CORONARY ARTERY DISEASE

Neopterin is associated with plaque inflammation and destabilisation in human coronary atherosclerotic lesions

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Background: Previous studies have shown that recent activation of the inflammatory response in coronary atherosclerotic lesions contributes to rapid progressive plaque destabilisation. Neopterin, a by-product of the guanosine triphosphate pathway, is produced by activated macrophages and serves as an activation marker for monocytes/macrophages.

Objective: To elucidate the role of neopterin in coronary plaque destabilisation by immunohistochemical study of the presence of neopterin in coronary atherectomy specimens obtained from patients with stable angina pectoris (SAP) and unstable angina pectoris (UAP).

Patients and methods: All patients underwent atherectomy of the primary atherosclerotic lesions responsible for SAP (n = 25) and UAP (n = 25). Frozen samples were studied with antibodies against smooth muscle cells, macrophages, T cells, neutrophils and neopterin.

Results: In 22/25 patients with UAP, abundant neopterin-positive macrophages were found at the sites of coronary culprit lesions. However, in 25 lesions from patients with SAP, only 11 lesions showed neopterin positivity. Quantitatively, the neopterin-positive macrophage score was significantly higher ($p < 0.001$) in patients with UAP than in patients with SAP. Moreover, the neopterin-positive macrophage score showed a significant positive correlation with the number of neutrophils or T cells, respectively (neutrophils, $r = 0.55$, $p < 0.001$; T cells, $r = 0.70$, $p < 0.001$).

Conclusions: Neopterin can be considered as one of the significant factors in the process of plaque inflammation and destabilisation in human coronary atherosclerotic lesions. Its exact role in the process needs to be investigated further.

Coronary vascular inflammation has a pivotal role in most cases of acute coronary syndromes. Inflammatory phenomena within vulnerable plaques may explain plaque rupture or erosion and superimposed thrombosis, which leads to vessel closure and ensuing myocardial ischaemic injury.¹ Previous studies have shown that macrophages and T lymphocytes are the dominant types of inflammatory cells in human coronary unstable plaques, such as ruptured or eroded plaques.^{2–6} Moreover, we have recently demonstrated that neutrophils have a role in mediating destabilisation of atherosclerotic plaques.^{7–8} Activated macrophages within plaques can produce a range of proteases that lead to proteolytic destruction of the connective tissue matrix and may contribute to the active phenomenon of plaque disruption.⁹ Unstable plaques also contain activated T cells that produce the cytokine interferon γ (IFN γ), which both activates macrophages present in the atherosclerotic lesions and interferes with matrix collagen synthesis.¹⁰

Neopterin, a pteridine derivative, is secreted by macrophages after stimulation by IFN γ ^{11–12} and has been shown to be raised in the serum of patients with unstable angina pectoris (UAP) and myocardial infarction as compared with control subjects and patients with stable angina pectoris (SAP).^{13–14} In addition, serum levels of neopterin are associated with the presence of angiographically demonstrated complex lesions in patients with UAP and represent a marker of coronary disease activity.^{15–16} However, the presence of neopterin in human coronary unstable plaques has not been reported. Therefore, we investigated the immunolocalisation of neopterin in coronary atherectomy specimens taken from the culprit lesions responsible for SAP and UAP.

PATIENTS AND METHODS

The study was approved by the hospital ethics committee, and informed consent was obtained from all patients before the study.

Patients

In the present study, coronary atherectomy specimens, obtained by directional coronary atherectomy (DCA) between April 2001 and March 2004 from the culprit lesion in 50 patients who presented with either SAP (n = 25) or UAP (n = 25), were examined. The patients with UAP comprised 15 patients in Braunwald's class I, and 10 in class III¹⁷ undergoing percutaneous coronary intervention for a single primary lesion at Osaka City General Hospital, Osaka. Restenosis specimens, vein graft specimens and specimens from patients who required multiple interventions at the same time were not included. All patients were receiving aspirin (81 mg) treatment, and anti-anginal treatment (β blockers, nitrates or calcium channel blockers, alone or in combination) was prescribed for 87% of the patients, but no one had received thrombolytic agents. The culprit lesion was identified from clinical, ECG and angiographic data. Patients with a culprit lesion that could not be identified were excluded from the study.

