

Neutrophil infiltration and oxidant-production in human atherosclerotic carotid plaques

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Summary. To clarify the clinical implications of neutrophils in vulnerable plaques we evaluated the function and activity of infiltrated neutrophils in an atherosclerotic plaque, focusing on oxidant production. A histopathological investigation was performed using carotid arterial samples obtained from seven patients. The atherosclerotic plaques were examined cytochemically for naphthol-ASD-chloroacetate esterase activity and oxidant-production, and immunohistochemically using N-formyl peptide receptor-like 1 (fPRL1)-, CD66b-, CD68- or p22^{phox}-specific antibodies. The cytoplasmic fPRL1 intensity value of the neutrophils in the plaque was estimated using an activity index.

Naphthol-ASD-chloroacetate esterase activity was found in cells located in the atherosclerotic plaque, indicating that the cells were neutrophils. The cytoplasmic fPRL1 intensity value of the neutrophils in the plaque decreased to approximately 60% of the intensity observed in the capillary vessels. Oxidant-production was also detected in the plaques, and both neutrophils and macrophages were observed at the corresponding oxidant-production sites. p22^{phox} expression was also located in the same areas in which oxidant-production was observed in these plaques. We could not directly evaluate how much ROS generated from the infiltrated neutrophils contributed the plaque vulnerability followed by its rupture. However, the infiltrated neutrophils in the atherosclerotic plaques morphologically appeared activated and were actively generating oxidant, implying that neutrophils, together with macrophages, infiltrate into atherosclerotic plaques and contribute to plaque vulnerability.

Key words: N-formyl peptide receptor-like 1 (fPRL1), Rupture of human atherosclerotic plaque, Inflammation, Serum amyloid A, Neutrophil

Introduction

Inflammation is a factor that both indicates and contributes to atherosclerosis (van der Wal et al., 1994; Hansson, 2005). Macrophages and leukocytes, including polymorphonuclear cells and T-cells, have been reported as the primary cellular components inherent to the formation of inflammatory plaques (Hansson, 2001). These inflammatory cells accumulate in the plaques from the circulating blood and induce the production of proinflammatory cytokines (Reape and Groot, 1999), recruit inflammatory cells and induce the production of acute-phase reactants on the local and systemic scale, which results in the growth of atheromatous plaques and subsequent plaque rupture.

Oxidative stress generated by reactive oxygen species produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase plays an important role in proinflammatory process of atherosclerosis (Azumi et al., 1999). The expression of the NADPH oxidase subunits p22^{phox}, p67^{phox} and p47^{phox} is increased in the macrophages and smooth muscle cells of advanced atherosclerotic plaques (Guzik et al., 2006). Since neutrophils contain NADPH oxidase, increase in number, and because neutrophil activation in the circulating blood has been reported in coronary artery diseases (CAD) (Nijm et al., 2005; Gach et al., 2006), significant attention has recently focused on the role of neutrophils in plaque rupture. Interestingly, neutrophils have only been observed in unstable plaques, suggesting that the presence of neutrophils is strongly correlated with plaque vulnerability (Naruko et al., 2002). In spite of this attention, the morphological characterisation of

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the neutrophils that have infiltrated into the plaque, especially in regards to their function, has not been reported. Neutrophils accumulated in the inflammatory site subsequently release reactive oxygen species (ROS) and proteolytic enzymes (Faurischou and Borregaard, 2003). N-formyl peptide receptor-like 1 (fPRL1), one of the seven transmembrane domain G-protein-coupled receptors, is highly expressed in the neutrophils and mediates some neutrophil functions, including release of granules and phagocytosis (Almkvist et al., 2001; Bylund et al., 2002, 2003). Here, we histopathologically investigated the neutrophils present in human carotid atherosclerotic plaques by evaluating the (fPRL1) staining profile and oxidant -production.

Materials and methods

Patients and samples

Carotid endarterectomy samples were obtained from seven patients (six males and one female with a mean age of 70 ± 8 years old) who underwent endarterectomy of the internal carotid artery subsequent to diagnosis with carotid artery (CA) stenosis from January 2005 to August 2007. All samples used in this study were obtained under the approval of the Hospital Ethics Committee of Chikamori Hospital in Kochi, Japan, and each participant provided informed consent. After routine histological evaluations we retrospectively performed further histological evaluations with fixed samples. All patients suffered severe carotid artery stenosis (three had right CA stenosis and four had left CA stenosis) with cerebral ischemic episodes, and none had any previous history of revascularisation surgery (Table 1). Four of the seven patients were referred to the hospital due to the need for a carotid endarterectomy. All patients were diagnosed with hypertension. Five patients had type 2 diabetes mellitus, three patients had Coronary artery disease and three patients had dyslipidemia. The severity of atherosclerotic lesion (AHA classification) of samples were as follows; type III, 1; type IV, 4; type V,

1; and type VI, 1 (Stary et al., 1994, 1995). Preoperative blood examinations revealed that the white blood cell and neutrophil counts were $6,200$ (mean) $\pm 1,180$ (SD) per mm^3 and $3,670 \pm 390$ per mm^3 , respectively. The average value for C-reactive protein was 0.3 mg/dl (ranging from 0.1 to 0.6 mg/dl).

All of the samples were fixed in 20% formaldehyde, and serial $4 \mu\text{m}$ -sections were cut. Each of the specimens was routinely examined following staining with both haematoxylin and eosin staining and naphthol-ASD-chloroacetate esterase (NASDCE) staining. The other serial sections were used for the immuno-histochemical detection of fPRL1, CD66b, CD68 or p22^{phox} and for the *in situ* detection of oxidant -production. A bone marrow specimen from a patient determined to have normocellular bone marrow was used as a control for the fPRL1 staining assays.

Cytochemical detection of oxidant-producing sites

Oxidant-producing sites were visualised using a modified cerium-based cytochemical method, as previously described (Okada et al., 1987; Kobayashi et al., 1999). The deparaffinised sections were washed with HEPES buffer (20 mM HEPES pH 7.4, 20 mM Tricine and 140 mM NaCl). The sections were then incubated for 30 minutes at 37°C in a buffer containing 20 mM HEPES (pH 7.4), 20 mM Tricine, 140 mM NaCl, 1 mM CaCl_2 , 1 mM CeCl_3 , 1 mM NADPH, $20 \mu\text{M}$ flavin adenine dinucleotide and 1 mM NaN_3 . The specimens were then washed with HEPES buffer before being rinsed in distilled water. After cytochemical incubation, the sections were immersed in an aqueous solution of 1% $\text{Pb}(\text{NO}_3)_2$ for 10 minutes at room temperature. They were then washed with distilled water and subsequently incubated in an aqueous solution of 1% ammonium sulfide for 10 minutes at room temperature. As a control, the tissues were incubated in the reaction buffer containing oxidant-scavenging agents (1 mM p-benzoquinone (Hirai et al., 1991) or $1 \mu\text{M}$ β -sitosterol (Yokota et al., 2006)). The stained sections were then

Table 1. Patients clinical characteristics.

