Expression of Interleukin-18 in Coronary Plaque Obtained by Atherectomy from Patients with Stable and Unstable Angina

Running head: Interleukin-18 expression in unstable coronary plaque

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Abbreviations

DCA = directional coronary atherectomy

IFN-γ = interferon-γ

IL = interleukin

TNF-α = tumor necrosis factor-α

Introduction

Increasing evidence supports the notion that inflammation is involved in the pathogenesis of atherosclerosis and plaque instability [1, 2]. We and other investigators have shown that many pro- and anti-inflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-10 and tumor necrosis factor (TNF)-α are expressed in human atherosclerotic plaques [1-3] and that a balance between pro- and anti-inflammatory responses is a major determinant of the onset of acute coronary events [4]. Interleukin-18 was originally identified as an interferon (IFN)-γ-inducing factor that might play a central role in the inflammatory cascade [5, 6]. This pro-inflammatory cytokine is expressed in human atherosclerotic plaques [7, 8], and is related to plaque progression and instability [7-10]. However, IL-18 localization in coronary plaques and its relationship to clinical types of angina have not been examined. We therefore immunohistochemically investigated IL-18 localization in culprit lesions obtained by directional coronary atherectomy (DCA) from patients with either unstable or stable angina.

Material and methods

Patients

We examined 31 consecutive patients who had undergone DCA for a *de novo* lesion. The study population comprised 16 patients with stable angina, classes 1-3 according to the Canadian Cardiovascular Society classification [11], and 15 with unstable angina, classes I-III according to Braunwald's classification [12]. Patients with serious infectious diseases, malignancies, or chronic inflammatory diseases were excluded. All of the included patients provided written informed consent to participate in the study, and the institutional ethics committees approved the study protocol. Hypertension (systolic pressure >140 mmHg and/or diastolic pressure >90 mmHg), hyperlipidemia (total cholesterol >220 mg/dl), diabetes mellitus,

smoking, obesity (body mass index >30 kg/m^2) and a family history of coronary artery disease represented risk factors for coronary artery disease. A culprit lesion was identified from a combination of electrocardiographic findings, angiographic lesion morphology and left ventriculographic or echocardiographic wall motion abnormalities.

Immunohistochemistry and quantitative methods

DCA specimens were immediately fixed in 4% paraformaldehyde and stained with hematoxylin and eosin. Sections were also immunohistochemically evaluated using primary antibodies against SM1 (smooth muscle cells, Kyowa Hakko Kogyo, Tokyo, Japan), CD68 (macrophages, DakoCytomation, Glostrup, Denmark), CD34 (endothelial cells, DakoCytomation), IFN-γ (DakoCytomation) and IL-18 (Medical & Biological Laboratories, Nagoya, Japan). For immunostaining of IFN-γ, 12 frozen sections which were obtained from 5 patients with stable angina and 7 with unstable angina also were used. The sections were stained using the EnVision+ kit (DakoCytomation). Horseradish peroxidase activity was visualized with 3, 3'-diaminobenzidine containing hydrogen peroxide. To identify which type of cells were immunopositive for IL-18, we also performed double immunostaining with IL-18 and the above antibodies. IL-18 was visualized using 3-amino-9-ethylcarbazole containing hydrogen peroxide (red; DakoCytomation) and other antibodies were visualized with 3, 3', 5, 5'-tetramethylbenzidine (blue; Vector Laboratories Inc, Burlingame, CA, USA). In general, specificity of staining by these antibodies was tested in comparison with the staining of negative control using non-immune mouse IgG_1 and non-immune rabbit or rat serum. Immunopositive areas were quantified using a color imaging analysis system (MacSCORP, Mitani, Fukui, Japan) and are expressed as the ratio of positively stained areas per total tissue as described [3]. In the present study, two investigators (T. I. and A. Y.) who were blinded to the patients' clinical

classification examined DCA specimens, and the inter- and intra- observer correlation for immunohistochemical scoring were high (r=0.986, $P<0.001$; r=0.989, $P<0.001$, respectively).

