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Relationship Between Coronary Plaque Formation and NAD(P)H Oxidase-derived Reactive Oxygen Species - Comparison of Intravascular Ultrasound Finding of Atherosclerotic Lesions with Histochemical Characteristics-

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Background: Oxidative stress induced by reactive oxygen species (ROS) in the vessel wall plays an essential role in atherogenesis. Recently, we demonstrated that the generation of ROS via NAD(P)H oxidase was correlated with plaque instability using coronary specimens obtained by directional coronary atherectomy (DCA). In this study, the relation between plaque formation and ROS generation was studied based on pre-interventional examination with intravascular ultrasound (IVUS) and histological analysis of the corresponding DCA specimens.

Methods: Pre-interventional IVUS images of 29 patients were analyzed. Vessel cross-sectional area (CSA), lumen CSA, plaque CSA and % plaque area were obtained at the minimal lumen site. ROS generation and expression of NAD(P)H oxidase p22^{phox} in DCA specimens were evaluated by the dihydroethidium method and immunohistochemistry as the ratio of positive area versus total surface area in each specimen.

Results: ROS positive area ratio in DCA specimens was correlated positively with vessel CSA, plaque CSA, and % plaque area ($r = 0.51$, $p = 0.0046$; $r = 0.62$, $p = 0.0004$; $r = 0.60$, $p = 0.0006$, respectively). Further, p22^{phox} positive area ratio in DCA specimens was also correlated with vessel CSA and plaque CSA ($r = 0.47$, $p = 0.0095$; $r = 0.47$, $p = 0.0103$, respectively).

Conclusions: This *in vivo* study clearly demonstrates that NAD(P)H oxidase-derived ROS plays a significant role in the development of atherosclerosis.

Changes in size of coronary arteries occur as a response to progression of atherosclerotic plaques (7). Autopsy studies have shown that extension of plaque burden and enlargement of vessel size were likely correlated with the frequency of acute coronary syndromes (6,25). Recently, intravascular ultrasound (IVUS) provides detailed images of the vessel wall with a high frequency, and enables assessment of the morphology and distribution of atherosclerotic plaque *in vivo* (22,23,30). *In vivo* studies using IVUS have conformed that target lesions in

patients who have acute coronary syndrome more frequently exhibit a large plaque area and increased vessel size (28,29).

On the other hands, oxidative stress induced by reactive oxygen species (ROS) in the vessel wall plays an important role not only in atherogenesis but also in the instability of atherosclerotic plaques (17). NAD(P)H oxidase is a major source of ROS in vascular cells (8). This oxidase system was originally identified as the defense against exogenous microorganisms in phagocytes. It has become evident that this oxidase is an important enzymatic origin of ROS in vasculature. The NAD(P)H oxidase system is composed of at least 6 components: plasma membrane spanning cytochrome b558 composed of gp91^{phox} and p22^{phox}, the 3 cytosolic components p67^{phox}, p47^{phox}, p40^{phox}, and the small G protein rac (1,16), and it was reported that p22^{phox} is a common component between phagocytic and vascular NAD(P)H oxidases (3) and this small component is essential for the generation of ROS.

To investigate clinically the role of this vascular oxidase system in the development of atherosclerosis, we investigated the correlation of extension of plaque burden and vessel sizes with ROS generation via NAD(P)H oxidase. For this aim, we measured the extension of plaque burden and vessel size using IVUS prior to directional coronary atherectomy (DCA). Furthermore, the generation of ROS and expression of NAD(P)H oxidase p22^{phox} in the corresponding specimens obtained by DCA were examined.

MATERIALS AND METHODS

Patient Population

The study population consisted of 29 patients with angina pectoris who were treated with percutaneous coronary intervention (PCI) using DCA for a *de novo* lesion in the native coronary artery from May 1, 2000, to March 31, 2001 at Miki City Hospital. Clinical profile including age, gender, risk factors for coronary disease (Hypertension, hypercholesterolemia, diabetes mellitus, and smoking) and angiographic data were available from patient charts and the interventional database at our institution. The diagnosis of hypertension, hyperlipidemia, or diabetes is based on the criteria of guideline of Japan Society of Hypertension, Japan Atherosclerosis Society, or Japan Diabetes Society, respectively. Unstable angina pectoris was defined according to Braunwald's criteria (10), and angiographic appearances before DCA were evaluated by the classification reported by Ambrose *et al* (2).

