Inhibitory effect of omega-3 fatty acids on alveolar bone resorption and osteoclast differentiation

Yu Ozaki1), Toshiya Morozumi1), Kiyoko Watanabe2), Yoshito Toyama3), Haruka Sasaki2), Takenori Sato4), Yuko Yamamoto5), Masahiro To5), Keitaro Inaba2), Keiichi Tsukinoki6), Nobushiro Hamada2), and Masato Minabe2)

1) Division of Periodontology, Department of Oral Interdisciplinary Medicine, Graduate School of Dentistry, Kanagawa Dental University, Yokosuka, Japan
2) Division of Oral Microbiology, Department of Oral Science, Graduate School of Dentistry, Kanagawa Dental University, Yokosuka, Japan
3) Division of Oral Biochemistry, Department of Oral Science, Kanagawa Dental University, Yokosuka, Japan
4) Division of Dental Anatomy, Department of Oral Science, Graduate School of Dentistry, Kanagawa Dental University, Yokosuka, Japan
5) Division of Environmental Pathology, Department of Oral Science, Graduate School of Dentistry, Kanagawa Dental University, Yokosuka, Japan

Abstract: In this study, a Porphyromonas gingivalis (P. g.)-infected mouse periodontitis model was used to investigate the effect of omega-3 fatty acid intake on differentiation and maturation of cultured osteoclast. Four-week-old C57BL/6Jccl mice were divided into four groups according to the diets they were fed from the beginning of the experiment (i.e., food containing omega-3 or omega-6 fatty acids) and whether they were orally administered P. g. Thirty-three days after beginning the experiment, bone marrow cells were sampled from the femoral bone of mice from each group and differentiated into osteoclasts; the effects of the ingestion of different fatty acids were subsequently investigated. There was no statistical interaction between the different fatty acids and P. g. infection on the number of osteoclasts (P = 0.6). However, the fatty acid type affected the number of osteoclasts in mice (P = 0.0013), with the omega-3 groups demonstrating lower osteoclast numbers than the omega-6 groups. Furthermore, the addition of resolvin E1 (RvE1), which is an omega-3 fatty acid-derived lipid mediator, suppressed the differentiation of mouse cultured osteoclasts (P < 0.0001). Therefore, the ingestion of omega-3 fatty acids may suppress osteoclast differentiation while inhibiting bone resorption and tissue destruction due to periodontitis.

Keywords: inflammation, mouse, omega-3 fatty acids, osteoclast, periodontitis

Introduction

In recent years in Japan, it has become clear that, in addition to age-related and genetic factors, lifestyle habits such as an inappropriate diet and lack of exercise are strongly involved in malignant neoplasms, heart disease, and cerebrovascular disease [1]. These three diseases account for more than half of the deaths nationally [2]. Chronic inflammation is the basis for lifestyle diseases including diabetes and periodontitis, which are involved in triggering and aggravating heart and cerebrovascular diseases. One cause of these diseases appears to be a disruption in the inflammation resolution mechanism, whereby the inflammatory response induced in vivo is not appropriately resolved [3-5].

Periodontitis is a type of localized, chronic inflammation that is caused by specific bacteria. It is characterized by dental tissue breakdown and alveolar bone resorption due to neutrophils and macrophages. Lipopolysaccharides (LPS) from periodontal pathogenic bacteria induce bone marrow cells to differentiate into osteoclasts [6]. Recently, it has been reported that Porphyromonas gingivalis (P. g.)-LPS is transported throughout the body to tissues via the circulatory system [7]. Furthermore, it has been elucidated that the majority of tissue destruction in periodontitis is caused by an inflammatory reaction as a part of the host immune response to infection, rather than direct action against the source of infection [8]. These reports have focused on the effect of periodontal disease on the condition of the body [6-8]. Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, attenuate inflammation by impeding the action of cyclooxygenases in the arachidonic acid cascade to inhibit the generation of prostaglandins [9]. Although it has been reported that orally and locally administered NSAIDs inhibit the alveolar bone resorption accompanying periodontitis [10], the use of NSAIDs for this purpose has not been applied clinically due to adverse reactions and the need for long-term administration [8]. Golub et al. reported that, in addition to its conventionally known antibacterial effects, tetracycline can suppress the progression of periodontitis and rheumatoid inflammation by inhibiting collagenase activity [11-13]. However, as these pharmaceutical effects are only temporary and cause adverse reactions, it is not appropriate to administer tetracycline for the long-term prevention of periodontitis.

