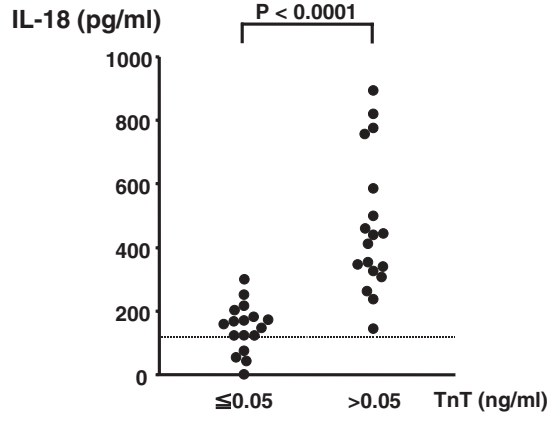


**Fig. 1.** Subgroup analysis of serum levels of interleukin-18 (*IL-18*) in patients with acute coronary syndrome. The median value of troponin-T (*TnT*) was 0.05 ng/ml in 35 patients. Seventeen patients had serum levels of TnT ≦0.05 ng/ml, whereas 18 patients had serum levels of TnT >0.05 ng/ml. Each point represents one subject; *dotted line* indicates the mean serum levels of IL-18 in control subjects

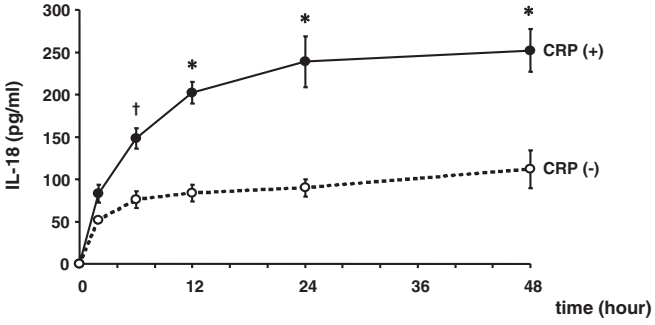


**Fig. 2.** The effect of human recombinant C-reactive protein (hr *CRP*) on the production of IL-18 in human umbilical vein endothelial cells (HUVECs). Time course of the release of IL-18 during incubation with (*closed circles*) or without (*open circles*) hr CRP (100 μg/ml). The values shown are mean ± SEM of triplicates. Production of IL-18 was compared with HUVECs incubated without hr CRP for the same time period. $*P < 0.001$, $^{\dagger}P < 0.05$. The data represent three independent experiments

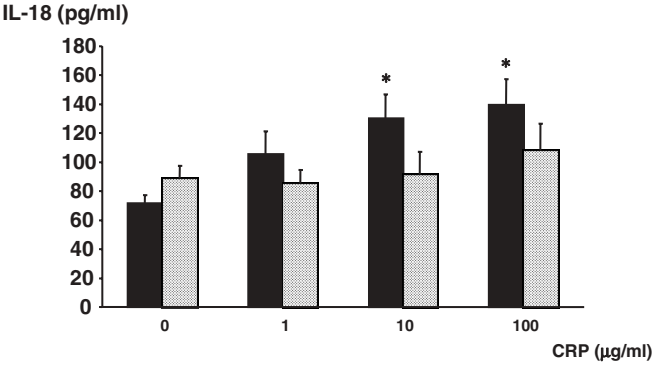


**Fig. 3.** The effect of CRP on IL-18 production and the inhibitory effect of IL-10 in HUVECs. Dose response of the effects of 24-h incubation with hr CRP on IL-18 production. HUVECs were stimulated with hr CRP (0, 1, 10, 100 μg/ml) with (*stippled bars*) or without (*closed bars*) hr IL-10 pretreatment (10 ng/ml, 1 h). The *error bars* indicate the SEM of the mean of triplicates. $*P < 0.01$ vs untreated control. The data represent three independent experiments

Moreover, the effect of 100 μg/ml of hr CRP on IL-18 release was also inhibited by 10 ng/ml of hr IL-10 (139.6 ± 17.7 to 108.1 ± 18.6 pg/ml, $P < 0.05$).

## Discussion

In this study, we demonstrated for the first time that measurement of IL-18 provides important information about the severity of myocardial damage in patients with acute coronary syndrome. This study also showed that hr CRP directly induces the expression of IL-18 in HUVECs. Furthermore, the incubation with hr IL-10 modulated the releasing of IL-18 in the cells treated with hr CRP.