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

IVUS system and procedure

Following baseline coronary angiography after intracoronary administration of 100 to 200 µg of nitroglycerine, IVUS imaging was performed before DCA using a commercially available 30- or 40-MHz ultrasound catheter (Boston Scientific Co., Natick, MA). The IVUS catheter was advanced > 10 mm beyond the lesion, and motorized pullback (0.5 mm/sec) was performed to a point > 10 mm proximal to the lesion during IVUS data acquisition. All IVUS images were recorded on half-inch, high resolution S-VHS videotape for off-line analysis.

IVUS analysis

IVUS images were digitized with commercially available software for IVUS image analysis, which runs on an Intel Pentium-based PC system with Windows NT (NetraIVUS TM, ScImage Inc., Los Altos, CA). Two independent operators, who were blinded to the clinical presentation and the histological results, analyzed the IVUS images. The target lesion was selected as the site with the smallest luminal area in the segment where DCA was

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performed. A pullback after DCA confirmed that the tissue was retrieved from this segment. At the site with the smallest luminal area, vessel, lumen, and plaque (vessel minus lumen) cross-sectional area (CSA) were measured (20). Percent plaque area was calculated as plaque CSA divided by vessel CSA. The intraobserver and interobserver correlation coefficients resulted in r-values of 0.99 and 0.97 for the lumen CSA, and r-values of 0.99 and 0.96 for the vessel CSA, respectively.

Histological analysis

After the DCA procedure, obtained tissues were immediately embedded in OCT compound (SAKURA Finetechnical Co.) in liquid nitrogen and stored at -80°C. To detect in situ generation of ROS in DCA specimens, fluorescence microtopography with dihydroethidium was performed as previously described (19). Briefly, unfixed frozen samples were cut into 5 µm-thick sections and placed on glass slides. Dihydroethidium (10 µmol/L) was applied to each tissue section, and then the sections were coverslipped. The slides were incubated in a light-protected humidified chamber at 37°C for 30 minutes. The image of dihydroethidium was obtained by a laser scanning confocal imaging system (MRC-1024, BioRad) with a 585-nm long-pass filter. Generation of ROS was demonstrated by red fluorescence labeling.

Immunofluorescence experiments were performed as previously described (3). Unfixed frozen samples were cut from a given sample and air-dried onto slides. Additional serial cryostat sections were stained with hematoxylin and eosin for analysis of morphological details by light microscopy. The tissue slices were fixed with 100% acetone at -20°C for 10 minutes. The sections were incubated with BSA (Dako LSAB kit, Dako A/S) for 60 minutes at room temperature and then incubated with primary antibody overnight at 4°C. The primary antibody used in the present study was rabbit polyclonal anti-human p22^{phox} antibody that was against synthetic peptide corresponding to its C-terminal region (residues 175 to 194). The specificity of this antibody has been reported previously (11,14). Texas red conjugated antiimmunoglobulin was applied as secondary antibody. The samples were examined by the laser scanning confocal imaging system. The presence of p22^{phox} was demonstrated by red immunofluorescence labeling.

Three independent pathologists who were blinded to the identities of patients examined the DCA samples. The intraobserver and interobserver variations were assessed by the method of Bland and Altman (5). The intraobserver and interobserver comparisons revealed high correlations (r=0.90 to 0.95), and there was no significant variation in intraobserver and interobserver data.

Statistical analysis

Statistical analysis was performed with StatView 5.0 (SAS Institute, Cary, NC). Continuous variables are expressed as mean ± 1 SD. Linear regression analyses were performed between IVUS measurements and histological parameters including ROS-positive and p22^{phox}-positive areas. P value <0.05 was considered statistically significant.

RESULTS

Patient Characteristics

Baseline patient characteristics for the 29 patients are shown in Table 1. In this cohort, 16 patients had unstable angina pectoris. Target lesion for DCA was left anterior descending in 21 patients, left circumflex in 2 patients, and right coronary in 6 patients.