Case	Age (y.o.)	Complications	CEA Samples	AHA Classification	Peripheral blood			Neutrophil No. in plaques (/mm ²)		
					Leukocyte No.	Neutrophil No.	CRP	Shoulder	Lipid core	Control
1	62	AP, DM, HT	Left ICA	V	8,000	4,800	0.6	355.5	161.6	48.5
2	76	CI, HT	Left ICA	IV	7,300	4,745	0.4	312.1	153.5	48.5
3	71	HT, DM, MI	Right ICA	IV	5,400	1,926	0.1	565	176.7	87.5
4	64	CI, HT, DM	Left ICA	III	5,900	3,009	0.2	300	60.2	20.2
5	83	CI, HT	Left ICA	VI	4,100	2,214	0.1	642.4	165.7	92.9
6	63	DM, HC, HT	Right ICA	IV	6,100	4,270	0.1	NA	NA	NA
7	73	DM, HT, AP, CI, HC	Right ICA	IV	6,600	4,158	0.3	NA	NA	NA

AP: angina pectoris; DM: diabetes mellitus; HT: hypertension; MI: myocardial infarction; CI: cerebral infarction; HC: hypercholesterolemia; CEA: carotid endarterectomy; ICA: internal carotid artery; NA: no available data by a reason of a lack of samples for evaluation; AHA Classification: the American Heart Association histological criteria; CRP: C-reactive protein (mg/dl); Control: area opposite the carotid lumen; Leukocyte count and Neutrophil counts: counts are expressed as the numbers of cells per legislative millimeter; No: numbers.

observed using a light microscope. The reliability of the cytochemical method used here was confirmed using X-ray spectrum spot -analysis of the reaction product (Okada et al., 1987).

Immunohistochemical analysis

For immunohistochemical analysis, tissues were fixed in 20% formaldehyde in PBS for 1 hour at room temperature and were subsequently dehydrated and embedded in paraffin. Sections were cut (4 μ m thick), dehydrated and subsequently used for immunohistochemistry according to previously described methods (Ohara et al., 2007). Briefly, sections were deparaffinised, hydrated and incubated for 10 minutes in 3% hydrogen peroxide diluted in distilled water to reduce endogenous peroxidase activity. The sections were then incubated with 10% normal goat serum for 10 minutes to prevent nonspecific binding of the secondary antibody. Immunostaining was then performed using an automated immunostainer (OptiMax Plus; Biogenex, San Ramo, CA). After washing in PBS, the sections were incubated with primary antibodies overnight at 4°C. The primary antibodies used in this study included the mouse monoclonal anti-human CD66b antibody for the detection of neutrophils (BioLegend, San Diego, CA; diluted 40-fold in PBS), the mouse monoclonal anti-human CD68 antibody for the detection of macrophages (Dako, Carpinteria, CA; diluted 50-fold in PBS), the rabbit polyclonal anti-human fPRL1 antibody for the detection of fPRL1 (generated in our laboratory; diluted 1,000-fold in PBS) and the rabbit monoclonal anti-human p22^{phox} antibody (Santa Cruz, Heidelberg, Germany; diluted 50-fold in PBS). After washing three times in PBS, the sections were incubated with a peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (1000-fold dilution) for 30 minutes and subsequently labelled with 3,3-diaminobenzidine tetrahydrochloride for 3 minutes at room temperature. The resulting sections were counterstained with haematoxylin. The specificity of this immunohistochemical procedure was verified using a negative control (lacking the primary antibody, and using rabbit preimmune serum and normal mouse IgG) and positive controls before evaluation.

Neutrophils were recognized by CD66b staining under x 400 magnification and their numbers in atherosclerotic tissues were counted in three region of interest (ROI); plaque shoulder, lipid core, and arterial wall distant from atherosclerotic plaques. Neutrophil numbers of each ROI were expressed as the numbers of cells per square millimeter of measured tissue (Table 1).

Quantification of neutrophil cytoplasmic fPRL1 staining

The immunohistochemical staining of neutrophils for fPRL1 was observed under a standard light microscope with x1000 magnification. The presence of neutrophils was confirmed using anti-CD66b staining of

identical cells in a serial section and by the shape of their nuclei. The intensity of the fPRL1 immunohistochemical staining of the neutrophils cytoplasm was quantitatively estimated using the ImageJ analysis program (National Institute of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij/>) according to a modified method previously reported (Ohara et al., 2007). The localised spot intensity values in the cytoplasm were arbitrarily graded from 0 to 225 for white and black, respectively. The neutrophil fPRL1 intensity was quantified by the mean value of three spots randomly selected within the cytoplasm of the neutrophil. The neutrophil fPRL1 intensity values were measured in at least ten neutrophils observed in the samples.

Statistical analysis

All data are presented as the mean \pm SD (standard deviation). The variables between the two groups were compared using the Mann-Whitney U test, and the variables between the three groups were compared using a one-way analysis of variance. *p* values less than 0.05 were considered significant. All computations were carried out with SPSS, version 11.0.1 J.

Results

Distribution of NASDCE-positive neutrophils in human atherosclerotic plaques

The presence of neutrophils in atherosclerotic plaques obtained from seven patients was investigated

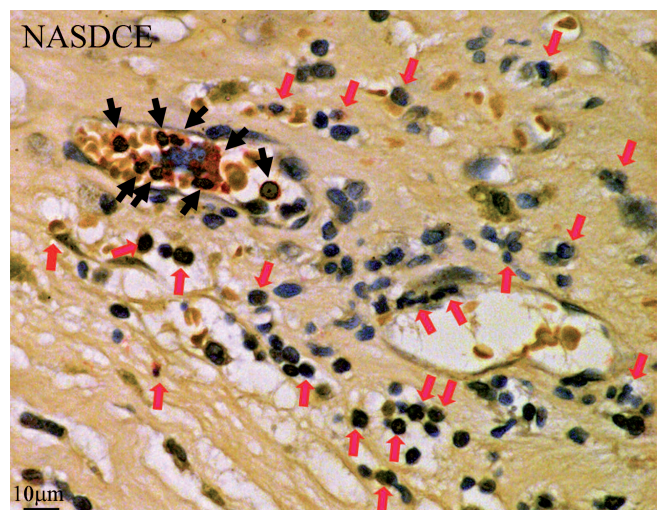


Fig. 1. Distribution of NASDCE-positive neutrophils in an atherosclerotic plaque. Following staining for NASDCE, we detected at least two kinds of neutrophils in the atherosclerotic plaque specimens. NASDCE-positive neutrophils (black arrows) were present within or in the perivascular areas of the capillary vessels, while NASDCE-weakly positive polymorphonuclear cells (red arrows) were present away from the capillary vessels. x 400

following NASDCE staining, a common method to identify neutrophils in tissue. The staining of NASDCE activity was intense in cells within the capillary vessels compared to that in cells localised to the outer vascular space (indicated with black and red arrows, respectively; Fig. 1). The staining of NASDCE activity became weak in cells located further from the capillary vessels (indicated with red arrows; Fig. 1).

Alteration of neutrophil cytoplasmic anti-fPRL1 staining pattern in atherosclerotic plaques

The cytoplasmic fPRL1 staining pattern of neutrophils was heterogeneous, from well to poorly stained, depending on their location relative to the capillary vessels in atherosclerotic plaques (Fig. 2). The CD66b positive cells were identified as neutrophils. Neutrophils inside the capillary vessels (red arrow-head) strongly expressed cytoplasmic fPRL1, whereas neutrophils outside of the capillary vessels (blue arrow-head) weakly expressed cytoplasmic fPRL1. The

representative findings of normal isotype antibody staining were shown in Fig. 2C,D.

