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Proteomic profiling of human neutrophils in relation to immunoglobulin G Fc receptor IIIb polymorphism

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Background and Objective: Neutrophils are essential in host defense against periodontopathic bacteria. Immunoglobulin G Fc receptor IIIb (FcγRIIIb) is a neutrophil-specific receptor for immunoglobulin G and bears the functional NA1–NA2 polymorphism. Accumulating evidence suggests a significant association between FcγRIIIb gene polymorphism and periodontitis. In this study, we employed a proteomic approach to evaluate the relevance of FcγRIIIb polymorphism to protein expression profiles of neutrophils.

Material and Methods: Neutrophils were collected from ten healthy subjects whose FcγRIIIb genotypes were determined by allele-specific PCRs. Expressions of proteins induced by interaction via FcγRIIIb were examined between the FcγRIIIb genotypes with two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins that were significantly different in expression levels between the FcγRIIIb genotypes were determined with computer image analysis, and identified with mass spectrometry and protein databases.

Results: A total of 757 protein spots were observed in the two-dimensional electrophoretograms of neutrophils from five FcγRIIIb-NA1/NA1 and five FcγRIIIb-NA2/NA2 donors. A statistical analysis revealed that the expression levels of five proteins were significantly different between the FcγRIIIb genotypes ($p < 0.05$). The FcγRIIIb-NA1/NA1 neutrophils exhibited two spots that were significantly underexpressed (protein-arginine deiminase type-4 and annexin VI) and three spots that were significantly overexpressed (Cdc42hs-Gdp complex, myosin light chain 12A and coactosin-like 1) when compared with FcγRIIIb-NA2/NA2 neutrophils. The same expression profiles of protein-arginine deiminase type-4 were obtained by ELISA.

Conclusion: Differential protein expression profiles were observed in neutrophils between FcγRIIIb genotypes.

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Neutrophils are crucial for host defense against periodontopathic bacteria in the periodontium (1). Neutrophils represent approximately 90% of immunocompetent cells in gingival crevicular fluid (2). Increased levels of immunoglobulin G (IgG) antibodies against periodontopathic bacteria are observed in gingival crevicular fluid from periodontitis patients (3). Dense infiltrates of neutrophils with high expression levels of IgG Fc receptors (FcγRs) are also detected in diseased periodontium (4). Furthermore, FcγR expression levels are associated with the phagocytic function of neutrophils (5,6). Therefore, neutrophil FcγRs may play a prominent role in the pathogenesis of periodontitis.

Human neutrophils constitutively express two different FcγRs: FcγRIIa and FcγRIIIb. FcγRIIa plays a role in triggering neutrophil function, and bears either an arginine (FcγRIIa-R131) or histidine (FcγRIIa-H131) at amino acid position 131 in the second extracellular Ig-like domain (7). FcγRIIIb is a neutrophil-specific receptor and bears the functional NA1–NA2 polymorphism, caused by four amino acid substitutions within the first extracellular Ig-like domain (8). This substitution affects receptor affinity for IgG1, altering neutrophil functions. Our previous study indicated frequencies of FcγRIIa and FcγRIIIb genotypes in Japanese healthy individuals to be 3.8% for -R/R131 and 60.0% for -H/H131, and 36.2% for -NA1/NA1 and 11.4% for -NA2/NA2 (9). It has been demonstrated that the NA2-bearing neutrophils interact less efficiently with IgG1 than the NA1-bearing cells (10). These functional differences among FcγRIIIb genotypes were also confirmed in neutrophils from healthy subjects as well as periodontitis patients (11).

We have previously demonstrated a significant association between FcγRIIIb gene polymorphisms and susceptibility to aggressive periodontitis. The FcγRIIIb-NA2/NA2 genotype was found to be increased in prevalence in Japanese subjects with aggressive periodontitis compared with healthy control subjects (12). These findings are in agreement with results of other studies in African-American and Brazilian populations (13,14), but are different from studies in Caucasian and Taiwanese populations (15,16). Recently, a meta-analysis suggested FcγRIIIb genotypes to be an important factor associated with susceptibility to periodontitis (17). These observations might be explained by differential expressions of neutrophil proteins induced by FcγRIIIb–IgG1 interaction. It is therefore hypothesized that neutrophil proteins could be screened for differences between FcγRIIIb-NA1/NA1 and -NA2/NA2 genotypes. To test our hypothesis, we employed a proteomic approach to evaluate the relevance of FcγRIIIb polymorphism to protein expression profiles of neutrophils by two-dimensional (2-D) gel electrophoresis and mass spectrometry of the proteins.