TABLE 1. Baseline patient and lesion characteristics

Patients, n	29
Age, y	63.7 ± 10.4
Men	25 (86.2%)
Hypertension*	12 (41.4%)
Hypercholesterolemia**	13 (44.8%)
Diabetes***	10 (34.5%)
Smoking	15 (51.7%)
Clinical presentation	
Unstable angina	16 (55.2%)
Stable angina	13 (44.8%)
Target coronary artery	
Left anterior descending	21 (72.4%)
Left circumflex	2 (6.9%)
Right	6 (20.7%)
Angiographic stenosis of DCA sites (%)	86.3 ± 8.2
Angiographic appearance	
Concentric narrowing	8 (27.6%)
Type 1 eccentric (asymmetric with smooth border)	11 (37.9%)
Type 2 eccentric (asymmetric with irregular border)	5 (17.2%)
Multiple irregular narrowing	5 (17.2%)

Data are presented as mean ± 1 SD or number of patients/arteries (percentage).

* Hypertension was defined as a systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or use of an antihypertensive drug.

** Hypercholesterolemia was defined as a total cholesterol level ≥240 mg/dl or medication use.

*** Diabetes was defined as diet-controlled and oral agent-treated or insulin-treated.

Quantitative IVUS Findings

Mean Vessel CSA, mean lumen CSA, mean plaque CSA, and mean % plaque CSA were 16.0 ± 5.3 mm², 2.7 ± 1.3 mm², 13.2 ± 4.9 mm², and 82.1 ± 8.2 %, respectively (Table 2).

TABLE 2. Intravascular ultrasound measurements of lesion

Vessel CSA (mm ²)	16.0 ± 5.3
Lumen CSA (mm ²)	2.7 ± 1.3
Plaque CSA (mm ²)	13.2 ± 4.9
% plaque CSA (%)	82.1 ± 8.2

Data are presented as mean ± 1 SD.

CSA: cross-sectional area

ROS generation and NAD(P)H oxidase p22^{phox} expression in DCA specimens

Figure 1 shows representative micrographs of fluorescence images with dihydroethidium for detection of in situ generation of ROS and of immunostaining of p22^{phox} in DCA specimens. Mean ROS positive area ratio in DCA specimens of all cases was 0.10 ± 0.09 , and mean p22^{phox} positive area ratio in DCA specimens was 0.08 ± 0.06 .

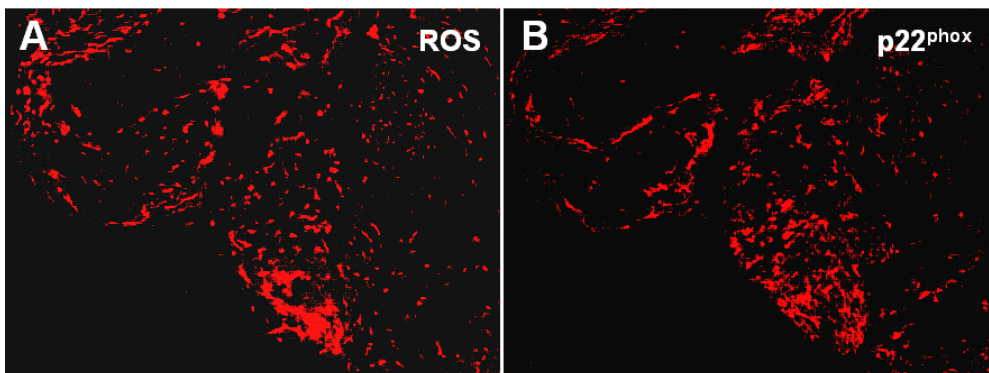


FIG 1. Example of immunohistochemical examinations of DCA specimens

A: fluorescence images with dihydroethidium for detection in situ generation of ROS in DCA specimens

B: immunostaining of p22^{phox} in DCA specimens.