The cytoplasmic fPRL1 intensity was quantified in the neutrophils. Fifteen neutrophils in the plaque were analysed in cases 1, 2 and 3, and 10 neutrophils were analyzed in case 4. The cytoplasmic fPRL1 intensity value of the neutrophils is shown in Figures 3A through 3D. The mean cytoplasmic fPRL1 intensity value of neutrophils in the capillary vessels of the plaques was 152.4 ± 12.2 arbitrary units (AU). However, this value was significantly lower in the neutrophils distal to the capillary vessels (102.8 ± 5.0 AU; $p < 0.001$). Furthermore, the mean cytoplasmic fPRL1 intensity value of 15 bone marrow neutrophils, which may have been quiescent, was 169.7 ± 15.2 AU, similar to that of neutrophils located inside the capillary vessels. Similar results were obtained from the other three patients (case 2, inside the capillary vessels 144.1 ± 7.4 AU vs. outside the vessels 115.6 ± 3.2 AU, $p < 0.001$; case 3, 149.7 ± 8.4 AU vs. 114.6 ± 5.0 AU, $p < 0.001$; and case 4, 155.8 ± 8.0 AU vs. 135.1 ± 4.5 AU, $p < 0.001$).

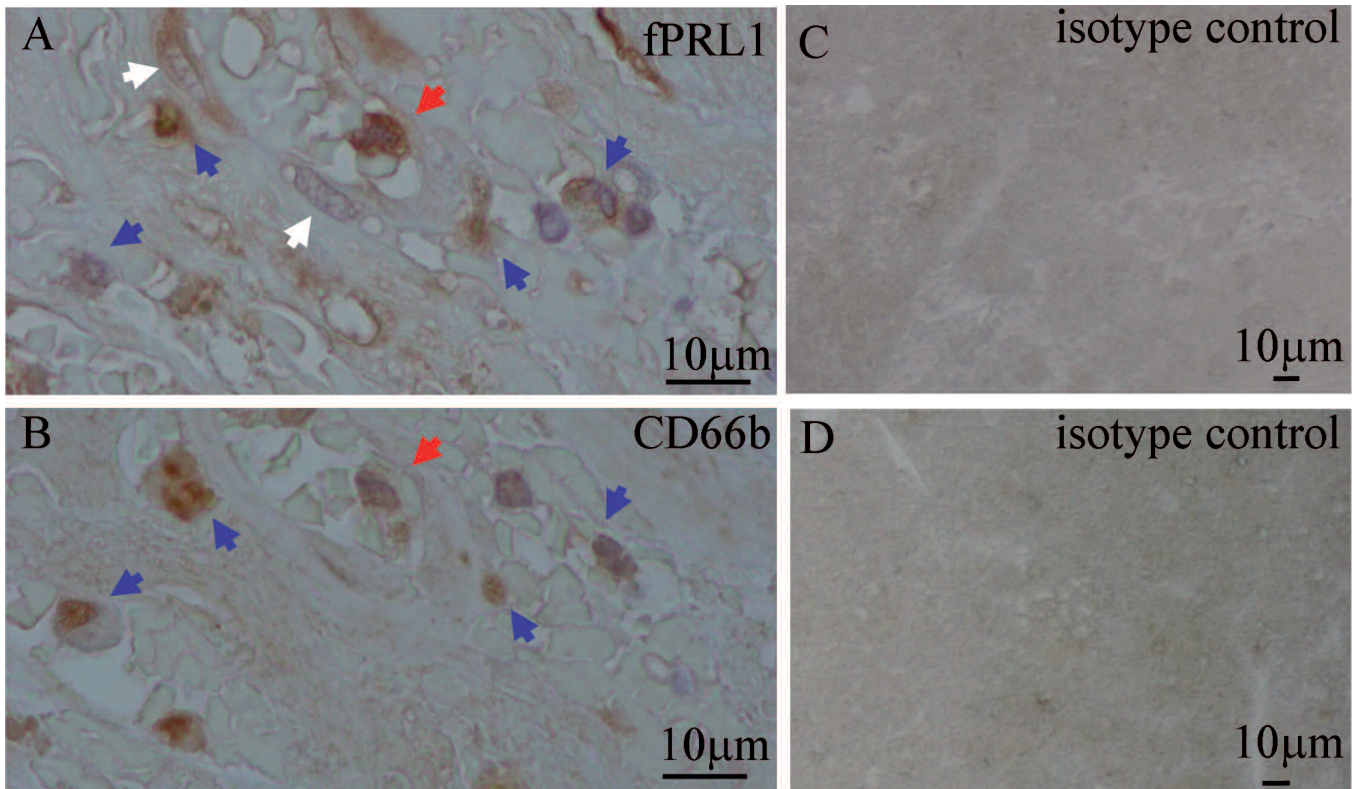


Fig. 2. Alteration of cytoplasmic anti-fPRL1 staining patterns in neutrophils located in atherosclerotic plaques. **Panel A** was anti-fPRL1 staining, and **panel B** was CD66b in serial sections of human atherosclerotic plaque. **Panel C and D** were normal isotype antibody controls staining: **C**, preimmune rabbit serum; **D**, normal mouse IgG. The CD66b positive cells were identified as neutrophils. The cytoplasmic fPRL1 staining of neutrophils was heterogeneous depending on the location relative to the capillary vessels. The neutrophils located inside the capillary vessels (red arrow) were stained strongly for fPRL1 (fPRL1 intensity was 173.7 AU), while the cytoplasmic staining of the extravasated neutrophils (blue arrow) was weak (fPRL1 intensities were 138.7-150.5 AU). The white arrow head indicated capillary endothelial cells. A, B, x 1,000; C, D, x 200