Patients were selected for DCA according to strictly defined angiographic criteria: a proximal located eccentric culprit lesion in a non-tortuous coronary artery > 3 mm in diameter. DCA was performed with the femoral approach using the Flexicut

Abbreviations: DCA, directional coronary atherectomy; IFN γ , interferon γ ; ox-LDL, oxidised low-density lipoprotein; SAP, stable angina pectoris; UAP, unstable angina pectoris

DCA catheter (Guidant Corporation, Santa Clara, CA, USA) of appropriate size to produce an approximate device-to-artery ratio of 1.1:1. The time interval between diagnostic coronary angiography and DCA ranged from 0 to 3 days in the two patient groups. Immediately after DCA, the tissue specimens were carefully oriented along their longest axis, snap frozen, and stored at -80°C . The snap-frozen samples obtained by DCA were subsequently serially sectioned to produce sections of 6 μm in thickness, and then fixed in acetone. Every first section was stained with haematoxylin-eosin; the other sections were used for immunohistochemical staining.

Coronary angiographic analysis

All coronary artery stenoses were selectively assessed by two experienced observers. Coronary stenoses were viewed in two orthogonal projections and classified as "complex" or "smooth" based on the Ambrose classification.¹⁸ Simple lesions were concentric or eccentric and characterised by a smooth border and a broad neck. Complex lesions were eccentric with a narrow neck, irregular borders or overhanging edges, including ulceration or the presence of thrombus. Intracoronary thrombus was identified by the presence of intraluminal filling defects surrounded by contrast on at least three sides, with or without contrast staining. In all patients, offline quantitative coronary angiography was conducted with the view revealing the highest degree of stenosis. Calculations were performed using the Cardiovascular Measurement System (CMS-MEDIS Medical Imaging System, Leiden, The Netherlands) by an investigator who was unaware of the study design.

Immunohistochemistry

Single staining

The cellular components were analysed using monoclonal antibodies against smooth muscle cells (1A4; DAKO, Glostrup, Denmark), macrophages (EBM11; DAKO), T cells (SK7; Becton Dickinson, San Jose, CA, USA), and neopterin (Biogenesis Inc, NH, USA). To identify neutrophils, the following antibodies were used: CD66b (80H3; Beckman Coulter, Fullerton, CA, USA), elastase (NP57; DAKO) and myeloperoxidase (MPO-7; DAKO). As we have already reported,⁷ double immunostaining analysis showed that CD66b positivity was detected in neutrophils but not in macrophages. In contrast, elastase and myeloperoxidase positivity was found in neutrophils but occasionally also in

macrophages. However, most elastase-positive and myeloperoxidase-positive cells within the plaque are neutrophils. Non-immune mouse IgG serum (DAKO) served as a negative control. Sections were incubated at 4°C overnight or for 1 hour at room temperature, and then subjected to a three-step staining procedure, using the streptavidin-biotin complex method for detection. Peroxidase activity was visualised with 3-amino-9-ethyl-carbazole (10 minutes, room temperature), and the sections were faintly counterstained with haematoxylin.