Statistical analysis

Data are expressed as means ± SE. Differences between 2 groups were analyzed using an unpaired Student's *t*-test or the Mann-Whitney *U* test when the variance was skewed. Categorical variables were compared by Fisher's exact probability test. The relationship between IL-18 and IFN-γ expression was evaluated by linear regression analysis. All tests were two-sided and a *P* value of <0.05 was considered statistically significant.

Results

Table 1 lists the clinical characteristics of the patients. Risk factors for coronary artery disease and administered drugs did not significantly differ between patients with stable and unstable angina. Fig. 1 shows representative immunohistochemical IL-18 findings in coronary plaque obtained from patients with stable (A) and unstable (B) angina. Immunopositive areas for IL-18 were significantly greater in patients with unstable, than with stable angina (22.4 ± 5.1 vs. 8.2 ± 3.5%, $P<0.05$; Fig. 2A). Furthermore, IL-18 expression was more increased in plaques with than without thrombus (24.3 ± 4.3 vs. 1.9 ± 1.2%, $P<0.01$; Fig. 1C and D, Fig. 2B). Double immunostaining demonstrated that IL-18 immunoreactive cells were mainly macrophages (Fig. 1E, F and G). And immunopositive areas for IL-18 significantly correlated with those for IFN-γ (r=0.891, $P<0.001$, Fig. 3).

Discussion

We demonstrated for the first time that IL-18 is present in coronary culprit plaques

obtained by DCA. The immunopositive area for IL-18 was greater in patients with unstable, than with stable angina, and the immunopositive cells were mainly macrophages.

Several cell types from the innate (macrophages and dendritic cells) and adaptive (T, B cells) immune systems produce IL-18 [5, 6]. This pleiotropic, pro-inflammatory cytokine possesses several physiological properties, such as the induction of IL-1β, TNF-α and chemokines, expression of adhesion molecules and metalloproteinases and cell death [5, 6, 8], all of which are involved in plaque progression, instability and thrombogenesis. Other investigators have reported that IL-18 expression is increased in human atherosclerotic plaques, and that it is localized mainly in macrophages [7, 8]. Our results are consistent with these previous reports and further indicate that immunoreactive IL-18 is more prominent in coronary plaques that are prone to rupture and in those with thrombus.

Experimental studies have demonstrated that IL-18 enhances atherosclerosis through the release of IFN-γ [9, 13], and that IL-18 inhibition by IL-18 binding protein reduces plaque progression in apolipoprotein E (apo-E)-knockout mice [10]. In addition, the extent of atherosclerosis is reduced in IL-18/apo-E double knockout mice [13]. In the present study, IL-18 expression significantly correlated with IFN-γ expression. Although we could not determine the physiological role of IL-18 in plaques, our results and these lines of evidence support the notion that IL-18 plays a proatherogenic role.

On the other hand, clinical studies relating IL-18 to atherosclerosis and cardiovascular events are limited and controversial [14-20]. Recent clinical studies have assessed the prognostic value of serum IL-18 for future coronary events in healthy individuals [14, 15]. The Prospective Epidemiological Study of the Myocardial Infarction study (PRIME) showed that elevated serum IL-18 is associated with an increased risk of coronary events [14], whereas the MONICA/KORA Augsburg case-cohort study did not find a significant independent association

between increased IL-18 concentrations and subsequent coronary events [15]. Meanwhile, some studies of patients with coronary heart diseases have found that the serum IL-18 level is a predictive marker for future cardiovascular events [16-18]. However, one large prospective study of patients with angiographically confirmed coronary diseases showed that increased IL-18 levels at baseline are independently associated with future cardiovascular death during a 3.9-year follow-up [19], but at 5.9 years, IL-18 concentrations were no longer predictive of outcome [20]. Although the evidence suggests that IL-18 could be a novel marker of future cardiovascular events in patients with coronary heart diseases, further studies are required to establish its role in predicting the risk of coronary events.

The present study has several limitations, the first of which is that it is a small study cohort. A study of far more specimens is necessary to establish the role of IL-18 and to evaluate the effects of drugs such as statin on IL-18 regulation. In addition, the relationship between IL-18 levels in serum and tissue could not be assessed, as blood samples from the patients were unavailable. Finally, a quantitative measure could not be done because each DCA sample was very small in amount.