Material and methods

Participants and cells

Forty Japanese nonsmoker healthy volunteers with no signs of systemic disease (20 males and 20 females; age range, 22–54 years; mean age, 32.4 years) were recruited into this study between July 2007 and January 2010. Written informed consent was obtained from all participants. The format of the study was reviewed and approved by the Ethical Committee for the use of Human Subjects in Research, Niigata University Faculty of Dentistry. All participants were confirmed to exhibit all sites with probing depth ≤ 3 mm, according to clinical periodontal examination. Neutrophils were isolated from heparinized peripheral blood using a double density gradient purification with Histopaque 1077 and 1119 (Sigma, St Louis, MO, USA) (18). Purified neutrophils were washed with phosphate-buffered saline (PBS) containing 1 mM phenylnethanesulfonyl fluoride and 1 mM ethylenediaminetetraacetic acid (PIPBS), and resuspended in PIPBS at 3×10^6 cells/mL.

Genotyping of FcγR

For a matched experimental design, all participants in each FcγRIIIb genotype group were also matched for their FcγRIIa genotype. Genomic DNA was isolated from peripheral blood in all participants using DNA extractor WB kit (WAKO, Osaka, Japan) according to the manufacturer's instructions. The FcγRIIIb and FcγRIIa genotypes were determined by PCR, as detailed previously (19,20).

FcγRIIIb — One-hundred nanograms of genomic DNA was added to a 50 μL reaction mixture containing 15 mM tromethamine (pH 8.0), 50 mM potassium chloride, 25 mM magnesium chloride, 25 μM dNTP, 400 nM of the sense or antisense primer and 1 U Taq polymerase. The PCR conditions were as follows: one cycle at 95°C for 9 min, followed by 35 cycles at 95°C for 30 s, 63°C for 30 s and 72°C for 30 s, ending at 72°C for 10 min. For the FcγRIIIB-NA2 genotyping, PCR assay was performed with the NA2 sense and antisense primer. The amplification protocol was as follows: one cycle at 95°C for 9 min, followed by 35 cycles at 95°C for 30 s, 64°C for 15 s and 72°C for 30 s, ending at 72°C for 10 min. The end-products of the specific NA1 and NA2 reactions were 141 and 169 bp, respectively.

FcγRIIa — One-hundred nanograms of genomic DNA was added to a 50 μL reaction mixture containing 15 mM tromethamine (pH 8.0), 50 mM potassium chloride, 25 mM magnesium chloride, 25 μM dNTP, 100 nM each of primer P63 and P52 and 1 U Taq polymerase. The PCR conditions were as follows: one cycle at 95°C for 5 min, 55°C for 5 min and 72°C for 5 min. This was followed by 35 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, ending with an extension step at 72°C for 10 min. The PCR product was subsequently reamplified using the combination of the common antisense primer located on intron 4 and the nucleotide (nt) 507 G-specific or A-specific primer. The second set of PCR conditions were as follows: 95°C for 5 min followed by 30 cycles of 95°C for 15 s, 62°C for 30 s and 72°C for 30 s, with an extension step at 72°C for 10 min. The PCR reactions yielded a 278 bp product for both FcγRIIa alleles.

All PCR products were analysed by electrophoresis on 2% agarose gels stained with ethidium bromide.