Double immunostaining

The simultaneous identification of smooth muscle cells and macrophages was performed based on two primary antibodies of a different IgG subclass (1A4/CD68).¹⁹ The following subsequent incubation steps were performed: normal goat serum (15 minutes), blotted off without washing; a cocktail of two primary monoclonal antibodies consisting of anti-smooth muscle actin, 1A4 (IgG2a) and anti-CD68, EBM11 (IgG1) (60 minutes); cocktail of two secondary antibodies consisting of alkaline phosphatase conjugated goat anti-mouse IgG specific (GAM-IgG1/AP) and biotinylated goat anti-mouse IgG2a specific (GAM-IgG2a/Bio) (30 minutes); and β -galactosidase-labelled streptavidin (30 minutes). Finally, the enzymatic activity of β -galactosidase for 1A4 was visualised in turquoise (BioGenex Kit; BioGenex, San Ramon, CA, USA) and that of alkaline phosphatase for CD68 in red (New Fuchsin Kit, DAKO). To identify cell types that stained positive for neopterin, we also performed double immunostaining with smooth muscle cells or macrophages and neopterin according to the method previously reported with minor procedural modifications.¹⁹ For this staining, alkaline phosphatase was visualised with fast blue BB (blue, smooth muscle cells or macrophages) and peroxidase was visualised with 3-amino-9-ethylcarbazole development (red, neopterin).

Quantitative methods

The tissue area occupied by immunostained macrophages and neopterin was quantified, using computer-aided planimetry and expressed as a percentage of the total surface area of the tissue section. In addition, on the basis of these quantifications, a "neopterin-positive macrophage score" was calculated as follows: neopterin-positive macrophage score = neopterin-positive area/macrophage-positive area. Numbers of CD3-positive T cells and myeloperoxidase-positive neutrophils were counted in the entire tissue sections, and expressed as the number of cells per mm^2 of the tissue. Neutrophils within thrombi or tissue-attached blood clots were excluded. The morphometric analysis was performed by a single investigator who was unaware of the patients' characteristics and histological classifications. Data are shown as the mean (SD). The two groups were compared with an unpaired Student's *t* test or with a Mann-Whitney U test when the variance was heterogeneous. Categorical variables were compared using the χ^2 test or Fisher exact test. Values of $p < 0.05$ were considered significant.

RESULTS

Clinical presentation and angiographic findings

Table 1 shows the patients' characteristics. There were no differences in age, gender, presence of risk factors, serum levels of total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides between the two groups. Detailed angiographic assessment of the target coronary artery lesions was performed in 47/50 patients before DCA. In the remaining three patients, technical problems precluded adequate angiographic analysis. For the quantitative coronary angiography analysis, there were no significant differences in pre-procedure minimal lumen diameter and

Table 1 Clinical and angiographic features of patients who underwent directional coronary atherectomy for treatment of symptomatic ischaemic heart disease

Characteristics	SAP (n = 25)	UAP (n = 25)	p Value
Age (years)	63 (11)	61 (12)	0.41
Male (%)	88	84	0.68
Hypertension (%)	40	60	0.16
Diabetes mellitus (%)	28	44	0.24
Smoking (%)	72	76	0.75
Total cholesterol (mmol/l)	4.70 (1.00)	5.20 (0.70)	0.07
HDL cholesterol (mmol/l)	1.25 (0.35)	1.20 (0.35)	0.59
LDL cholesterol (mmol/l)	2.65 (0.90)	3.20 (0.50)	0.06
Triglycerides (mmol/l)	1.72 (0.86)	1.88 (0.90)	0.58
QCA analysis			
MLD (mm)	0.80 (0.44)	0.76 (0.45)	0.72
DS (%)	76 (11)	82 (10)	0.06
Stenosis morphology			<0.0001
Smooth (%)	95	32	
Complex (%)	5	68	

Values are mean (SD) or percentages.

DS, diameter stenosis; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MLD, minimal lumen diameter; QCA, quantitative coronary angiography; SAP, stable angina pectoris; UAP, unstable angina pectoris.