DCA: directional coronary atherectomy; ROS: reactive oxygen species

Correlations of IVUS parameters of the lesions with ROS generation and p22^{phox} expression in DCA specimens

ROS positive area ratio in DCA specimens was correlated positively with vessel CSA, plaque CSA, and % plaque area ($r = 0.51$, $p = 0.0046$; $r = 0.62$, $p = 0.0004$; $r = 0.60$, $p = 0.0006$, respectively, Figure2). Further, p22^{phox} positive area ratio in DCA specimens was also correlated with vessel CSA and plaque CSA, although correlations were weak in comparison with those of ROS positive area ratio ($r = 0.47$, $p = 0.0095$; $r = 0.47$, $p = 0.0103$, respectively, Figure3). Neither ROS positive area ratio nor p22^{phox} positive area ratio in DCA specimens was correlated with lumen CSA. Representative cases are shown in Figure 4. ROS positive area ratio and p22^{phox} positive area ratio in DCA specimens from the lesion with larger vessel CSA and plaque CSA were greater than those in DCA specimens from the lesion with smaller vessel CSA and plaque CSA.

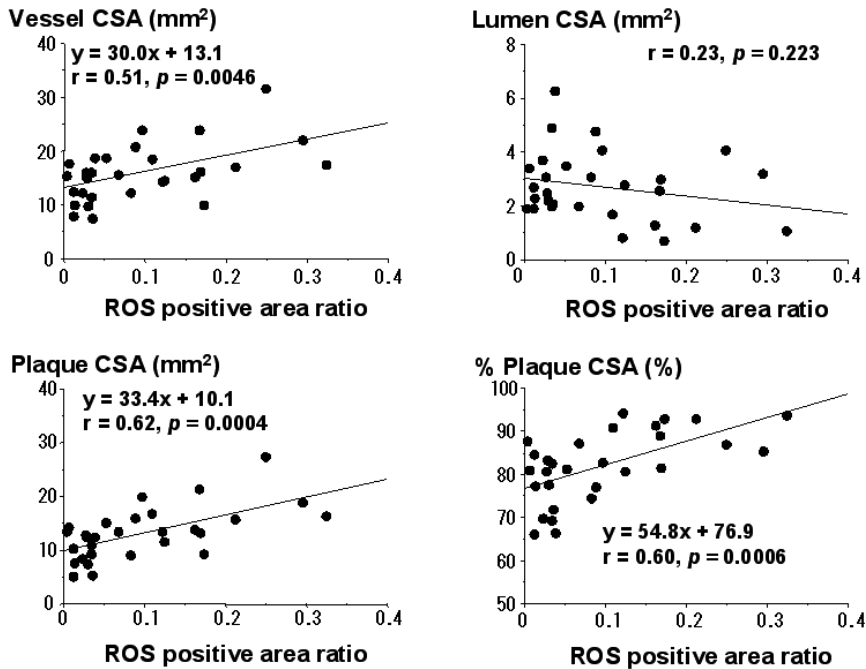


FIG 2. Correlations of ROS positive area ratio in DCA specimens with IVUS measurements
 ROS: reactive oxygen species; DCA: directional coronary atherectomy; IVUS: intravascular ultrasound; CSA: cross sectional area