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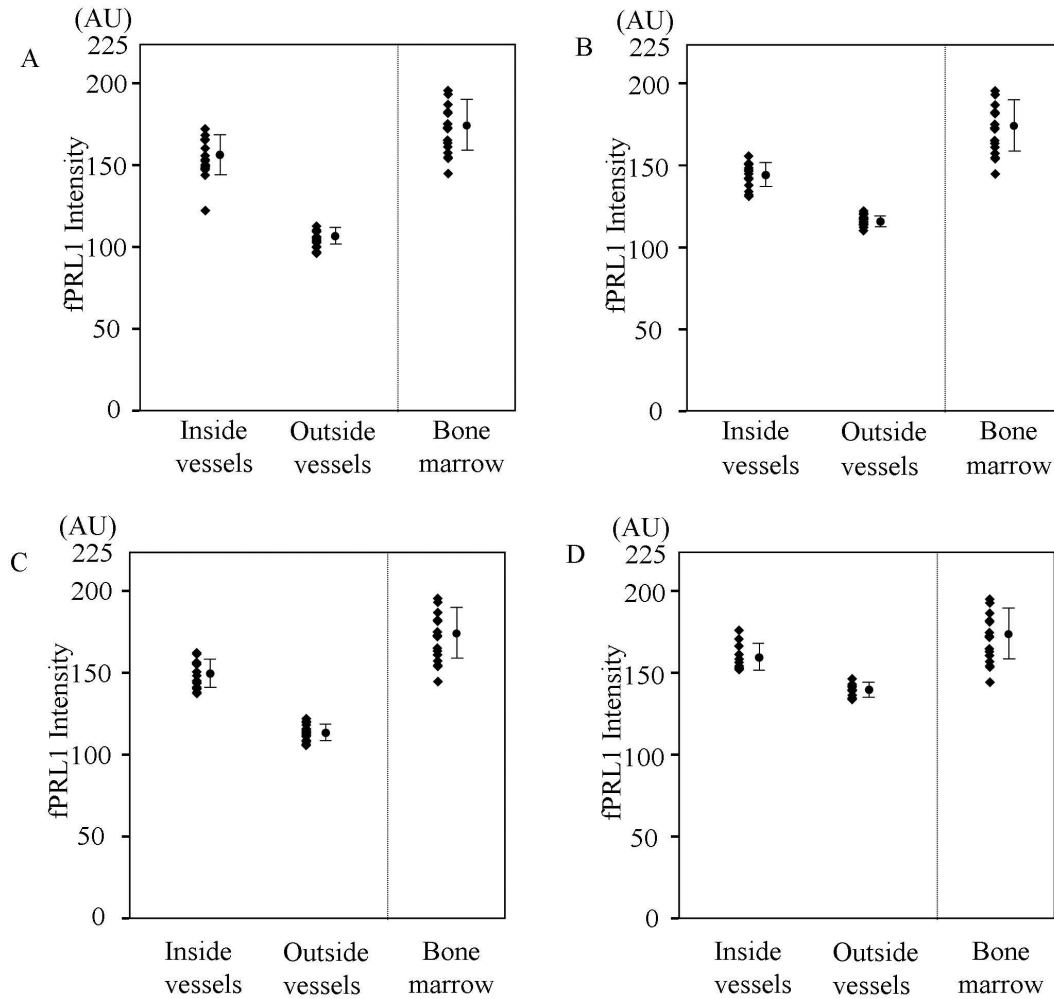


Fig. 3. fPRL1 intensity compared by the location of neutrophils in atherosclerotic plaques. The immunohistochemically determined cytoplasmic fPRL1 intensity of the neutrophils expressed in arbitrary units (AU) and plotted for four of the cases. In **3A** (case 1), the cytoplasmic fPRL1 intensity value of the neutrophils located outside the capillary vessels (102.8 ± 5.0 AU, $n=15$) was significantly lower ($p < 0.001$) than the intensity of the cells inside the capillary vessels (152.4 ± 12.2 , $n=15$). The cytoplasmic fPRL1 intensity value of bone marrow neutrophils (169.7 ± 15.2 , $n=15$) was used as a control. The results of the three patients shown in **Figures B** (case 2), **C** (case 3) and **D** (case 4) were similar to the result shown in Figure 3A, 144.1 ± 7.4 AU (inside) vs. 115.6 ± 3.2 AU (outside, $n=15$, $p < 0.001$) for case 2, 149.7 ± 8.4 AU (inside) vs. 114.6 ± 5.0 AU (outside, $n=15$, $p < 0.001$) for case 3 and 155.8 ± 8.0 AU (inside) vs. 135.1 ± 4.5 AU (outside, $n=10$, $p < 0.001$) for case 4. Each error bar in figures indicates mean \pm SD.

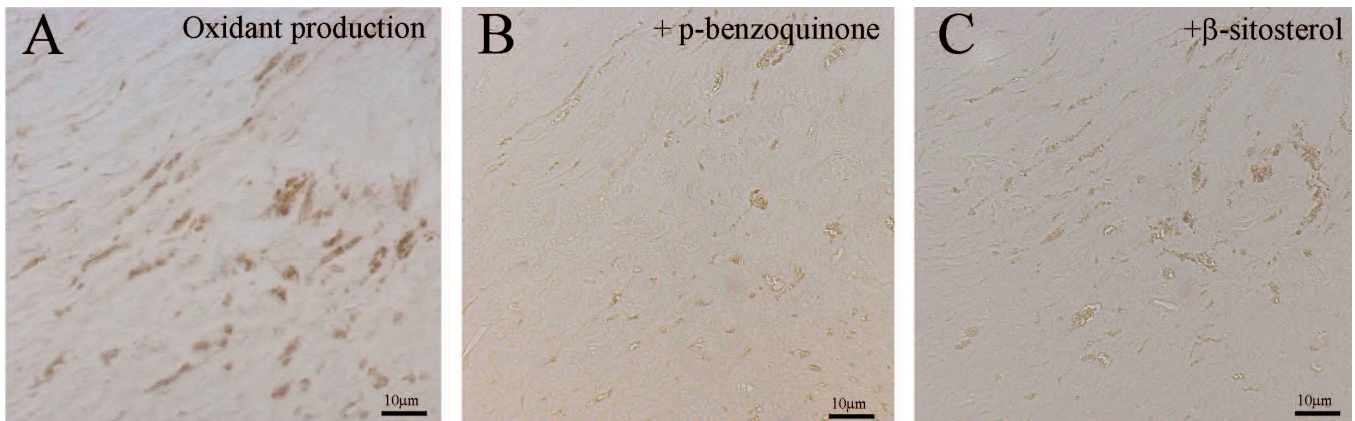


Fig. 4. *In situ* detection of oxidant-producing sites in an atherosclerotic plaque. The detection of oxidant production was performed in specimens fixed in formaldehyde and embedded in paraffin. **Panels A-C** are serial sections. **Panel A:** no inhibitor added. **Panel B:** treated with *p*-benzoquinone. **Panel C:** treated with β -sitosterol. The detection was clearly inhibited by oxidant scavengers. $\times 400$

Cytochemical detection of oxidant-producing sites in human atherosclerotic plaques

To confirm whether the oxidant-producing sites could be specifically detected in specimens fixed in formaldehyde and embedded in paraffin, a preliminary experiment was performed using an oxidant-scavenger. The reaction products were localised in cells distributed

throughout the atherosclerotic plaque of the arterial wall (Fig. 4A). The specificity of the histochemical reaction was verified by inhibiting this reaction using oxidant-scavengers, such as *p*-benzoquinone (Fig. 4B) or β -sitosterol (Fig. 4C). Oxidant-production was inhibited by the addition of these oxidant-scavengers. Oxidant production in our system was not quantitatively detected. We estimated the results of oxidant production in the