Figure 1 Micrographs of an atherectomy specimen obtained from a culprit lesion in a patient with unstable angina pectoris. (A) Fragment of atherosclerotic plaque tissue. Double immunostaining (smooth muscle cell, turquoise/macrophage, red) reveals abundant macrophages. (B) The adjacent section stained with anti-neopterin antibody shows the presence of abundant neopterin-positive cells. (C) The anti-myeloperoxidase antibody shows the myeloperoxidase positivity of neutrophils. (D) The anti-T cell CD3 antibody shows a large number of T cells. (E) Double immunostaining for macrophages (blue) and neopterin (red) discloses that most cells show double staining (purple), indicating that neopterin is expressed in the vast majority of macrophages. (F) Double immunostaining for smooth muscle cells (blue) and neopterin (red) shows that these smooth muscle cells are negative for neopterin. Bar: A–F, 100 µm.

diameter stenosis between the two groups. Of the 47 target coronary artery stenosis analysed, 18 (38%) were complex lesions and 29 (62%) were smooth. Target stenoses were complex in 1/22 (5%) patients with SAP and in 17/25 (68%) with UAP ($p < 0.001$).

Histological findings

In the culprit lesions of patients with SAP, 11/25 (44%) lesions contained foci of macrophages, and only 1/25 (4%) lesions contained neutrophils. In contrast, all 25 lesions obtained from patients with UAP contained macrophages, and 14 (56%) showed distinct neutrophil infiltration. T cells were present in 36/50 lesions of patients with SAP and UAP. Only 12/25 (48%) patients with SAP had T cells compared with 24/25 (96%) patients with UAP. In the specimens of patients with UAP, 22/25 (88%) lesions showed the presence of neopterin-positive cells. In contrast, in the lesions of patients with SAP, only 11/25 (44%) lesions stained positive for neopterin (figs 1 and 2). Double immunostaining for neopterin and macrophages or smooth muscle cells demonstrated that the vast majority of neopterin-positive cells were macrophages in patients with UAP (fig 1E and F). In contrast, in the atherectomy specimens of patients with SAP, neopterin positivity was sparse and when

present, only a few macrophages were positive for neopterin (fig 2F).

Morphometric analysis

Figure 3 shows the morphometric results. The neopterin-positive macrophage score was significantly higher ($p < 0.001$) in patients with UAP than in patients with SAP. In addition, the neopterin-positive macrophage score was significantly higher ($p < 0.01$) in patients with angiographic complex lesions than in patients with angiographic smooth lesions. Moreover, the neopterin-positive macrophage score showed a significant positive correlation with the number of myeloperoxidase-positive neutrophils or CD3-positive T cells, respectively (neutrophils, $r = 0.55$, $p < 0.001$; T cells, $r = 0.70$, $p < 0.001$).

DISCUSSION

Neopterin, a molecule secreted by activated macrophages on stimulation by $\text{IFN}\gamma$,^{11 12} has previously been reported to be raised in the serum of patients with acute coronary syndrome compared with patients with SAP^{13 14} and to correlate with the presence of angiographically detected complex lesions, and increased cardiovascular risk.^{15 16} Recently, rapid coronary artery disease progression was shown to be associated with raised serum neopterin concentrations both in patients with

Figure 2 Micrographs of an atherectomy specimen obtained from a culprit lesion in a patient with stable angina pectoris. (A) Fragment of atherosclerotic plaque tissue. Double immunostaining (smooth muscle cell, turquoise/macrophage, red) shows scattered macrophages in the lesion. The area indicated by the asterisk is shown in higher magnification in B–F. (B) Double immunostaining (smooth muscle cell, turquoise/macrophage, red) shows scattered macrophages in the lesion. (C) The adjacent section stained with anti-neopterin antibody shows that macrophages have scant positivity for neopterin. (D) The anti-myeloperoxidase antibody shows that there are no myeloperoxidase-positive neutrophils. (E) The anti-T cell CD3 antibody shows a few T cells. (F) Double immunostaining for macrophages (blue) and neopterin (red) reveals that only a few neopterin-positive macrophages are present (compare with fig 1E). Bar: A, 500 μ m; B–F, 100 μ m.