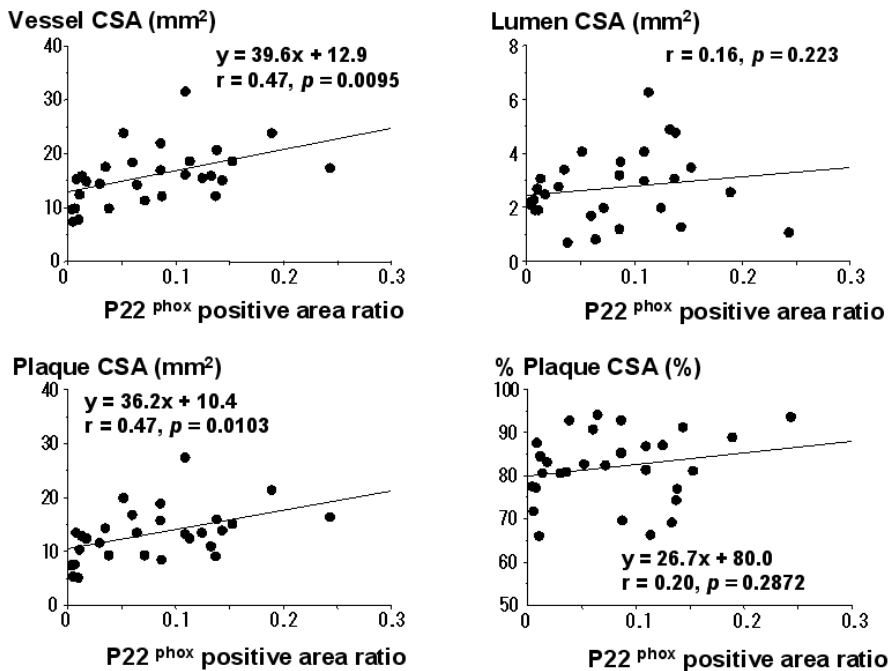


FIG 3. Correlations of p22^{phox} positive area ratio in DCA specimens with IVUS measurements
 ROS: reactive oxygen species; DCA: directional coronary atherectomy; IVUS: intravascular ultrasound; CSA: cross sectional area

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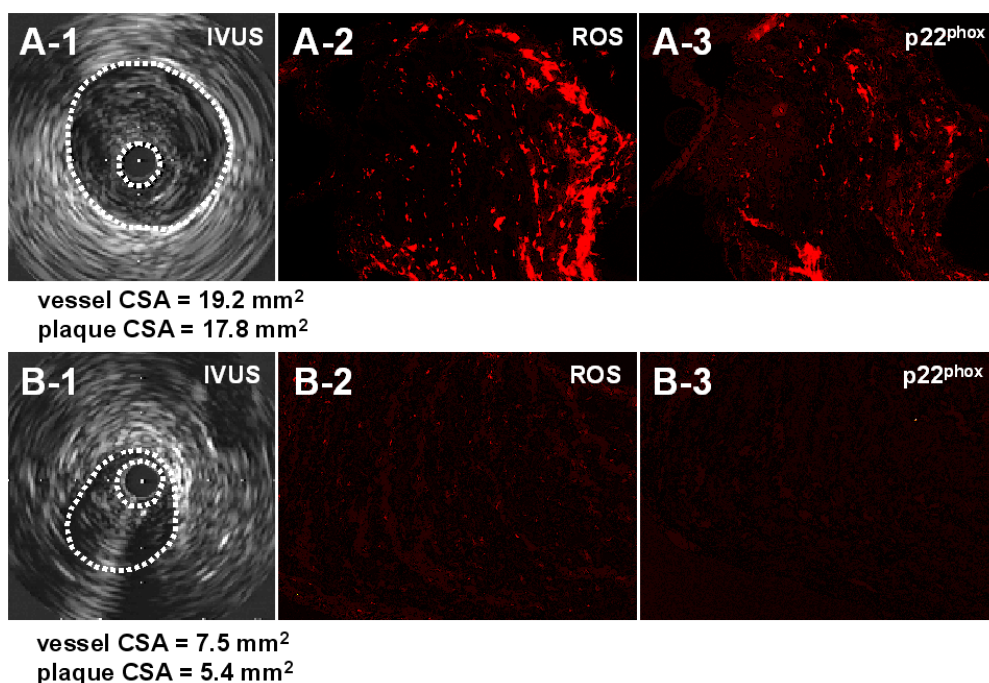


FIG 4. Representative IVUS images and immunohistochemical examinations of DCA specimens

A-1: IVUS image of the lesion with larger vessel and plaque CSA

A-2: fluorescence image with dihydroethidium for detection in situ generation of ROS in a DCA specimen from the lesion of A-1

A-3: immunostaining of p22^{phox} in DCA specimens in a DCA specimen from the lesion of A-1

B-1: IVUS image of the lesion with smaller vessel CSA and plaque CSA.

B-2: fluorescence image with dihydroethidium for detection in situ generation of ROS in a DCA specimen from the lesion of B-1

B-3: immunostaining of p22^{phox} in DCA specimens in a DCA specimen from the lesion of B-1

DCA: directional coronary atherectomy; ROS: reactive oxygen species; IVUS: intravascular ultrasound; CSA: cross sectional area

DISCUSSION

In the present study, atherosclerotic plaque specimens from 29 patients, obtained by DCA that performed after pre-interventional IVUS examination, were immunohistochemically analyzed. We found that ROS positive area ratio in DCA specimens was positively correlated with vessel CSA, plaque CSA, and % plaque area. There are accumulating evidences that the redox state is a critical determinant for cellular response and oxidative stress plays an important role in pathogenesis of various diseases. Our observations confirm significant roles of oxidase stress induced by ROS in atherosclerotic plaque burden and atherogenesis in patients with coronary artery disease.