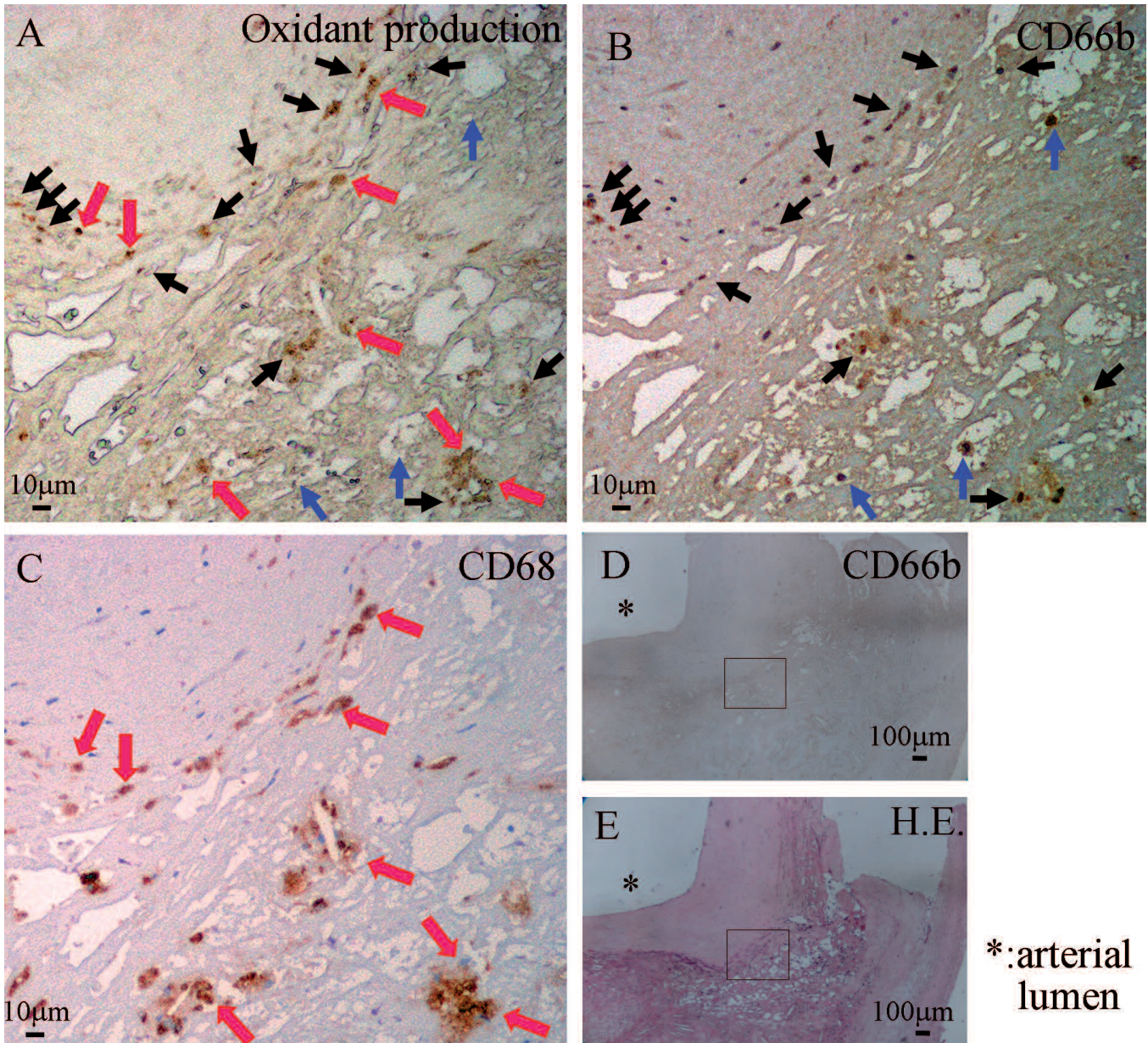


Fig. 5. Oxidant-producing sites and the expression pattern of CD66b and CD68 in human atherosclerotic plaques. **Panel A:** An oxidant-producing site. **Panel B and Panel C:** CD66b and CD 68 staining in serial sections of human atherosclerotic plaques, respectively. **Panel D and Panel E:** CD66b and H.E. staining in low magnification. In **panels A and B**, the black arrows indicate the cells with oxidant-producing sites and CD66b expression, and the blue arrows indicate CD 66b-positive cells without oxidant production. In **panels A and C**, the red arrows indicate the cells with oxidant production and CD68 expression. A-C, x 200; D, E, x 40

*:arterial lumen

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same experiment, in which the steps described in this method must be carried out exactly as described. Oxidant production was not observed in the normal area of the arterial wall adjacent to the lesion.

Oxidant-producing sites and CD66b and CD68 staining pattern in human atherosclerotic plaques

The comparison of oxidant-producing sites and CD66b- and CD68- positive cells in serial sections of human atherosclerotic plaques is shown in Figure 5. Some oxidant-producing sites coincided with CD66b-positive cells (black arrows), while others coincided with CD68-positive cells (red arrows). Some CD66b-positive cells (blue arrows) did not coincide with oxidant-producing-sites, indicating that both neutrophils and

monocytes/macrophages contribute to oxidant-production in human atherosclerotic plaques.

NADPH oxidase subunit (p22^{phox}), CD66b and CD68 expression in human atherosclerotic plaques

A number of CD66b- or CD68- positive cells were observed in the atherosclerotic plaques (Fig. 6). A large proportion of these neutrophils might also participate in oxidant-production in atherosclerotic plaques.

Oxidant-producing sites and p22^{phox}-positive cells in human atherosclerotic plaques

The expression pattern of p22^{phox} was similar to the pattern of oxidant-producing sites (Fig. 7). As p22^{phox}-