In conclusion, we demonstrated that IL-18 expression is increased in unstable coronary plaques obtained by DCA. This finding supports the suggestions derived from experimental studies that the inhibition or reduction of IL-18 expression in culprit plaques would be a novel therapeutic strategy for plaque stabilization and for improving the clinical outcome of acute coronary events.

Summary

Interleukin (IL)-18 plays a central role in the inflammatory cascade. We immunohistochemically assessed specimens obtained by directional coronary atherectomy

(DCA) from patients with stable and unstable angina to determine whether IL-18 expression in coronary atherosclerotic lesions is related to plaque instability. We detected IL-18 immunoreactivity in coronary culprit plaques, especially in macrophages. More areas were immunopositive for IL-18 in DCA specimens from patients with unstable angina than in those with stable angina and IL-18 expression was increased in plaques containing thrombus. Furthermore, IL-18 expression significantly correlated with IFN-γ expression. These findings suggest that IL-18 is involved in the pathogenesis of plaque instability in human coronary arteries.

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References

1. Fuster V, Moreno PR, Fayad ZA, Corti R, Badimon JJ. Atherothrombosis and high-risk plaque part I: evolving concepts. *J Am Coll Cardiol.* 2005;46:937-954.
2. Lutgens E, van Suylen RJ, Faber BC, Gijbels MJ, Eurlings PM, Bijnen AP, Cleutjens KB, Heeneman S, Daemen MJAP. Atherosclerotic plaque rupture. Local or systemic process? *Arterioscler Thromb Vasc Biol.* 2003;23:2123-2130.
3. Nishihira K, Imamura T, Yamashita A, Hatakeyama K, Shibata Y, Nagatomo Y, Date H, Kita T, Eto T, Asada Y. Increased expression of interleukin-10 in unstable plaque obtained by directional coronary atherectomy. *Eur Heart J.* 2006;27:1685-1689.
4. Chalikias GK, Tziakas DN, Kaski JC, Hatzinikolaou EI, Stakos DA, Tentes IK, Kortsaris A, Hatseras DI. Interleukin-18: interleukin-10 ratio and in-hospital adverse event in patients with acute coronary syndrome. *Atherosclerosis.* 2005;182:135-143.
5. Mühl H, Pfeilschifter J. Interluekin-18 bioactivity: a novel target for immuno-pharmacological anti-inflammatory intervention. *Eur J Pharmacol.* 2004;500:63-71.
6. Dinarello CA. Interleukin-18: a proinflammatory cytokine. *Eur Cytokine Netw.* 2000;11:483-486.
7. Mallat Z, Corbaz A, Scoazec A, Besnard S, Leséche G, Chvatchko Y, Tedgui A. Expression of interleukin-18 in human atherosclerotic plaques and relation to plaque instability. *Circulation.* 2001;104:1598-1603.
8. Gerdes N, Sukhova GK, Libby P, Reynolds RS, Young JL, Schönbeck U. 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.* 2002;195:245-257.
9. Whitman SC, Ravisankar P, Daugherty A. Interleukin-18 enhances atherosclerosis in

apolipoprotein E(-/-) mice through release of interferon-gamma. *Circ Res.* 2002;90:e34-e38.