FcγRIIIb expression

Surface expression levels of FcγRIIIb were analysed by flow cytometry (6). In short, purified neutrophils were incubated with phycoerythrin-conjugated anti-CD11b monoclonal antibody (Becton Dickinson, San Jose, CA, USA) for 30 min at 4°C. After washing with PBS, samples were incubated with fluorescein isothiocyanate (FITC)-labelled monoclonal antibody 3G8 (Medarex, Annandale, NJ, USA), or isotype-matched FITC-labelled mouse IgG (Coulter, Hialeah, FL, USA) for 30 min at 4°C. The mixture was washed with PBS containing 0.2% ethylenediaminetetraacetic acid and 0.1% sodium azide, and analysed with FACScan and CELLQuest software (Becton Dickinson). The FITC fluorescence intensity was expressed as mean log fluorescence.

Cell culture and protein sample preparation

Purified neutrophils were pre-incubated in PIPBS in an atmosphere of 5% CO_2 in air at 37°C for 10 min. As a stimulant for neutrophil FcγRIIIb, human monoclonal IgG1 antibodies to *Porphyromonas gingivalis* 381 recombinant 40 kDa outer membrane protein (hMAb) were constructed (21). The neutrophils were then incubated with hMAb (100 ng/mL) at 37°C for 30 min, washed twice with PIPBS and subjected to protein extraction. One millilitre of cell pellet sample was mixed with 1 mL of lysis buffer consisting of 5 M urea, 2 M thiourea, 2% w/v CHAPS (3-[(3-cholamidopropyl) dimethyl-ammonio] propanesulfonic acid), 2% w/v SB3-10 (*N*-decyl-*N*,*N*-dimethyl-3-ammonio-1-propane-sulfonate), and 1% w/v dithiothreitol, and then vortexed vigorously. This was followed by centrifugation of the solution at 20,000*g* for 30 min while maintaining the temperature at 20°C, to prevent precipitation of the lysis buffer. The supernatant was removed from the cell debris and stored at −80°C until use. Protein concentration in the supernatant was quantified with a protein assay kit (Bio-Rad, Hercules, CA, USA).

Two-dimensional gel electrophoresis

After the samples were concentrated and desalted with an Ultrafree-0.5 centrifugal filter device (Millipore, Billerica, MA, USA), the first-dimensional separation of proteins was carried out on immobilized pH gradient (IPG) strips, in accordance with published procedures (22,23). Briefly, the protein samples (100 μg of total protein) were applied overnight to Immobiline Drystrips (pH 3–10; GE Healthcare Bio-Science, Chalfont St Giles, UK) by in-gel rehydration. The rehydrated gels were then gently dried with sheets of tissue paper to remove excess fluid, and were subjected to isoelectric focusing (IEF) in a CoolPhoreStar IPG-IEF Type P electrophoresis unit (Anatech, Tokyo, Japan), according to the manufacturer's instructions. This was followed by second-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 9–18% acrylamide gradient gels using a Hoefer Iso-Dalt electrophoresis chamber (GE Healthcare Bio-Science). The proteins separated on the 2-D acrylamide gels were stained with fluorescent dye SYPRO Ruby (Invitrogen, Carlsbad, CA, USA) and detected with a Molecular Imager FX (Bio-Rad). ImageMaster 2D Platinum image analysis software (GE Healthcare Bio-Sciences) was used to identify matched and unmatched protein spots on gel images of stimulated neutrophils with FcγRIIIB-NA1/NA1 and -NA2/NA2. Over ninety per cent of the spots were coincident in the four replicates, when the densitometric volume (related to the quantity of protein) of the spots was > 200. The over- and underexpression of the matched protein spot was identified when difference in the spot volume was significantly different between FcγRIIIb-NA1/NA1 and -NA2/NA2 neutrophils.