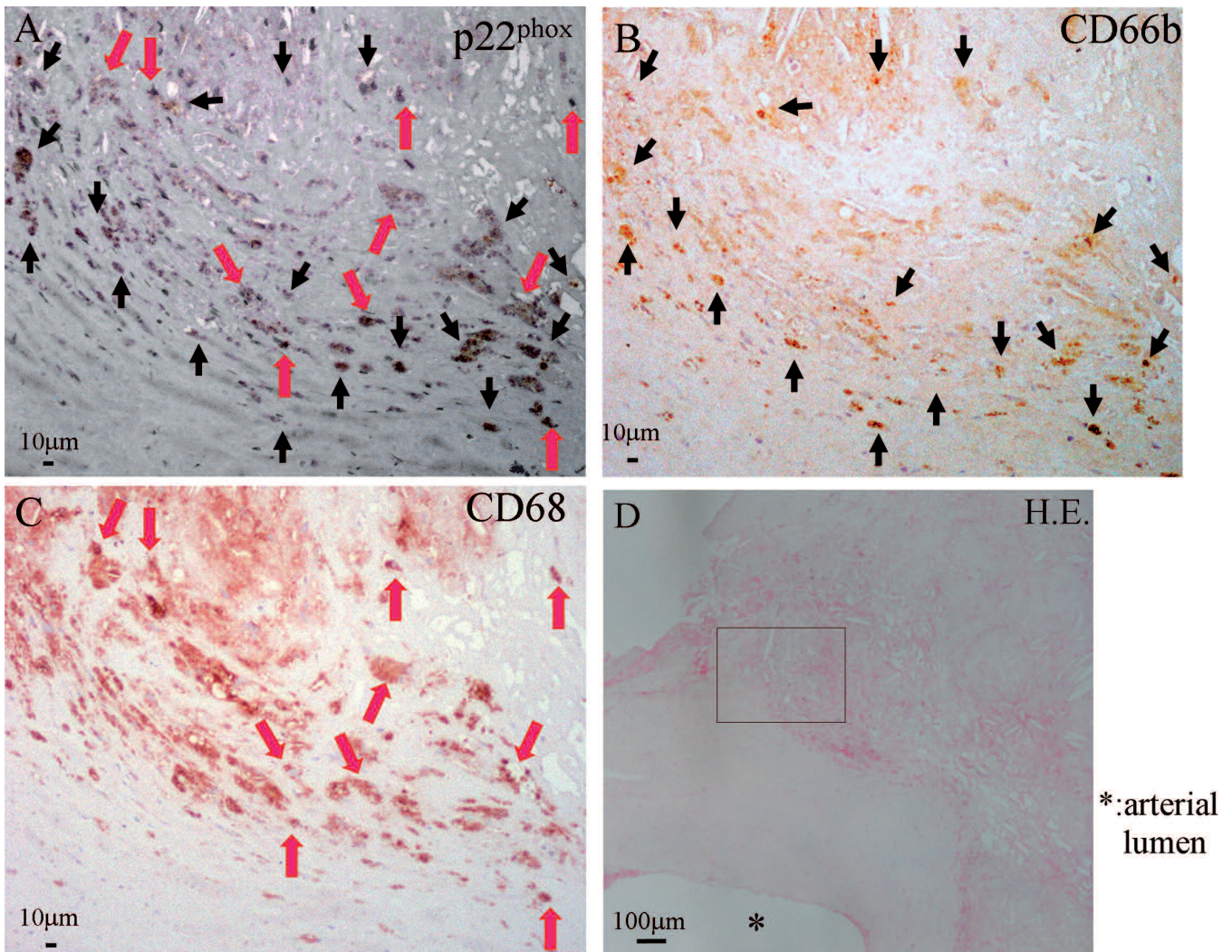


Fig. 6. Expression pattern of p22^{phox}, CD66b and CD68 in atherosclerotic plaques. **Panel A:** Expression of the p22^{phox} NADPH oxidase subunit staining pattern. **Panels B and C:** CD66b and CD 68 expression, respectively, in serial sections of human atherosclerotic plaques. **Panel D:** H.E. staining. In **panels A and B**, the black arrows indicate cells coexpressing p22^{phox} and CD66b. In **panels A and C**, the red arrows indicate cells coexpressing p22^{phox} and CD68. A-C, x 200; D, x 40

positive cells were more frequently detected than oxidant-producing sites, only a fraction of the p22^{phox}-positive cells contributed to oxidant production. In addition, since the cell size and the shapes of the nucleus of the p22^{phox}-positive cells was not homogeneous, p22^{phox}-positive cells most likely represented a mix of monocytes/macrophages and neutrophils.

Discussion

Recent studies have shown that inflammation plays a key role in the progression of atherosclerosis and coronary artery disease. Macrophages and leukocytes are the primary cells responsible for inflammation, and a large number of these cells reside in the atherosclerotic lesion (Schaffne et al., 1980; Gerrity, 1981a,b; Jonasson et al., 1986). The progressive increase of inflammatory cells in the plaque induces a massive secretion of proteolytic enzymes from these cells, which leads to the destabilisation of the plaque and plaque rupture (Brown et al., 1995). Moreover, neutrophil infiltration has been observed in unstable plaques but not in stable plaques, establishing neutrophil infiltration as a pathognomonic feature of plaque vulnerability (van der Wal et al., 1994; Naruko et al., 2002; Leclercq et al., 2007). While these observations directly indicate that neutrophil infiltration into the plaques facilitates plaque vulnerability, the clinical importance of the neutrophils in plaque vulnerability has not been documented.

In the present study, NASDCE-positive cells were detected within and around capillary vessels in human atherosclerotic plaques, while a number of polymorphonuclear cells weakly positive for NASDCE were present outside the capillary vessels. As CD66b-positive cells were detected both inside and outside the

capillary vessels, it is possible that some neutrophils in human atherosclerotic plaques were not detected with NASDCE staining. We have previously demonstrated that the ability to detect neutrophils in human purulent dermatitis based on cytoplasmic fPRL1 staining decreased in parallel with the activation state of the neutrophils. Both oxidant production and the change in neutrophil cytoplasmic fPRL1 intensity were examined to investigate morphology and function of the neutrophils, especially the NASDCE weakly positive neutrophils in human atherosclerotic plaques.

It has been reported that fPRL1 is contained in secondary and tertiary granules of mature neutrophils, and that the activated neutrophils at inflammatory sites lose their round configuration, transmigrate across the endothelial barrier under exocytosis of granules, and phagocytosis of foreign particles there (Almkvist et al., 2001; Bylund et al., 2002, 2003). These extravasated neutrophils are considered to be bioactive and morphologically different from the neutrophils within the vessel, although the close relation between the activation of neutrophil function including ROS production and morphological changes including fPRL1 staining in the inflammatory process has not been clarified. It has been shown that neutrophil cytoplasmic fPRL1 intensity decreases with neutrophil activation due to exocytosis of fPRL1 to the cell surface. Therefore, we considered neutrophils with a decreased cytoplasmic fPRL1 intensity to be activated neutrophils (Ohara et al., 2007). In the present study examining human atherosclerotic plaques, the cytoplasmic fPRL1 intensity of neutrophils outside the capillary vessels was lower compared to cells inside the vessels. The alteration in neutrophil cytoplasmic fPRL1 intensity was identical to the previously reported alteration in alkaline

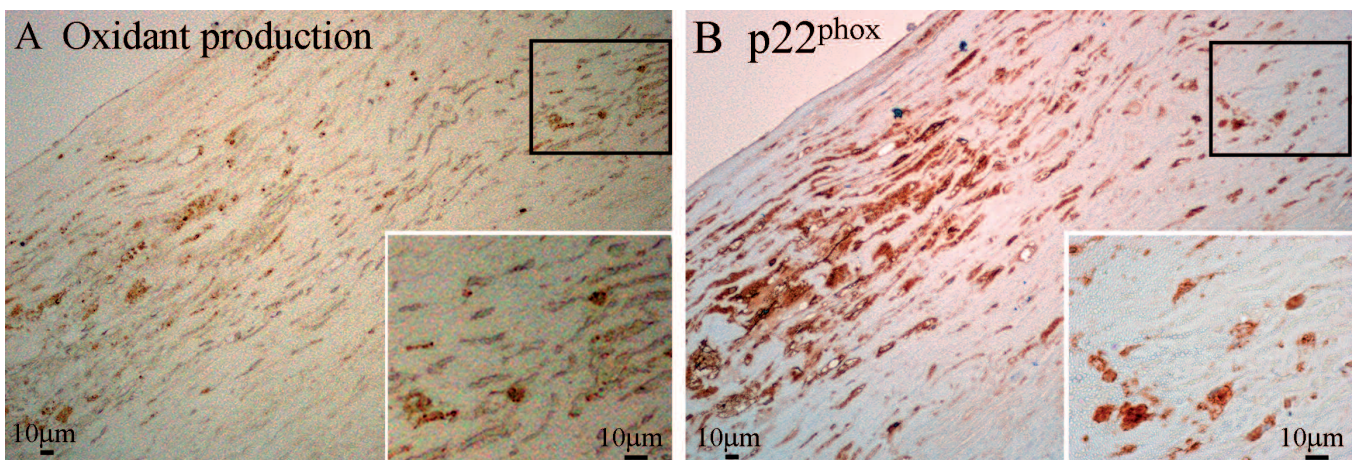


Fig. 7. Oxidant-producing sites and the p22^{phox} expressing pattern in human atherosclerotic plaques. **Panel A:** An oxidant producing site. **Panel B:** p22^{phox} staining in serial sections of a human atherosclerotic plaque. The expression pattern of the phagocytic NADPH oxidase p22^{phox} subunit was similar to oxidant-producing sites. As the area positive for p22^{phox} expression was larger than the oxidant-producing area, a proportion of p22^{phox}-positive phagocytes most likely contributed to oxidant production. In addition, since the cell size and nucleus shape of the p22^{phox}-positive cells were not homogeneous, these cells seemed to be not only monocytes/macrophages, but also neutrophils. x 200