10. Mallat Z, Corbaz A, Scoazec A, Graber P, Alouani S, Esposito B, Humbert Y, Chvatchko Y, Tedgui A. Interleukin-18/interleukin-18 binding protein signaling modulates atherosclerotic lesion development and stability. *Circ Res*.2001;89:e41-e45.
11. Campeau L. Grading of angina pectoris. *Circulation*. 1976;54:522-533.
12. Braunwald E. Unstable angina: a classification. *Circulation.* 1989;80:410-414.
13. Elhage R, Jawien J, Rudling M, Ljunggren H-G, Takeda K, Akira S, Bayard F, Hansson GH. Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice. *Cardiovasc Res.* 2003;59:234-240.
14. Blankenberg S, Luc G, Ducimetière P, Arveiler D, Ferrières J, Amouyel P, Evans A, Cambien F, Tiret L, on behalf of the PRIME study group. Interleukin-18 and the risk of coronary heart disease in European men. The prospective epidemiological study of myocardial infarction (PRIME). *Circulation.* 2003;108:2453-2459.
15. Koenig W, Khuseyinova N, Baumert J, Thorand B, Loewel H, Chambless L, Meisinger C, Schneider A, Martin S, Kolb H, Herder C. Increased concentrations of c-reactive protein and IL-6 but not IL-18 are independently associated with incident coronary events in middle-aged men and women. Results from the MONICA/KORA Augsburg case-cohort study, 1984-2002. *Arterioscler Thromb Vasc Biol.* 2006;26:2745-2751.
16. Mallat Z, Henry P, Fressonnet R, Alouani S, Scoazec A, Beaufils P, Chvatchko Y, Tedgui A. Increased plasma concentrations of interleukin-18 in acute coronary syndromes. *Heart.* 2002;88:467-479.
17. Baiidya SG, Zeng QT, Wang X, Guo H-P. T helper cell related interleukins and the angiographic morphology in unstable angina. *Cytokine*. 2005;30:303-310.

18. Kawasaki D, Tsujino T, Morimoto S, Fujioka Y, Naito Y, Okumura T, Masutani M, Shimizu H, Yuba M, Ueda A, Ohyanagi M, Kashiwamura S, Okamura H, Iwasaki T. Usefulness of circulating interleukin-18 concentration in acute myocardial infarction as a risk factor for late restenosis after emergency coronary angioplasty. *Am J Cardiol.* 2003;91:1258-1261.

19. Blankenberg S, Tiret L, Bickel C, Peetz D, Cambien F, Meyer J, Rupprecht HJ; for the Athero*Gene* investigators. Interleukin-18 is a strong predictor of cardiovascular death in stable and unstable angina. *Circulation.* 2002;106:24-30.

20. Tiret L, Godefroy T, Lubos E, Nicaud V, Tregouet DA, Barbaux S, Schnabel R, Bickel C, Espinola-Klein C, Poirier O, Perret C, Munzel T, Rupprecht HJ, Lackner K, Cambien F, Blankenberg S; Athero*Gene* Investigators. Genetic analysis of the interleukin-18 system highlights the role of the interleukin-18 gene in cardiovascular disease. *Circulation.* 2005;112:643-50.

Table 1. Clinical characteristics of study patients.

	Stable angina n = 16	**Unstable angina** n = 15	***P***
Age (years ± SD)	62.8±12.7	62.3±10.2	NS
Men (%)	13 (81)	11 (73)	NS
Risk factors			
Hypertension (%)	11 (69)	10 (67)	NS
Hyperlipidemia (%)	10 (63)	5 (33)	NS
Diabetes mellitus (%)	4 (25)	4 (27)	NS
Smoker (%)	8 (50)	9 (60)	NS
Obesity (%)	5 (31)	3 (20)	NS
Family history (%)	2 (13)	3 (20)	NS
Medication (on admission)			
Aspirin (%)	15 (94)	12 (80)	NS
Beta-blockers (%)	3 (19)	2 (13)	NS
Nitrates (%)	5 (31)	6 (40)	NS
Calcium antagonists (%)	8 (50)	4 (27)	NS
Statins (%)	6 (38)	3 (20)	NS

Hypertension: systolic pressure >140 mmHg and/or diastolic pressure >90 mmHg.

Hyperlipidemia: total cholesterol >220 mg/dl or patient on lipid-lowering therapy.

Obesity: body mass index >30 kg/m^2). Values of $P<0.05$ were considered significant. NS, not significant.

Figure legends

Figure 1. Representative light and immunohistochemical microphotographs of DCA specimens from patients with stable (A) and unstable (B, C and D) angina. Immunohistochemical stainig for IL-18 (A, B and D). Hematoxylin and eosin stain (C). Double immunostaining for endothelial cells (E, blue), smooth muscle cells (F, blue) and macrophages (G, blue) with IL-18 (red). Double immunostaining shows that IL-18 immunoreactive cells are mainly macrophages (G, arrows). Negative control using non-immune rabbit serum (H). T, thrombus.