Mass spectrometric analysis

The over- or underexpressed proteins were cut for in-gel digestion and were subsequently subjected to mass spectrometric analysis, as described by Gerashchenko *et al*. (23). The fluorescently stained 2-D gels were further stained with silver to visualize spots. Briefly, the selected matched protein spots were excised from the dried, silver-stained 2-D gels (24) and rehydrated in 100 mM ammonium bicarbonate for 20 min. The gel spots were then destained in a solution of 15 mM potassium ferricyanide and 50 mM thiosulfate for 20 min, rinsed twice in deionized water and finally dehydrated in 100% acetonitrile until they turned opaque white. The spots were then dried in a vacuum centrifuge, followed by overnight rehydration in digestion solution consisting of 50 mM ammonium bicarbonate, 5 mM calcium chloride and 0.01 μg/μL modified sequence-grade trypsin (Promega, Madison, WI, USA) at 37°C. The digestion of spots was terminated by treatment with 5% trifluoroacetic acid for 20 min. Peptides were extracted from the spots three times with 5% trifluoroacetic acid in 50% acetonitrile for 20 min, and were pooled and dried in a vacuum centrifuge. This was followed by their collection/concentration with Zip-Tip C18 pipette tips (Millipore), according to the manufacturer's protocol. The peptide mass spectra were measured using an ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Identification of protein was performed with MASCOT search engine (Matrix Science, London, UK) using the National Center for Biotechnology Information (NCBI) database.

ELISA

Concentrations of protein-arginine deiminase type-4 (PADI4) in the subject samples were determined by ELISA with commercially available kits (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. Briefly, each of the 96-well microtitre plates was coated with 50 μL of the protein samples and PADI4 peptides (Abcam, Tokyo, Japan) in coating buffer (pH 9.5) overnight at 4°C. After removal of the sample solution, 200 μL of a blocking reagent

was added to each well for 2 h at room temperature. After washing twice, 100 μL of goat anti-human PADI4 IgG antibodies (1:50,000 dilution; Abcam) were added and incubated for 2 h at room temperature. After washing three times, horseradish peroxidase-conjugated rabbit anti-goat IgG antibodies (1:10,000 dilution; Abcam) were further added and incubated for 2 h at room temperature. After washing three times, colour development was performed with 100 μL of substrate reagent containing 3,3′,5,5′-tetramethylbenzidine dihydrochloride and hydrogen peroxide for 10 min at 37°C, and stopped by addition of 100 μL of stop solution. Optical density at 450 nm (OD 450 nm) was measured with an automated microplate reader (Bio-Rad Japan Laboratories, Tokyo, Japan). The appropriate dilution of protein sample was determined from preliminary experiments involving serial dilutions of samples and measurements of OD 450 nm. Concentrations of PADI4 in the samples from FcγRIIIb-NA1/NA1 and -NA2/NA2 neutrophils were expressed as means ± standard error (SE) of experiments with four different donor pairs ($n = 5$, for both FcγRIIIb-NA1/NA1 and -NA2/NA2 genotypes).

Statistical analyses

Differences in the spot volume of matched proteins between FcγRIIIb-NA1/NA1 and -NA2/NA2 neutrophils were assessed using Student's unpaired *t*-tests. The same tests were also used to assess differences in PADI4 concentrations between FcγRIIIb-NA1/NA1 and -NA2/NA2 neutrophils. Statistical significance was accepted at 5% ($p < 0.05$).

Results

In a group of 40 Japanese healthy individuals, we confirmed the previously reported data on the distribution of FcγRIIa and FcγRIIIb genotypes (2.5% for FcγRIIa-R/R131, 42.5% for -R/H131 and 55.0% for -H/H131; 35.0% for FcγRIIIb-NA1/NA1, 52.5% for -NA1/NA2 and 12.5% for -NA2/NA2) (9). We then determined the two FcγRIIIb genotype groups: five FcγRIIIb-NA1/NA1 donors (3 males and 2 females; mean age, 35.8 years) and five FcγRIIIb-NA2/NA2 donors (3 males and 2 females; mean age, 39.2 years), all of whom exhibited FcγRIIa-R/H131.

A total of 757 protein spots were observed in the 2-D electrophoretograms of FcγRIIIb-NA1/NA1 and -NA2/NA2 neutrophils. The number of protein spots observed in all 2-D gels was 330 for the FcγRIIIb-NA1/NA1 group and 353 for the -NA2/NA2 group. The mean and standard deviation (SD) for the number of spots was 534.8 ± 27.0 for the FcγRIIIb-NA1/NA1 group and 542.0 ± 8.3 for -NA2/NA2 group. Figure 1A shows representative 2-D electrophoretograms of proteins from FcγRIIIb-NA1/NA1 and -NA2/NA2 neutrophils. Most of the protein spots were distributed between p*I* 3 and 10, and between 10 and 225 kDa. A statistical analysis revealed that the expression levels of five protein spots were significantly different between the FcγRIIIb genotypes ($p < 0.05$; spot nos 1–5; Fig. 1B). We found no protein spots that were only identified in FcγRIIIb-NA1/NA1 or -NA2/NA2 neutrophils.