phosphatase, contained in the granules of the neutrophil cytoplasm (Kobayashi and Robinson, 1991). These data indicate that the alterations in neutrophil cytoplasmic fPRL1 expression can detect changes in neutrophil activation, similar to neutrophil granules containing fPRL1. The ratio of the cytoplasmic fPRL1 intensity of neutrophils in the extravascular space to the intensity of cells within the capillary vessels was 65-85%. These values could be a measure of the inflammation of the atherosclerotic plaques, as this ratio has been shown to be 55-75% in bacterial purulent dermatitis (Ohara et al., 2007). The NASDCE activity pattern of cells located in the extravascular space was weaker than in cells in the capillary vessels (Fig. 1). This observation suggests that NASDCE activity is released from the cells following stimulation.

Oxidant production was specifically detected in specimens fixed in formaldehyde and embedded in paraffin using a cytochemical reaction. Because frozen samples are predominantly used in the evaluation of oxidant production in atherosclerotic specimens (Miller et al., 1998), a luminometer and a skilled technique may be required. Our method for the detection of oxidant production in paraffin sections, using a cytochemical reaction, was simple to implement, which could make retrospective studies possible after other immunohistochemical staining.

The oxidant-producing sites mostly colocalized with CD66b- or CD68-positive cells. Moreover, CD66b-positive cells were just as frequent as CD68-positive cells. Oxidant production was detected in a subset of the p22^{phox}-positive cells. As the cell size and nucleus shape of these p22^{phox}-positive cells were not homogeneous, it is possible that both macrophages and neutrophils expressed p22^{phox}. The p22^{phox} subunit of NADPH oxidase was detected in both CD66b- and CD68-positive cells, indicating that both macrophages/monocytes and neutrophils containing NADPH oxidase contribute to oxidant production in human atherosclerosis. To our knowledge this is the first study detailing the direct relationship between infiltrated neutrophils and *in situ* oxidant production in atherosclerotic plaques. Therefore, this is the first study to demonstrate that neutrophils infiltrating into atherosclerotic plaques appear active cytologically and actively produce oxidant. This study confirmed that neutrophils significantly contribute to oxidant production in the plaques, particularly due to NADPH-oxidase, which is abundant in neutrophils. Since oxidant production is related to plaque vulnerability (Azumi et al., 1999; Guzik et al., 2006), we speculate that neutrophil-infiltrated plaques become vulnerable, thereby facilitating the rupture of the plaque.

Quiescent neutrophils, as judged by NASDCE expression and cytoplasmic fPRL1 staining, were present inside and around the capillary vessels of the plaque, although these neutrophils were notably not detected in the arterial subendothelial space. The blood supply to the plaque has been reported to be mostly delivered through the *vasa vasorum* that originates from

the *adventitia* (Kumamoto et al., 1995; Moreno et al., 2006). The cause of neutrophil activation in the perivascular region of the capillary vessels, however, remains to be precisely elucidated. Neutrophils stimulated via fPRL1 are known to increase Ca²⁺ influx, migrate and release inflammatory mediators (Rabiet et al., 2005; Boldt et al., 2006; Fu et al., 2006; Migeotte et al., 2006; Selvatici et al., 2006). In addition, a phagocytosed mitochondrial protein derived from damaged cells under oxidative stress has been reported to be a ligand for fPRL1 (Carp, 1982). Serum amyloid A, an acute-phase reactant that is synthesised and produced not only by hepatocytes, but also by vascular smooth muscle cells and macrophages, is also known to mediate the chemotactic migration of neutrophils and vascular smooth muscle cells (Su et al., 1999; Kumon et al., 2001, 2002). Furthermore, there are numerous types of cytokines and bioactive substances, including those mentioned above, which are secreted as a consequence of the various infiltrating cells and their restructuring of the matrix components in the plaques. Although the precise mechanism of neutrophil activation in the plaque remains unclear, the inhibition of neutrophil infiltration into the plaque may be a potential strategy to prevent the progression of atherosclerosis.

In the present study, we retrospectively performed cytochemical and immunohistochemical evaluation of neutrophil infiltration and oxidant production in human atherosclerotic plaques. As in our study the sample numbers were small, and the treatments and complications of the patients were heterogeneous, the conclusions of this study may be limited. We believe that a large-scale study will strengthen our results.

In conclusion, it is reported that the macrophages in atherosclerotic plaques contribute to instability of the plaque by producing ROS (Zalba et al., 2007). As the direct cause of additional infiltration of neutrophils into the plaques is unknown, the role of infiltrating neutrophils in plaque rupture is suggested by the observation of oxidant-production; the neutrophils in the plaques appear to be functionally active. Since NADPH oxidase activity is thought to account for a significant proportion of oxidant-production in neutrophils, neutrophils that have infiltrated into an atherosclerotic plaque should be considered a significant source of oxidant-production in plaques.

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References

- Almkvist A., Fäldt J., Dahlgren C., Leffler H. and Karlsson A. (2001). Lipopolysaccharide-induced gelatinase granule mobilization primes