A previous clinical study showed significant expression of IL-18 in human carotid atherosclerotic plaques.[8] Indeed, human atheroma in situ expressed IL-18 and its receptor compared with nondiseased arterial tissue.[11] A previous study of IL-18 concentration in control subjects showed that the level of IL-18 in age- and sex-matched controls was 26 ± 10 pg/ml.[12] We were able to demonstrate for the first time the association between serum levels of IL-18 and troponin-T in patients with acute coronary syndrome. The patients ranged in age from 39 to 79 years. The relation between IL-18 and troponin-T was independent of clinical features such as ejection fraction and remained unaffected by another inflammatory marker, hs-CRP. Cardiac troponin-T, a sensitive marker of myocardial necrosis, is known as a predictive marker for short-term prognosis in patients with acute coronary syndrome.[13] According to a report that the focus of systemic inflammation in patients with unstable angina is the result of low-grade myocardial necrosis,[14] circulating IL-18 may also reflect the myocardial damage seen in patients with acute coronary syndrome. Thus, the effects of IL-18 on myocardial damage in ischemia are much less well understood. A recent study showed that the endogenous inhibitor of IL-18, IL-18 binding protein, modulates the development and stability of atherosclerosis in ApoE knockout mice.[15] These findings identify inhibitors of IL-18 signaling as new important therapeutic targets to prevent atherosclerotic plaque development and to limit plaque complications.

IL-10, which is produced by various inflammatory cells, is a major inhibitor of cytokine synthesis which suppresses macrophage function, and inhibits the production of proinflammatory cytokines.[16,17] Moreover, the protective role of IL-10 in atherosclerosis has been reported.[18] Pretreatment with hr IL-10 was effective in decreasing IL-18 release induced by hr CRP in our in vitro study. In a previous study, serum levels of IL-10 were decreased in patients with acute coronary syndrome.[19] The therapeutic modulation of endothelial inflammation by IL-10 could be a future target for acute coronary syndrome.

CRP is strongly associated with the occurrence of new cardiovascular events in patients with unstable angina,[20] and it is also an important risk factor for cardiac mortality in normal subjects.[21] Although CRP can merely be a marker of an underlying inflammatory/atherosclerotic process, it is possible that CRP may contribute to the pathogenesis of atherosclerosis/inflammation.[2,22,23] Taking this epidemiological and experimental background into account, human endothelial cells were incubated with hr CRP as a model of endothelial inflammation in acute coronary syndrome. We demonstrated a direct important role for CRP, the release of IL-18 in human endothelial cells. These findings reveal that CRP may contribute to the mechanism of endothelial inflammation in acute coronary syndrome by activation of the IL-18 system, which may amplify the inflammatory cascade in tissue injury in addition to initiating endothelial damage and atherogenesis promoted through the recruitment of leukocytes. Although all mechanisms of the effects of CRP have not been clarified in this study, CRP may contribute to the mechanism of atherosclerosis, partially via IL-18 activation.

In conclusion, we demonstrated a new step proximal to IL-18 in the inflammatory pathways of acute coronary syndrome. Circulating CRP could induce IL-18 directly in human endothelial cells. These results identify an important

role for CRP in the pathophysiology of plaque development/progression, and provide a direction for more specific immunomodulating therapy for atherosclerosis.

**Acknowledgments** This study was supported in part by grants for scientific research from the Ministry of Education, Science and Culture of Japan (No. 11838015 and 1538691).