Mean florescence intensities of neutrophils labelled with anti-FcγRIIIb monoclonal antibody proved comparable between FcγRIIIb genotype groups (mean ± SD arbitrary scale: 17.4 ± 2.4 for FcγRIIIb-NA1/NA1 group and 16.4 ± 6.8 for -NA2/NA2 group). In addition, the percentages of FcγRIIIb-positive neutrophils were comparable between the FcγRIIIb genotype groups (mean ± SD percentages: 93.5 ± 6.3 for FcγRIIIb-NA1/NA1 group and 96.0 ± 3.0 for -NA2/NA2 group).

The FcγRIIIb-NA1/NA1 neutrophils exhibited two spots (spot nos 1 and 2) that were significantly underexpressed compared with FcγRIIIb-NA2/NA2 neutrophils ($p < 0.05$; Fig. 2). The database search revealed that spot nos 1 and 2 corresponded to protein-arginine deiminase type-4 (PADI4) and annexin VI, respectively (Table 1). Another three spots (spot nos 3–5) were significantly overexpressed in FcγRIIIb-NA1/NA1 neutrophils compared with FcγRIIIb-NA2/NA2 neutrophils ($p < 0.05$; Fig. 2). Spot nos 3 4, and 5 were identified as Cdc42hs-Gdp complex, myosin light chain 12A and coactosin-like 1, respectively (Table 1).

To confirm reliability of our proteomic results, a representative PADI4 protein that was shown to be associated with human inflammatory disease (25–27) was selected, and evaluated in the protein samples with ELISA experiments. Concentrations of PADI4 in FcγRIIIb-NA1/NA1 neutrophils were significantly lower than those in FcγRIIIb-NA2/NA2 neutrophils ($p = 0.03$; Fig. 3).

Discussion

Our results indicated five proteins to be differentially expressed in neutrophils between the FcγRIIIb-NA1/NA1 and -NA2/NA2 genotypes. When compared with FcγRIIIb-NA2/NA2 neutrophils, two proteins (PADI4 and annexin VI) were underexpressed, while another three proteins (Cdc42hs-Gdp complex, myosin light chain 12A and coactosin-like 1) were overexpressed in FcγRIIIb-NA1/NA1 neutrophils. To exclude confounding factors responsible for the observed levels of proteins, we confirmed FcγRIIIb expression levels on neutrophils to be comparable between FcγRIIIb genotypes. In addition, because FcγRIIa genotypes regulate neutrophil functions (28), all participants in each FcγRIIIb genotype group were matched for their FcγRIIa genotype in this study. Therefore, it is conceivable that differential expression of the five proteins cannot be explained by underlying differences in FcγRIIIb expression levels or FcγRIIa genotypes. To best of our knowledge, this is the first proteomic evidence to show the differentially expressed proteins associated with FcγRIIIb polymorphism.

Protein-arginine deiminase type-4 is a citrullinating enzyme, converting peptidylarginine into citrulline via post-translational modification. Of five identified isotypes (PADI1–4 and 6), PADI4 is expressed in white blood cells, including neutrophils (29), and has been shown to be genetically