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- neutrophils for activation by galectin-3 and formylmethionyl-Leu-Phe. *Infect. Immun.* 69, 832-837.
- Azumi H., Inoue N., Takeshita S., Rikitake Y., Kawashima S., Hayashi Y., Itoh H. and Yokoyama M. (1999). Expression of NADH/NADPH oxidase p22^{phox} in human coronary arteries. *Circulation* 100, 1494-1498.
- Boldt K., Rist W., Weiss S.M., Weith A. and Lenter M.C. (2006). FPRL-1 induces modifications of migration-associated proteins in human neutrophils. *Proteomics* 6, 4790-4799.
- Brown D.L., Hibbs M.S., Kearney M., Loushin C. and Isner J.M. (1995). Identification of 92-kD gelatinase in human coronary atherosclerotic lesions association of active enzyme synthesis with unstable angina. *Circulation* 91, 2125-2131.
- Bylund J., Karlsson A., Boulay F. and Dahlgren C. (2002). Lipopolysaccharide-induced granule mobilization and priming of the neutrophil response to *Helicobacter pylori* peptide Hp(2-20), which activates formyl peptide receptor-like 1. *Infect. Immun.* 70, 2908-2914.
- Bylund J., Samuelsson M., Collins L.V. and Karlsson A. (2003). NADPH oxidase activation in murine neutrophils via formyl peptide receptors. *Exp. Cell Res.* 282, 70-77.
- Carp H. (1982). Mitochondrial N-formylmethionyl proteins as chemoattractants for neutrophils. *J. Exp. Med.* 155, 264-275.
- Faurschou M., Borregaard N. (2003). Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect.* 14, 1317-1327.
- Fu H., Karlsson J., Bylund J., Movitz C., Karlsson A. and Dahlgren C. (2006). Ligand recognition and activation of formyl peptide receptors in neutrophils. *J. Leukoc. Biol.* 79, 247-256.
- Gach O., Nys M., Deby-Dupont G., Chapelle J.P., Lamy M., Piérard L.A. and Legrand V. (2006). Acute neutrophil activation in direct stenting: comparison of stable and unstable angina patients. *Int. J. Cardiol.* 112, 59-65.
- Gerrity R.G. (1981a). The role of the monocyte in atherogenesis I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am. J. Pathol.* 103, 181-190.
- Gerrity R.G. (1981b). The role of the monocyte in atherogenesis II. Migration of foam cells from atherosclerotic lesions. *Am. J. Pathol.* 103, 191-200.
- Guzik T.J., Sadowski J., Guzik B., Jopek A., Kapelak B., Przybylowski P., Wierzbicki K., Korb R., Harrison D.G. and Channon K.M. (2006). Coronary artery superoxide production and nox isoform expression in human coronary artery disease. *Arterioscler. Thromb. Vasc. Biol.* 26, 333-339.
- Hansson G.K. (2001). Immune mechanisms in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 21, 1876-1890.
- Hansson G.K. (2005). Inflammation, atherosclerosis, and coronary artery disease. *N. Engl. J. Med.* 352, 1685-1695.
- Hirai K., Moriguchi K. and Wang G.Y. (1991). Human neutrophils produce free radicals from the cell-zymozan interface during phagocytosis and from the whole plasma membrane when stimulated with calcium ionophore A23187. *Exp. Cell Res.* 194, 19-27.
- Jonasson L., Holm J., Skalli O., Bondjers G. and Hansson G.K. (1986). Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis* 6, 131-138.
- Kobayashi T. and Robinson J.M. (1991). A novel intracellular compartment with unusual secretory properties in human neutrophils. *J. Cell Biol.* 113, 743-756.
- Kobayashi T., Garcia del Saz E., Hendry J. and Seguchi H. (1999). Detection of oxidant producing-sites in glutaraldehyde-fixed human neutrophils and eosinophils stimulated with phorbol myristate-acetate. *Histochem. J.* 31, 181-194.
- Kumamoto M., Nakashima Y. and Sueishi K. (1995). Intimal neovascularization in human coronary atherosclerosis: its origin and pathophysiological significance. *Hum. Pathol.* 26, 450-456.
- Kumon Y., Suehiro T., Hashimoto K. and Sipe J.D. (2001). Dexamethasone, but not IL-1 alone, upregulates acute-phase serum amyloid A gene expression and production by cultured human aortic smooth muscle cells. *Scand. J. Immunol.* 53, 7-12.
- Kumon Y., Hosokawa T., Suehiro T., Ikeda Y., Sipe J.D. and Hashimoto K. (2002). Acute-phase, but not constitutive serum amyloid A (SAA) is chemotactic for cultured human aortic smooth muscle cells. *Amyloid* 9, 237-241.
- Leclercq A., Houard X., Philippe M., Ollivier V., Sebbag U., Meilhac O. and Michel J.B. (2007). Involvement of intraplaque hemorrhage in atherothrombosis evolution via neutrophil protease enrichment. *J. Leukoc. Biol.* 82, 1420-1429.
- Migeotte I., Communi D. and Parmentier M. (2006). Formyl peptide receptors: A promiscuous subfamily of G protein-coupled receptors controlling immune responses. *Cytokine Growth Factor Rev.* 17, 501-519.
- Miller F.J. Jr, Gutterman D.D., Rios C.D., Heistad D.D. and Davidson B.L. (1998). Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. *Circ. Res.* 82, 1298-1305.
- Moreno P.R., Purushothaman K.R., Sirol M., Levy A.P. and Fuster V. (2006). Neovascularization in human atherosclerosis. *Circulation* 113, 2245-2252.
- Naruko T., Ueda M., Haze K., van der Wal A.C., van der Loos C.M., Itoh A., Komatsu R., Ikura Y., Ogami M., Shimada Y., Ehara S., Yoshiyama M., Takeuchi K., Yoshikawa J. and Becker A.E. (2002). Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation* 106, 2894-2900.
- Nijm J., Wikby A., Tompa A., Olsson A.G. and Jonasson L. (2005). Circulating levels of proinflammatory cytokines and neutrophil-platelet aggregates in patients with coronary artery disease. *Am. J. Cardiol.* 95, 452-456.
- Ohara E., Kumon Y., Kobayashi T., Takeuchi H. and Sugiura T. (2007). Exocytosis of neutrophil formyl peptide receptor-like 1 (fPRL1) results in downregulation of cytoplasmic fPRL1 in patients with purulent dermatitis. *Clin Vaccine Immunol.* 14, 678-684.
- Okada T., Kobayashi T., Hakoi K. and Seguchi H. (1987). A simple method for light microscopical visualization of phosphohydrolase activities in materials processed for cerium based ultra-cytochemistry. *Acta Histochem. Cytochem.* 20, 105-110.
- Rabiet M.J., Huet E. and Boulay Fx. (2005). Human mitochondria-derived N-formylated peptides are novel agonists equally active on FPR and FPRL1, while *Listeria* monocytogenes-derived peptides preferentially activate FPR. *Eur. J. Immunol.* 35, 2486-2495.
- Reape T.J. and Groot P.H. (1999). Chemokines and atherosclerosis. *Atherosclerosis* 147, 213-225.
- Schaffne T., Taylor K., Bartucci E.J., Fischer-Dzoga K., Beeson J.H., Glagov S. and Wissler R.W. (1980). Arterial foam cells with distinctive immunomorphologic and histochemical features of macrophages. *Am. J. Pathol.* 100, 57-80.
- Selvatici R., Falzarano S., Mollica A. and Spisani S. (2006). Signal transduction pathways triggered by selective formylpeptide

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- analogues in human neutrophils. *Eur. J. Pharmacol.* 534, 1-11.
- Stary H.C., Chandler A.B., Glagov S., Guyton J.R., Insull W., Rosenfeld M.E., Schaffer S.A., Schwartz C.J., Wagner W.D. and Wissler R.W. (1994). A definition of initial, fatty streak, and intermediate lesions of atherosclerosis: a report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler. Thromb. Vasc. Biol.* 14, 840-856.
- Stary H.C., Chandler A.B., Dinsmore R.E., Fuster V., Glagov S., Insull W., Rosenfeld M.E., Schwartz C.J., Wagner W.D. and Wissler R.W. (1995). A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis: a report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler. Thromb. Vasc. Biol.* 15, 1512-1531.
- Su S.B., Gong W., Gao J.L., Shen W., Murphy P.M., Oppenheim J.J. and Wang J.M. (1999). A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic cells. *J. Exp. Med.* 189, 395-402.
- van der Wal A.C., Becker A.E., van der Loos C.M. and Das P.K. (1994). Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation* 89, 36-44.
- Yokota J., Takuma D., Hamada A., Onogawa M., Yoshioka S., Kusunose M., Miyamura M., Kyotani S. and Nishioka Y. (2006). Scavenging of reactive oxygen species by *Eriobotrya japonica* seed extract. *Biol. Pharm. Bull.* 29, 467-471.

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