Omega-3 fatty acids are a group of unsaturated fatty acids with a double bond three carbon atom from the terminal methyl group. Omega-3 fatty acids include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [14]. Arachidonic acid, an omega-6 fatty acid (unsaturated fatty acid with a double bond six carbon atoms away from the terminal methyl group), is a precursor for activated eicosanoids such as prostaglandins, thromboxane, and leukotrienes [15]. It has been reported that these eicosanoids aggravate conditions such as periodontitis, diabetes, and arteriosclerosis [16,17]. Omega-3 fatty acids are also garnering attention as oral treatments because they do not cause marked adverse reactions like those seen with NSAIDs and tetracycline [18]. It has been reported that omega-3 fatty acids and their metabolites guide the inflammatory response toward the resolution stage, thereby preventing the development of chronic inflammatory diseases such as arteriosclerosis and diabetes [19]. In periodontal treatment, clinical trials are underway for the combined use of omega-3 fatty acids with host-modulation therapy. This combined therapy aims to improve the patient’s immune response rather than remove oral bacteria, which is the focus of conventional therapy [8].

In recent years, resolvins and protectins have been identified as novel compounds originating from omega-3 fatty acids that exhibit strong anti-inflammatory activities. These compounds are gaining attention as anti-inflammatory fat mediators responsible for the inflammatory suppression mechanism of omega-3 fatty acids [20]. Resolvins and protectins are in vivo metabolites derived from EPA and DHA, both of which are omega-3 fatty acids. In acute periodontitis caused by yeast zymosan, these substances suppress neutrophil invasion and inhibit the production of inflammatory cytokines while promoting the phagocytic activity of macrophages and their movement into lymph; thus, these substances promote the resolution of acute inflammation [21].

Many reports have indicated that EPA and DHA, found in large amounts in fish oil, exhibit anti-inflammatory activity in diseases such as periodontitis, for which the cardinal symptom is chronic inflammation. Naqvi et al. reported that, in 9,182 subjects, the prevalence of periodontitis was lower following consumption of DHA and EPA, whereas that

Correspondence to Dr. Toshiya Morozumi, Division of Periodontology, Department of Oral Interdisciplinary Medicine, Graduate School of Dentistry, Kanagawa Dental University, 82 Inaoka-Cho, Yokosuka, Kanagawa 238-8580, Japan
Fax: +81-46-822-8855 E-mail: morozumi@kdu.ac.jp
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for alpha-D-configured locked nucleic acid (Alpha-LNA) was higher [22]. However, Eberhard et al. reported that the local administration of EPA and DHA to models of experimental gingivitis did not significantly reduce inflammation [23]. Moreover, in a periodontitis model in which P. g. was administered to the gingival sulcus of rabbits, the administration of resolvin E1 (RvE1) suppressed local periodontal inflammation and alveolar bone resorption [24,25]. These reports suggest that omega-3 fatty acids promote the resolution of inflammation including the inhibition of cytokine production, by being absorbed within the body and undergoing metabolism, thereby suppressing osteoclast differentiation.

In the present study, mice fed a diet containing omega-3 and omega-6 fatty acids were used to investigate the effect of ingesting different fatty acids on the state of periodontitis and the induction of osteoclast differentiation in the alveolar bone. In addition, the effects of P. g. infection and intake of different fatty acids on osteoclast differentiation in bone marrow cells were examined. To clarify whether omega-3 fatty acids suppress bone resorption, the suppression of the differentiation of cultured osteoclasts by RvE1 (a metabolite of EPA) was also investigated.