Figure 2. Ratio of the IL-18 immunopositive area in DCA specimens from patients with stable and unstable angina (A) and in those with and without thrombus (B).

The ratio is significantly higher in patients with unstable than with stable angina (A) and IL-18 immunoreactivity is also more intense in specimens containing thrombus (B).

Figure 3. Correlation between ratio of IL-18 immunopositive areas and that of IFN-γ immunopositive areas.

IL-18 expression significantly correlate with IFN-γ expression (n=12).

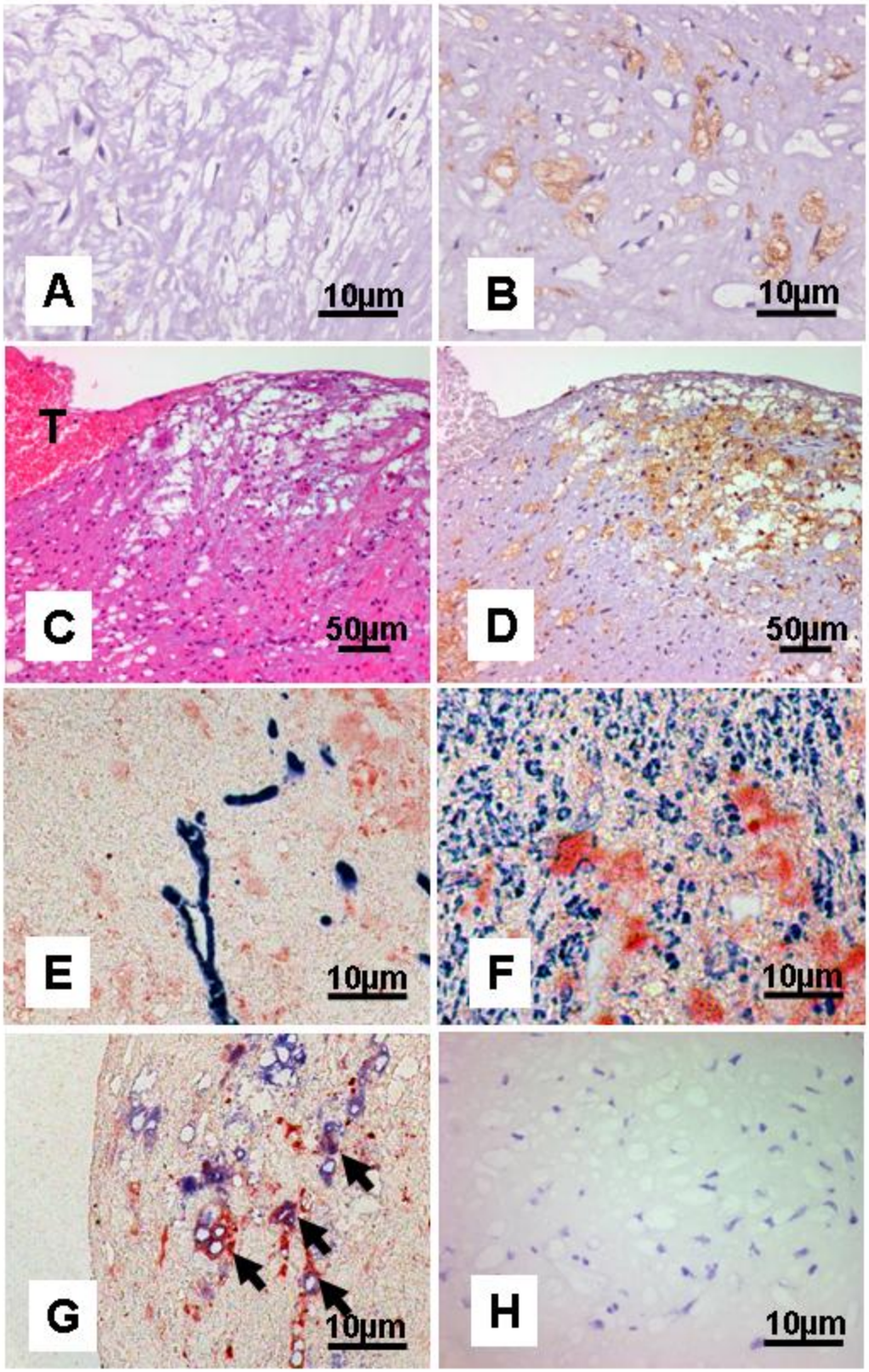
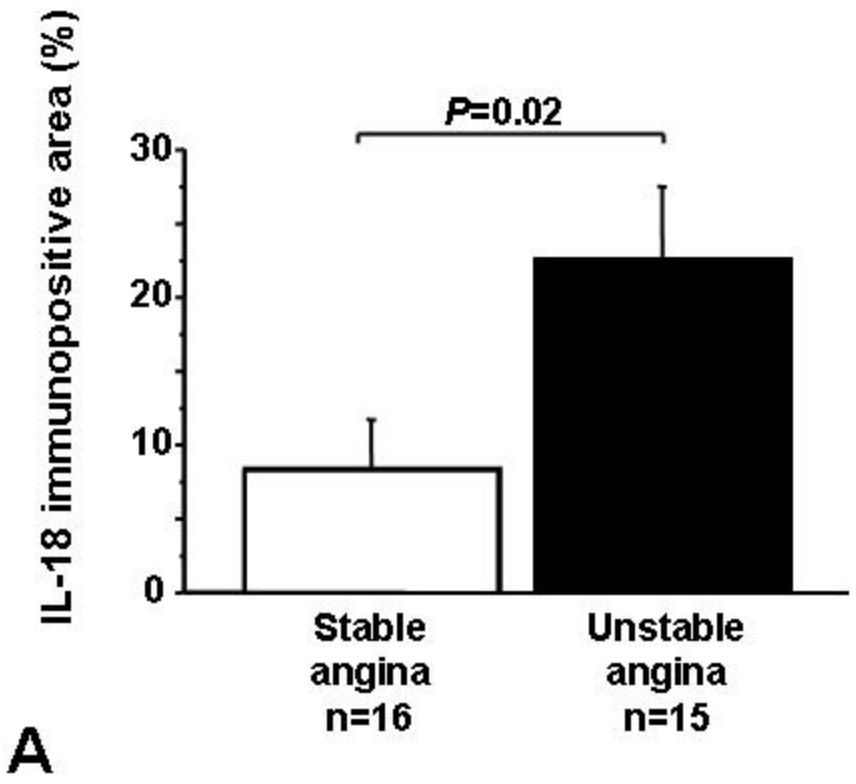