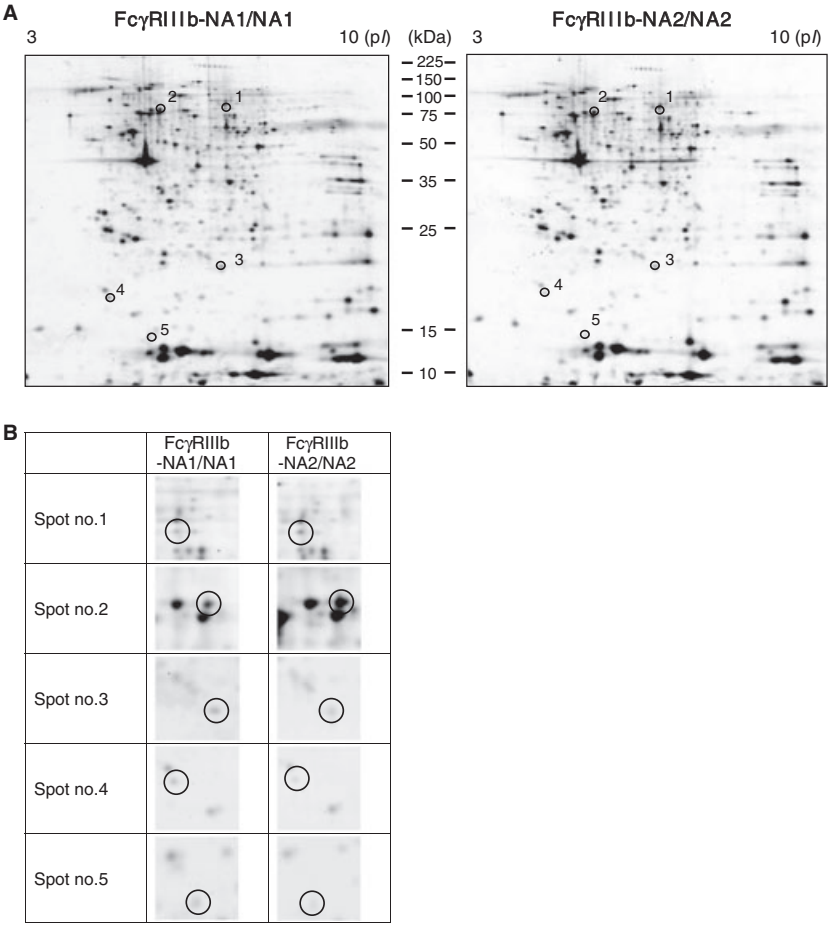

Fig. 1. Representative protein expression profiles in neutrophils obtained from FcγRIIIb-NA1/NA1 and -NA2/NA2 donors. (A) Two-dimensional gel electrophoresis of protein samples obtained from stimulated neutrophils in FcγRIIIb-NA1/NA1 and -NA2/NA2 donors. The vertical axis represents the molecular weight of proteins (in kilodaltons). The horizontal axis represents the range of p*I* values of proteins between 3 and 10. The circles indicate matched five protein spots (spot nos 1–5) with significant difference in the expression levels between FcγRIIIb-NA1/NA1 and -NA2/NA2 neutrophil groups ($n = 5$ for both genotype groups), as assessed by Student's unpaired *t*-test ($p < 0.05$). (B) The regions of the five matched spots (spot nos 1–5) are shown enlarged. These proteins were cut for in-gel digestion and were subsequently subjected to mass spectrometric analysis. The protein assignments are presented in Table 1.

associated with susceptibility to rheumatoid arthritis (25). Co-localization of PADI4, citrullinated protein and fibrin deposit was suggested to be responsible for fibrin citrullination, resulting in a potential antigen of rheumatoid arthritis autoimmunity (26). It has also been documented that overexpression of PADI4 and subsequent citrullination of intracellular proteins via anti-PADI4 response constitute an aetiological factor for chronic inflammatory diseases (27). Recent literature indicates that PADI4 was released from the cells when they exposed to excessive extracellular calcium ions (30). The FcγRIIIb-NA1/NA1 neutrophils were shown to deliver more signals for calcium ion mobilization via cross-linking with FcγRIIa than FcγRIIIb-NA2/NA2 neutrophils (31). Therefore, it is likely that underexpression of PADI4 was present in FcγRIIIb-NA1/NA1 neutrophils.