### Materials and Methods

**Animals**

C57BL/6Jcl mice [4-week-old, male, CLEA Japan (Tokyo, Japan)] were used. Thirty-two mice were randomly allocated into an omega-3 group and an omega-6 group. Both groups were then further subdivided into two groups, P. g.-infected and non-infected, forming a total of four groups. For the experiment, the mice were housed using 12 h light and dark cycles with the room temperature adjusted to 22 ± 3°C. The mice had ad libitum access to food and water. In addition to these 32 mice, 2 C57BL/6Jcl mice (5-week-old, male; CLEA, Japan) were used for investigating the differentiation-inhibiting effects of RvE1, a metabolite of EPA, on osteoclasts. This experiment conformed to the Kanagawa Dental University Animal Experiment Standards and the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain. The protocol for this study was screened and approved by the Kanagawa Dental University Animal Experiment Institutional Review Board (approval no. 15-026).

**Diets**

The compositions of the regular diet and experimental diets are presented in Table 1. Menhaden oil (fish oil) was added to a D12450K base (Research Diets) for preparing the omega-3 fatty acid diet, and sunflower oil was added to the same base for preparing the omega-6 fatty acid diet. The diets were sealed in a vacuum pack at the production stage and stored at −20°C.

| Ingredient                        | Omega-3 fatty acid diet | Omega-6 fatty acid diet |
|-----------------------------------|-------------------------|-------------------------|
| Sucrose                           | 0.00                    | 0.00                    |
| Casein                            | 203                     | 203                     |
| L-Cystine                         | 3.00                    | 3.00                    |
| Corn starch                       | 434                     | 434                     |
| Malodextrin                       | 152                     | 152                     |
| Cellulose                         | 50.6                    | 50.6                    |
| Mineral mixture                   | 10.1                    | 10.1                    |
| Dicalcium phosphate               | 13.0                    | 13.0                    |
| Calcium carbonate                 | 5.60                    | 5.60                    |
| Potassium citrate                 | 16.7                    | 16.7                    |
| Vitamin mixture                   | 10.0                    | 10.0                    |
| Choline bitartrate                | 2.0                     | 2.0                     |
| Menhaden oil                      | 100                     | -                       |
| Sunflower oil                     | -                       | 100                     |
| Total (g)                         | 1,000                   | 1,000                   |
| Omega-3 (g/kg fat)                | 407                     | 5                       |
| Omega-6 (g/kg fat)                | 27                      | 636                     |

**Bacterial strains and culture conditions**

The P. g. ATCC 33277 strain was cultured in brain heart infusion broth (BHI broth; Difco, Detroit, MI, USA) containing yeast extract (5 mg/mL), hemin (5 μg/mL), and vitamin K1 (0.5 μg/mL) or in BHI blood agar medium containing 5% sheep defibrinated blood under anaerobic conditions (85% N2, 10% H2, 5% CO2) at 37°C.

**Induction of experimental periodontitis**

The P. g.-infected group was treated according to the schedule in Fig. 1. Before inducing experimental periodontitis, all mice were provided drinking water (1 mg/mL sulfamethoxazole and 200 μg/mL trimethoprim in distilled water) from day 8 after acquisition of the animals. This drinking water was administered for 4 days to reduce normal oral bacterial counts. Next, distilled water that did not contain antibiotics was provided for 3 days to remove all remaining antibiotic substances in the animals’ mouths. Following this, a suspension containing P. g. (1.0 × 10^9 CFU/mL) that was adjusted with 2.5% carboxymethyl cellulose (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) was directly administered into the oral cavities of the mice for a total of three doses. The non-infected group was only administered the 5% carboxymethyl cellulose solution.

**Measurement of alveolar bone resorption**

The right side of the mouse maxillary bones was used to measure the amount of alveolar bone resorption. The maxillary bones were excised from the mice, heated for 10 min in 1.522 mmHg [26], and soaked in 3% sodium hypochlorite solution to remove soft tissue. The maxillary bones were then dried after the alveolar bone was stained with 1% methylene blue. The distance from the upper molar cementoenamel junction to the alveolar crest of each sample was measured seven times, and the mean value of those measurements was used as an index for the amount of alveolar bone resorption [27]. Measurements were performed using a dissecting microscope (40× magnification) with a high-resolution digital measurement system (Digital HD microscope VH-7000; Keyence, Osaka, Japan). All measurement units are in millimeters.