## References

1. Ross R (1999) Atherosclerosis—an inflammatory disease. N Engl J Med 340:115–126
2. Pasceri V, Willerson JT, Yeh ET (2000) Direct proinflammatory effect of C-reactive protein on human endothelial cells. Circulation 102:2165–2168
3. Okamura H, Tsutsui H, Komatsu T, Yutsudo M, Haruka A, Tanimoto T, Torigoe K, Okura T, Nukada Y, Hattori K, Alita K, Namba M, Tanabe F, Konishi K, Fukuda S, Kurimoto Y (1995) Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature 378:88–91
4. Tsutsui H, Nanishi K, Katsui K, Higashino K, Okamura H, Miyazaki Y, Kaneda K (1996) IFN-gamma-inducing factor upregulates Fas ligand-mediated cytotoxic activity of murine natural killer cell clones. J Immunol 157:3967–3973
5. Gracia JA, Forsey RJ, Chan WL, Glimour A, Leung BP, Greer MR, Kennedy K, Carter R, Wei X-Q, Xu D, Field M, Foulis A, Liew FY, McInnes IB (1999) A proinflammatory role for IL-18 in rheumatoid arthritis. J Clin Invest 104:1393–1401
6. Shigehara K, Shijubo N, Ohmichi M, Yamada G, Takahashi R, Okamura H, Kurimoto M, Hiraga Y, Tatsuno T, Abe S, Sato N (2000) Increased levels of interleukin-18 in patients with pulmonary sarcoidosis. Am J Respir Crit Care Med 162:1979–1982
7. Kanda T, Tanaka T, Sekiguchi K, Seta Y, Kurimoto M, Wilson McManus JE, Nagai R, Yang D, McManus BM, Kobayashi I (2000) Effect of interleukin-18 on viral myocarditis: enhancement of interferon-gamma and natural killer cell activity. J Mol Cell Cardiol 32:2163–2171
8. Mallat Z, Corbaz A, Scoazec A, Besnard S, Leseche G, Chvatchko Y, Tedgui A (2001) Expression of interleukin-18 in human atherosclerotic plaques and relation to plaque instability. Circulation 104:1598–1603
9. Mallat Z, Henry P, Fressonnet R, Scoazec A, Chvatchko Y, Tedgui A (2001) Plasma levels of interleukin (IL)-18 are increased in patients with acute coronary syndromes and are associated with increased mortality at follow-up. Circulation 104:II-390
10. Blankenberg S, Tiret L, Bickel C, Peetz D, Cambien F, Meyer J, Rupprecht HJ (2002) Interleukin-18 is a strong predictor of cardiovasucular death in stable and unstable angina. Circulation 106:24–30
11. Gerdes N, Sukhova GK, Libby P, Reynolds RS, Young JL, Schonbeck U (2002) Expression of interleukin (IL)-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for atherogenesis. J Exp Med 195:245–257
12. Seta Y, Kanda T, Tanaka T, Arai M, Sekiguchi K, Yokoyama T, Kurimoto M, Tamura J, Kurabayashi M (2000) Interleukin 18 in acute myocardial infarction. Heart 84:668
13. Aviles RJ, Askari AT, Lindahl B, Wallentin L, Jia G, Ohman M, Mahaffey KW, Newby LK, Califf RM, Simoons ML, Topol EJ, Lauer MS (2002) Troponin T levels in patients with acute coronary syndromes, with or without renal dysfunction. N Engl J Med 346:2047–2052
14. Cusack MR, Marber MS, Lambiase PD, Bucknall CA, Redwood SR (2002) Systemic inflammation in unstable angina is the result of myocardial necrosis. J Am Coll Cardiol 39:1917–1923
15. Mallat Z, Corbaz A, Scoazec A, Graber P, Alouani S, Esposito B, Humbert Y, Chvatchko Y, Tedgui A (2001) Interleukin-18/interleukin-18 binding protein signaling modulates atherosclerotic lesion development and stability. Circ Res 89:e41–e45
16. De Waal Malefyt R, Abrams J, Bennett B, Figdor CG, De Vries JE (1991) Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 174:1209–1220
17. Fiorentino DF, Zlotnik A, Mossman TR, Howard M, O'Garra A (1991) IL-10 inhibits cytokine production by activated macrophages. J Immunol 147:3815–3822
18. Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F, Bureau MF, Soubrier F, Espoisito B, Duez H, Fievet C, Staels B, Duverger N, Scherman D, Tedgui A (1999) Protective role of interleukin-10 in atherosclerosis. Circ Res 85:e17–e24
19. Smith DA, Irving SD, Sheldon J, Cole D, Kaski JC (2001) Serum levels of the anti-inflammatory cytokine interleukin-10 are decreased in patients with unstable angina. Circulation 104:746–749
20. Liuzzo G, Biasucci LM, Gallimore JR, Grillo RL, Rebuzzi AG, Pepys MB, Maseri A (1994) The prognostic value of C-reactive protein and serum amyloid A protein in severe unstable angina. N Engl J Med 331:417–424
21. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH (1997) Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. N Engl J Med 336:973–979
22. Shah PK (2000) Circulating markers of inflammation for vascular risk prediction: are they ready for prime time? Circulation 101: 1758–1759
23. Lagrand WK, Visser CA, Hermens WT, Niessen HW, Verheugt FW, Wolbink GJ, Hack CE (1999) C-reactive protein as a cardiovascular risk factor: more than an epiphenomenon? Circulation 100:96–102