Atherosclerosis is a complex process and atherosclerotic lesion is composed of various cell types such as smooth muscle cells, fibroblasts, and inflammatory cells such as macrophages and activated T lymphocytes. Besides these cells, there is extracellular matrix in atherosclerotic lesions, and dysregulation of extracellular matrix protein plays a role in atherogenesis. In the present investigation, the generation of ROS in coronary arteries is significantly correlated with % plaque area. This finding suggests the possibility that ROS

promote the growth and proliferation of cell types described above. Indeed, previous investigations demonstrated that enhanced ROS activate redox-sensitive signal transduction pathways, which induce expression of atherogenic gene products such as adhesion molecules and other vascular pro-inflammatory gene products (27). Furthermore, clinical investigations demonstrated that well-established coronary risk factors, including hyperlipidemia and diabetes, are associated with enhanced vascular ROS (9,24). Taken together, it is interesting to speculate that ROS induced by these coronary risk factors turns on redox-sensitive pathway, which results in the formation of atherosclerotic plaques. It is also demonstrated that generated ROS activate MMPs in cultured vascular cells (13). In human or animal models of atherosclerosis, varying MMPs have been demonstrated to increase in the atherosclerotic lesions. Several reports suggested that MMPs contribute to progression of atherosclerosis and vessel enlargement (15). Given a significant role of ROS in MMPs regulation, dysregulation of extracellular matrix by MMPs might contribute to plaque burden in patients with coronary artery disease.

Recent evidences suggest that NAD(P)H oxidase plays a crucial role in the generation of ROS in vascular cells (21,26). We previously reported that polymorphism of the NADPH oxidase p22^{phox} gene is a novel coronary risk marker for coronary artery disease (12). Furthermore, we demonstrated that the production of ROS was closely associated with the expression of p22^{phox} by means of double staining of ROS and p22^{phox} in DCA specimens, and that the ROS generation via NAD(P)H oxidase in the vessel wall plays an important role not only in the progression of coronary atherosclerosis but also in the instability of atherosclerotic plaques (4). Thus, NAD(P)H oxidase p22^{phox} is likely a key molecule linking oxidative stress and cardiovascular diseases. In the present investigation, NAD(P)H oxidase p22^{phox} positive area ratio in DCA specimens was significantly correlated with vessel CSA and plaque CSA although these correlations were weak in comparison with those of ROS positive area ratio. These results suggest that NAD(P)H oxidase-derived ROS generation might be associated with development of atherosclerotic plaque and vessel enlargement in patients with coronary artery disease (8,18). Potential explanations for relative weakness of correlations between p22^{phox} positive area ratio and IVUS parameters in comparison with those of ROS positive area ratio include several enzymatic origins of ROS other than NAD(P)H oxidase, such as cyclooxygenase, lipoxygenase, xanthine oxidase, myeloperoxidase, and NO synthase. These other enzymes might contribute to atherogenesis to some extents.

Our investigation presents some limitations. First, samples of this study were obtained from lesions with clinically significant stenosis, and may not always reflect focal processes in such as a rupture-prone plaque without clinically significant stenosis. Second, in the present study, the generation of ROS was assessed by microtopography with dihydroethidium. Several other ways for the detection of ROS, e.g., lucigenin-enhanced chemiluminescence, electron spin resonance, and the cytochrome c reduction method, have been reported. Each has merit and demerit regarding its sensitivity, specificity, and convenience to handle. Although it is ideal to evaluate the generation of ROS by several different methods, we confirmed that there was a good correlation between values measured by microtopography with dihydroethidium and values measured by lucigenin-enhanced chemiluminescence (4).

In conclusion, this *in vivo* study suggested that NAD(P)H oxidase-derived ROS may have a significant role in the development of atherosclerosis. Our *in vivo* findings indicate that vascular NAD(P)H oxidase might be one of potential therapeutic targets for coronary artery disease.

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