**Tissue preparation for tartrate-resistant acid phosphatase (TRAP) staining**

The tissue of the first to third molars, alveolar bone, and surrounding tissue from the left maxilla were soaked overnight in 10% formalin (pH 7.4), washed with 0.1 M PBS, and decalcified for approximately 4 weeks in 15% EDTA-30% sucrose solution. The tissue slices were again washed with 0.1 M PBS and embedded in paraffin at appropriate positions so that the tooth and sectioning surfaces were parallel. Serial, 4 μm-thick sections in the mesiodistal direction were then produced with a microtome.

Staining was performed for TRAP using the most buccolingually central section of each tooth. Staining was performed using a TRAP staining kit (TRAP Staining Kit; COSM0 BIO, Tokyo, Japan) according to the manufacturer’s instructions. Cells that were darkly stained red (TRAP-positive cells) were defined as osteoclasts. An optical microscope (Nikon, Tokyo, Japan) was used to examine the sections at 40× magnification to analyze alveolar bone resorption and the distribution of TRAP-positive cells. Cells that were TRAP-positive and had a nuclear number of ≥3 were deemed...
to be osteoclasts. The number of osteoclasts distributed on the alveolar surface adjacent to the first molar distal root was determined.

Analysis of osteoclast differentiation capacity
Differences in bone marrow cells collected from the mice in each group to osteoclasts was induced according to the method reported by Rahman et al. [28]. Fourteen days after the third Pg. infection (day 33 of the experiment), bone marrow cells sampled from the femoral bones of the mice from each group were cultured in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) in order to induce differentiation to osteoclasts. In a previous mouse study investigating osteoporosis, bone marrow cells were collected from mice tibia 14 days following ovarietomies [29]. Furthermore, it has been reported that EPA and DHA concentrations in rat plasma increased from mice tibia 14 days following ovariectomies [29]. Moreover, it has been assumed that EPA and DHA concentrations in rat plasma increased following the intake of fish oil [30]. Therefore, in the present study, bone marrow cells were collected from the femurs of mice 14 days after the third Pg. infection. Mouse bone marrow cells adjusted to 1.0 × 10^6 cells/mL in a 48-well plate were cultured in Minimum Essential Medium Eagle-Alpha Modification (α-MEM, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone, GE Healthcare, Chicago, IL, USA). M-CSF (100 ng/mL) and RANKL (200 ng/mL; Sigma-Aldrich) were added, and the cells were cultured for 5 days at 37°C in a 5% CO₂ environment.

This investigation of the differentiation-inhibiting effects of RvE1, a metabolite of EPA, on osteoclasts was conducted in accordance with the method reported by Herrera et al. [31]. The bone marrow cells of 2 C57BL/6J mice (5-week-old, male) were differentiated into osteoclasts in the presence of various concentrations of RvE1. Specifically, the bone marrow cells (1.0 × 10^6 cells/mL) were seeded in a 96-well plate with 100 ng/mL of M-CSF and RANKL in α-MEM containing 10% fetal bovine serum. RvE1 (Resolvin E1 Sodium Salt; TRC, North York, Canada) was added to reach final concentrations of 0, 0.01, 0.1, and 1 ng/mL, and the cells were cultured for 5 days. The final concentration of methanol carrier was ≤1%.

TRAP staining of cultured cells
After culturing, the differentiated osteoclasts were fixed in 10% paraformaldehyde. TRAP staining was performed using the method mentioned in section 6 above, and the osteoclasts, defined as TRAP-positive cells with three or more nuclei, were counted.

Statistical analysis
All statistical analyses were performed using JMP version 12 (SAS Institute Japan, Tokyo, Japan). The results are expressed as the mean ± standard errors. Outliers were rejected based on quartiles. Levene’s test was used to confirm equal variances, and factorial analysis of variance (ANOVA) was used to identify the two main effects and the interaction effects. Tukey’s multiple comparisons for the interaction were used for post hoc analysis when the interaction was significant. For statistical analysis related to the inhibitory effects of RvE1 on osteoclast differentiation, multiple comparisons between the control and experiment groups were performed using Dunnett’s multiple comparison test. A P < 0.05 was set as the significance level.