Figure. 1


IL-18 immunopositive area (%)
P=0.02
30
20
10
0
Stable angina n=16
Unstable angina n=15
A

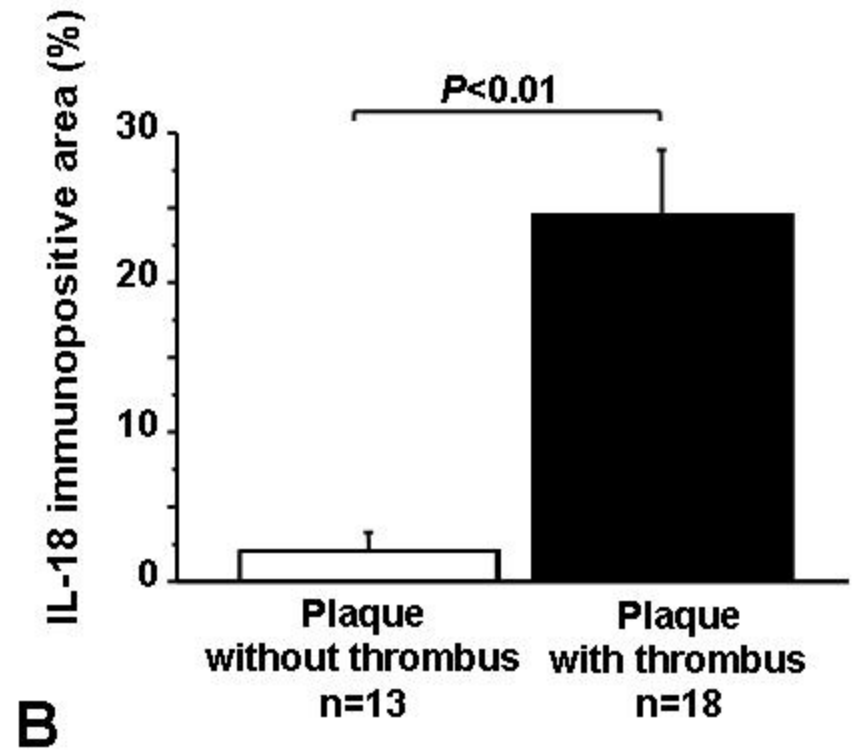

IL-18 immunopositive area (%)
P<0.01
30
20
10
0
Plaque without thrombus n=13
Plaque with thrombus n=18
B

Figure. 2

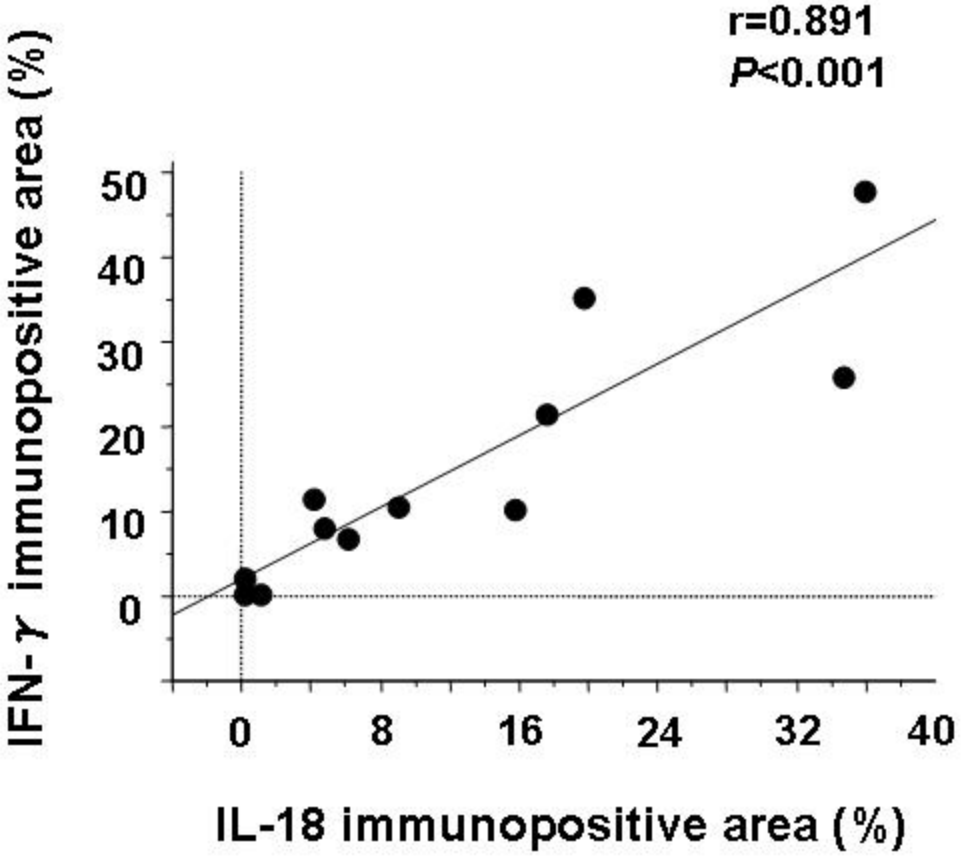

r=0.891
P<0.001
IFN-γ immunopositive area (%)
50
40
30
20
10
0
0
8
16
24
32
40
IL-18 immunopositive area (%)

Figure. 3