Figure 3 (A) Graphs showing the neopterin-positive macrophage score in atherectomy specimens obtained from culprit lesions in patients with stable and unstable angina pectoris. (B) Graphs showing the neopterin-positive macrophage score in atherectomy specimens obtained from culprit lesions in patients with angiographic simple and complex lesions.

UAP and SAP.²⁰ These findings suggest that raised serum levels of neopterin may be a useful marker for the severity of acute coronary syndromes. However, previous work did not directly assess the presence of neopterin in human atherosclerotic lesions in patients with SAP and UAP.

To the best of our knowledge, the present study is the first to demonstrate the immunolocalisation of neopterin in coronary atherectomy specimens taken from the culprit lesions responsible for SAP and UAP. Coronary plaques in patients with UAP have more extensive macrophage-rich areas than those in patients with SAP.^{2–4} These macrophages seem to have a key role in weakening the fibrous cap of atherosclerotic plaques by secreting protease that contributes to the active phenomenon of plaque disruption. In this study, double immunostaining for neopterin and macrophages or smooth muscle cells demonstrated that the great majority of neopterin-positive cells were macrophages. Moreover, the neopterin-positive macrophage score was significantly higher in patients with UAP than in patients with SAP. In addition, the neopterin-positive macrophage score was significantly higher in patients with angiographic complex lesions than in patients with angiographic

smooth lesions. These findings suggest that enhanced expression of neopterin in coronary plaques is closely related to plaque instability. Vulnerable plaques also have activated T cells that produce the cytokine IFN γ and activate macrophages.^{5,6} Importantly, IFN γ -stimulated macrophages produce neopterin.¹¹ In this study, the neopterin-positive macrophage score area showed a significant positive correlation with the number of T cells. These findings suggest that T cells produce the cytokine IFN γ and activate macrophages, which enhances neopterin expression in human unstable plaques obtained from DCA specimens.

The role of neopterin localisation in unstable plaques is unknown. Studies in vitro have shown that neopterin can enhance the oxidative potential of reactive oxygen species produced from immunocompetent cells.²¹⁻²³ Likewise, neopterin concentrations have been found to be associated with measures of oxidative stress in various diseases such as infection, autoimmunity and malignancy,²⁴ and neopterin concentrations correlate with prognosis in these diseases.^{25,26} Previously, we developed a new and highly sensitive method to measure oxidised low-density lipoprotein (ox-LDL) in circulating plasma, and demonstrated that plasma levels of ox-LDL relate directly to the severity of acute coronary syndromes. In addition, ox-LDL-positive macrophages were significantly higher in patients with UAP than in those with SAP in atherectomy specimens.²⁷ Moreover, our previous study demonstrated that an infiltration of myeloperoxidase, a strong pro-oxidant enzyme released from activated neutrophils, occurs in the culprit lesions of patients with UAP.⁷ In this study, the neopterin-positive macrophage score showed a significant positive correlation with the number of myeloperoxidase-positive neutrophils. These findings suggest that neopterin may have an important role in promoting the oxidative potential of reactive oxygen species, which may lead to plaque instability and the development of acute coronary syndromes.

There are a number of limitations to this study. First, coronary atherectomy specimens provide a unique source of plaque tissue because they make it possible to correlate features of plaque biology with the clinical status of the patient. However, only part of the entire plaque is excised and thus in studies based on examination of these specimens, sampling bias has to be considered. Second, the relationship between neopterin levels in serum and tissue could not be assessed as blood samples from patients were unavailable. Third, plaque instability is an extremely complex phenomenon that involves the interaction of many factors. Neopterin is only one of these factors and its functional significance cannot be determined without taking other factors into account.

In conclusion, neopterin can be considered as one of the significant factors in the process of plaque inflammation and destabilisation in human coronary atherosclerotic lesions. However, its exact role in the process needs to be investigated further.

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