Results
Effects of different fatty acids and Pg. infection on alveolar bone resorption in mice
Based on Levene’s test, the homoscedasticity between the data was assumed (P > 0.083; Fig. 2a). Statistical interactions between the effects of fatty acid composition and Pg. infection were observed for alveolar bone loss (P = 0.0035; two-way ANOVA, Fig. 2). Alveolar bone loss was higher in the omega-6 Pg. + group than in the other groups (P < 0.05, Tukey’s multiple comparisons, Fig. 2a, 2b). In the omega-3-fed groups, no difference was found between the Pg. + and Pg. − groups (Fig. 2a, b).

Mesiodistal histopathological analysis of the upper first molar root
Figure 3 shows TRAP-stained images of decalcified maxillary bone sections. The alveolar bone surfaces of the non-infected groups were found to have relatively smooth borders. Only a small number of TRAP-positive cells and bone resorption pits (approximately the same number) were observed. The TRAP-stained images of the infected groups revealed markedly more TRAP-positive cells and bone resorption pits in the omega-6 Pg. − group compared with those in the omega-6 Pg. + group. Additionally, the alveolar bone surfaces were found to have irregular borders. However, approximately the same number of TRAP-positive cells and bone resorption pits were observed in the omega-3 Pg. + group and the omega-3 Pg. − group, and the alveolar surface borders were smooth. Representative images are shown.
Effects of different fatty acids and \textit{P. g.} infection on osteoclast numbers in mice

Based on Levene’s test, the homoscedasticity between data was assumed ($P > 0.19$, Fig. 4a). No statistically significant interactions were observed between the effects of fatty acid composition and \textit{P. g.} infection on the number of osteoclasts ($P = 0.6$, two-way ANOVA, Fig. 4a), which indicated that the fatty acid composition and \textit{P. g.} infection did not elicit synergistic or offset effects on the number of osteoclasts. However, there was a difference in the number of osteoclasts between the two fatty acid treatments ($P = 0.0013$, two-way ANOVA, Fig. 4a), with fewer osteoclasts in the omega-3 groups than in the omega-6 groups (Fig. 4a, b).

Effects of RvE1 treatment on mouse osteoclast numbers

The number of osteoclasts was lower in the 0.01, 0.1, and 1 ng/mL RvE1 groups than in the group without RvE1 treatment ($P < 0.0001$, $P < 0.0001$, and $P < 0.0001$, respectively, Dunnett’s test, Fig. 5a). Images of TRAP-stained osteoclasts demonstrated that treatment with RvE1 at concentrations of 0.01, 0.1, and 1 ng/mL inhibited osteoclast fusion and multinucleation compared with when RvE1 was not added (Fig. 5b).

Discussion

It has been reported that the ingestion ratio of omega-3 fatty acids to omega-6 fatty acids, the latter of which are inflammatory eicosanoid substances, greatly affects atherosclerosis and inflammatory diseases [25,32]. The modern Western diet is composed of omega-3 and omega-6 fatty acids, the latter of which are inflammatory eicosanoid sub-

Bone resorption [33-35]. In the present study, the comparison of alveolar bone resorption resulting from periodontitis experimentally induced by \textit{P. g.} infection in mice fed a diet containing omega-3 versus omega-6 fatty acids indicated that alveolar bone resorption was markedly inhibited in the omega-3 group (Fig. 2a, b). There are two possible mechanisms of action for the inhibition of alveolar bone resorption by omega-3 fatty acid ingestion. The first possibility is that orally ingested omega-3 fatty acids act directly on the local periodontal site of inflammation to resolve such inflammation or that alveolar bone resorption is inhibited by the antimicrobial activity of the omega-3 fatty acids against the orally administered \textit{P. g.} bacteria. The second possibility is that orally ingested omega-3 fatty acids or their metabolites inhibit inflammation or osteoclast differentiation at periodontal sites of inflammation. Bendyk et al. reported that the amounts of EPA and DHA in the oral soft tissue of mice fed food containing omega-3 fatty acids were significantly increased compared with those in mice fed a diet containing omega-6 fatty acids [35]. It has also been reported that, in an \textit{in vivo} experiment, adding EPA or DHA, which are both omega-3 fatty acids, to cultured osteoclasts at concentrations of 20, 40, or 80 μM inhibited osteoclast differentiation [36]. In contrast, Eberhard et al. reported that administering EPA or DHA to intraoral sites of inflammation by means of gargling did not inhibit inflammation [23]. Choi et al. reported that the amount of omega-3 fatty acids contained in food ingested in 1 day is insufficient for direct antimicrobial activity against oral bacteria [37]. The present study also revealed no significant antibacterial activity against \textit{P. g.} by omega fatty acids in food (data not shown). Accordingly, this study’s results suggest that the inhibition of experimental periodontitis by omega-3 fatty acids is caused by the inhibition of \textit{in vivo} alveolar bone resorption by the ingested fatty acids and/or their metabolites.