Annexins constitute a family of calcium- and phospholipid-binding proteins, and have been implicated in intracellular calcium mobilization and regulation of cellular function. Annexin VI has been shown to concentrate in the phagosomal region of neutrophils during phagocytosis of mycobacteria (32). A proteomic study demonstrated annexin VI to be preferentially associated to late phagosomes, suggesting that this molecule could exert its function at a late step of endocytosis or phagocytosis (33). Considering our observations that overexpression of annexin VI was identified in FcγRIIIb-NA2/NA2 neutrophils, it would be conceivable that differential phagosome maturation may be partly responsible for the difference in neutrophil function between FcγRIIIb genotypes.

The Cdc42hs-Gdp complex is a member of the Rho family of GTPases (34), whereas coactosin-like 1 is a human filamentous actin-binding protein (35). These proteins are involved in regulation of the actin cytoskeleton in neutrophils, which is essential for phagocytosis and accompanying superoxide generation (34,35). Our results indicated an underexpression of Cdc42hs-Gdp complex and coactosin-like 1 in FcγRIIIb-NA2/NA2 neutrophils, which is in accordance with results of other studies showing a decreased function of neutrophils from FcγRIIIb-NA2-carrying donors (10,11). Further studies would be needed to evaluate the association between these molecule levels and cellular functions. Myosin light chain 12A plays a role in regulation of cytokinesis and cell locomotion. The levels of myosin light chain 12A were increased in FcγRIIIb-NA1/NA1 neutrophils. However, its role in host immune response has not been fully clarified.

Most of these proteins have also been identified in neutrophils by other proteomic analyses (36–38). Annexin VI was detected as an actin protein in the phagosome (36). Myosin light chain were identified as a cytoskeletal protein in the plasma membrane vesicles (36,37), while Cdc42hs was identified on neutrophil granules (38). Recently, a proteomic study indicated that highly abundant proteins, such as heat shock protein 70 and enolase 1,

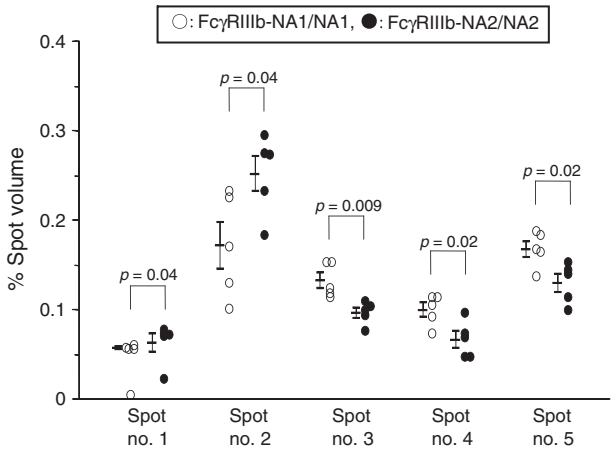

Fig. 2. Comparison of volume of five differentially expressed protein spots between FcγRIIIb-NA1/NA1 and -NA2/NA2 neutrophils (spot nos 1–5). Each circle represents the spot volume (%) for one donor. The mean and standard error are indicated for the FcγRIIIb-NA1/NA1 and the -NA2/NA2 groups. The vertical axis represents the spot volume (%), which was normalized to give a fractional volume of total spot volume per gel for intergel comparisons. The *p*-values mark significance of differences between FcγRIIIb genotype groups, as identified by Student's unpaired *t*-test.

were frequently identified in a diverse number of proteomic analyses (39). The cellular stress response may be the universal reason why these proteins were generally detected differentially (39), suggesting that it is important to check the specificity of proteins. We confirmed that six differentially expressed proteins in this study were not matched for the frequently detected proteins in general (39).

Caution is necessary when interpreting our results, because detection of protein spots on the 2-D gels was influenced by the employed techniques. We identified five differentially expressed proteins that were distributed on the gels between p*I* 3 and 10, and between 10 and 225 kDa. However, it is technically difficult to detect the spots that appeared in the lower or higher range of pH and molecular weight, which were not visible on the gel.