The bone marrow cells that were sampled from mice in each group after 33 days were induced to differentiate into osteoclasts with M-CSF and RANKL. The number of induced osteoclasts was significantly lower in the mice fed omega-3 fatty acids than in those fed omega-6 fatty acids (Fig. 4a, 4b). In an experiment using ovariectomized rats, Nakanishi et al. reported...
that the tibias of the rats fed diets containing fish oil exhibited suppressed expression of M-CSF and receptor activator of NF-κB (RANK) mRNA, which play important roles in osteoclast differentiation [30]. While further investigation is necessary, our study’s results appear to support this finding, indicating the possible suppression of osteoclast differentiation factors. In the present study, no statistical interactions were observed between the different fatty acids and Pg. infection with respect to the number of osteoclasts differentiated from bone marrow cells collected from the femur (Fig. 4a, b). However, in the alveolar bone, there were more osteoclasts in the omega-6 Pg− group than in the omega-6 Pg+ group (Fig. 3). Moreover, in the Pg−-infected mouse model, Pg. infection did not affect systemic osteoclast differentiation; however, it may promote alveolar bone osteoclast differentiation. Thus, intake of omega-3 fatty acids should serve to inhibit alveolar bone osteoclast differentiation through systemic effects.

Finally, it is important to note that although RvE1 acts as an anti-inflammatory agent, it is also inhibitory in a rat activity. Rather, it exhibits osteoclast differentiation model, the local administration of RvE1, an anti-inflammatory metabolite of EPA, to sites of inflammation locally inhibited inflammation and alveolar bone resorption [24]. Accordingly, the inhibition of alveolar bone resorption and osteoclast differentiation in experimental periodontitis by ingestion of a diet containing omega-3 fatty acids appeared to be related to the anti-inflammatory action of RvE1. Therefore, based on previously reported concentrations at which neuphil infiltration in response to acute inflammation in the peripheral cavities of mice was inhibited [20], the inhibitory effect of 0.01, 0.1, or 1 mg/mL RvE1 against the differentiation of cultured osteoclasts was investigated. The results revealed that RvE1, which is synthesized from EPA by vascular endothelial cells, inhibits the release of phosphatidyl in the resolution phase of inflammation [20,38], actually does have anti-inflammatory activity. It inhibits osteoclast differentiation (Fig. 5a, 5b). Gao et al. reported that RvE1 acts via osteoblast chemokine-like receptor 1 to promote osteoprotegerin (OPG) production, thereby suppressing osteoclast differentiation [39]. Zhu et al. reported that the expression of dendirtic cell specific transmembrane protein (DC-STAMP), an osteoclast fusion protein, is downregulated by the action of RvE1 on leuoktrine B4 receptor 1 (BLTL), thereby inhibiting the multinucleation of osteoclasts [40]. The results of the present study suggest that similar effects were involved in the inhibition of osteoclast differentiation by RvE1.

In this study’s experiments, natural menhaden oil and sunflower oil were added to the omega-3 fatty acid diet and omega-6 fatty acid diet, respectively. The oil could be contaminated with small amounts of impurities, which may have affected this study’s results. Although this is a potential study limitation, because a previous study also used menhaden oil impurities are an unlikely concern [41].

Collectively, the above results indicate that the ingestion of omega-3 fatty acids may inhibit periodontitis-induced differentiation into osteoclasts while also inhibiting the bone resorption associated with inflammation.

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Conflict of interest
The authors declare no conflict of interest.

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