These proteomic approaches may be effective for defining risk markers associated with disease susceptibility, but have problems with interindividual differences. Differences between donors were observed in the protein expression levels, even within the same FcγRIIIb genotype group. However, our statistical analyses revealed differences in the expressions between FcγRIIIb genotype groups to be greater than those among donors

Table 1. Identification of proteins corresponding to the differentially expressed spots in stimulated neutrophils between FcγRIIIb-NA1/NA1 and -NA2/NA2 geneotypes

	Protein name	Mean spot volume ratio (NA1/NA2)	Accession number (NCBI)	Molecular mass (Da)	MOWSE score	Matched peptide sequences
Spot no. 1	Protein-arginine deiminase type-4 (PADI4)	0.6	gi|12230488	74,079	182	TLPVVFDSPR VMGPDFGYVTR ILFGDSCYPSNDSR GPQTGGISGLDSFGNLEVSPPVTVR
Spot no. 2	Annexin VI	0.7	gi|34533483	75,873	153	ALIEILATR LVFDEYLK FMTILCTR SEIDLLNIRR ILISLATGHREEGGENLDQAR
Spot no. 3	Cdc42hs-Gdp complex	1.4	gi|4389379	21,258	78	YVECSALTQK WVPEITHHCPK QKPITPETAEKLAR
Spot no. 4	Myosin light chain 12A	1.5	gi|5453740	19,794	124	GNFNYIEFTR KGNFNYIEFTR FTDEEVDELYR EAPIDKKGNFNYIEFTR
Spot no. 5	Coactosin-like 1	1.3	gi|21624607	15,944	194	KELEEDFIK DDGSAVWVTFK ELEEDFIKSELK ELEEDFIKSELKK FALITWIGENVSGLQR SKFALITWIGENVSGLQR

The proteins correspond to five spots (numbered in Fig. 1) in which a significant difference in the expression levels between FcγRIIIb-NA1/NA1 and -NA2/NA2 neutrophils was found ($p < 0.05$). Probability-based MOWSE scores showed significant matches, with ion scores > 37 for the spot nos 1, 2 and 5 ($p < 0.05$), and > 38 for spot nos 3 and 4 ($p < 0.05$) (40).

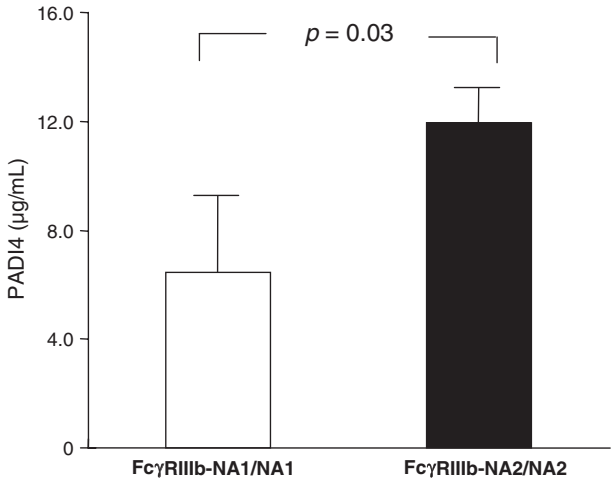

Fig. 3. Concentrations of protein-arginine deiminase type-4 (PADI4) in stimulated neutrophils form FcγRIIIb-NA1/NA1 and -NA2/NA2 donors. Results are expressed as means ± SEM of experiments with four different donor pairs ($n = 5$ for both FcγRIIIb-NA1/NA1 and -NA2/NA2 genotype groups). The p-value is the significance of difference between FcγRIIIb-NA1/NA1 and -NA2/NA2 neutrophils, as assessed by Student's unpaired t-test.

within the FcγRIIIb genotype group, which was also confirmed by results of ELISA experiments. In addition, the 2-D gel image analysis revealed more than 90% of the spots to be coincident in the four replicates within the same donor. Therefore, these observations support the reliability of our findings of differentially expressed proteins associated with FcγRIIIb polymorphism. To further elucidate a role of FcγRIIIb genotypes in the pathogenesis of periodontits, it would be necessary also to study patients with periodontitis.

In conclusion, this study provides the possibility of differential protein expression profiles of neutrophils between FcγRIIIb genotypes.

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