Antibodies to Proteinase 3 Prime Human Oral, Lung, and Kidney Epithelial Cells To Secrete Proinflammatory Cytokines upon Stimulation with Agonists to Various Toll-Like Receptors, NOD1, and NOD2\textsuperscript{V}

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Antineutrophil cytoplasmic antibodies (ANCA) are autoantibodies, the detection of which in serum can be used in the diagnosis of Wegener’s granulomatosis (WG). Proteinase 3 (PR3) is a major target antigen of ANCA in WG patients, and the interaction of PR3 ANCA with leukocytes causes a debilitating autoimmune disease. The first signs and symptoms in WG patients are observed in the oral cavity, lungs, and kidneys. Human epithelial cells generally do not secrete proinflammatory cytokines upon stimulation with pathogen-associated molecular patterns (PAMPs). In this study, anti-PR3 antibodies (Abs) and PR3 ANCA-containing sera from WG patients endowed human oral, lung, and kidney epithelial cells with responsiveness to PAMPs in terms of the production of proinflammatory cytokines, such as interleukin-6 (IL-6), IL-8, monocyte chemoattractant protein-1, and tumor necrosis factor alpha. Protease-activated receptor-2 (PAR-2) agonist peptides mimicked the priming effects of PR3 ANCA against PAMPs. Furthermore, the anti-PR3 Ab-mediated cell activation was significantly abolished by RNA interference targeting PAR-2 and NF-κB. This is the first report of priming effects of anti-PR3 Abs (PR3 ANCA) on epithelial cells. The results suggest that anti-PR3 Abs (PR3 ANCA) prime human epithelial cells to produce cytokines upon stimulation with various PAMPs, and these mechanisms may be involved in severe chronic inflammation in WG.

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cretory forms in human oral epithelial cells and that the addition of anti-PR3 Abs to cytokine-primed oral epithelial cells in culture induces the aggregation of PR3, followed by the activation of protease-activated receptor-2 (PAR-2), which results in remarkable secretion of IL-8 and MCP-1 (31). PAR family members are G-protein-coupled receptors characterized by a proteolytic cleavage of the N terminus that exposes tethered ligands and autoactivates the receptor function (6, 8, 24). There are four members of this family. PAR-2 is activated by trypsin and mast cell tryptase, as well as coagulation factors VIIa and Xa. Because PARs are expressed on a wide variety of cell types, including neutrophils, they are believed to play important roles in several pathophysiological processes, including growth, development, inflammation, tissue repair, and pain.

The innate immune system recognizes microorganisms through a series of pattern recognition receptors that have been highly conserved during evolution and are specific for common motifs found in microorganisms but not in eukaryotes, designated pathogen-associated molecular patterns (PAMPs) (1, 18, 20). Representative microbial PAMPs are the lipid A moiety of LPS, lipopeptides, peptidoglycans (PGNs), and viral double-stranded and single-stranded RNAs. Akira et al. demonstrated that these PAMPs are recognized specifically by the respective Toll-like receptor (TLR) (2). In addition, some NOD-like receptor family members were demonstrated previously to be intracellular receptors for partial structures of PGNs; NOD1 and NOD2 recognize a diaminopimelic acid (DAP)-containing peptide moiety (5, 11, 29) and a muramyl dipeptide (MDP) moiety (12, 19), respectively.

The first signs and symptoms in WG patients are in the oral, lung, and kidney epithelia. Human epithelial cells, including those of the oral cavity, lungs, and kidneys, generally do not secrete proinflammatory cytokines upon stimulation with PAMPs (28). In this study, we examined whether anti-PR3 Abs (PR3 ANCA) were capable of priming human oral, lung, and kidney epithelial cells. If they are, the mechanism may be involved in the pathogenesis of ANCA-related inflammatory diseases represented by WG.

MATERIALS AND METHODS

Reagents. Synthetic MDP (MurNac-L-Ala-L-isogln) and an Escherichia coli-type lipid A (LA-15-PP) were purchased from the Protein Research Foundation Peptide Institute (Osaka, Japan). Double-stranded poly(I-C) was obtained from Sigma-Aldrich (St. Louis, MO). Single-stranded poly(U) was purchased from Invivogen (San Diego, CA). A conventional CpG DNA, CpG DNA 1826 (TCC ATGACGTTCTACGACGT [the CpG motif is underlined], was provided by Sigma-Genosys (Tokyo, Japan). A synthetic Mycoplasma-type diacyl lipopeptide, FSL-1 [S-(2-[3-bis(palmitoyl)oxy]-(2R,3S)-propyl)-cysteinyl-GDPKPSF], was purchased from EMC Microcollections (Tübingen, Germany). Two synthetic desmuramylpeptides—DAP-containing PGN fragment FK156 (o-lactoyl-ω-Ala-γ-Glu-meso-DAP-Gly) and the FK156 derivative FK565 (heptanyloyl-ω-Glu-meso-DAP-ω-Ala)—were supplied by Astellas Pharmaceutical Co. (Tokyo, Japan). Recombinant human IL-1α and TNF-α were provided by Dainippon Pharmaceutical Co. (Osaka, Japan). A PAR-2 agonist peptide (PAR-2AP; SLIGKV) was synthesized by Takara (Otsu, Japan). Nonenzymatic cell dissociation solution (CDS) was obtained from Sigma-Aldrich. All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Serum samples. ANCA-containing sera were obtained from four patients with WG at Tohoku University Hospital, Sendai, Japan. The samples were immediately purified by centrifugation, aliquoted, and frozen at −70°C until being used. PR3 ANCA and MPO ANCA titers were reconfirmed by the enzyme immuno-assay method by BML Co. (Sendai, Japan). All of the ANCA-containing sera were PR3 ANCA-positive and MPO ANCA-negative specimens. Normal serum from a healthy adult donor was used as a control.

Cells and cell culture. The human oral epithelial cell line HSC-2, the human lung epithelial cell line A549, and the human kidney epithelial cell line Caki-1 were obtained from the Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The cells were cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated fetal calf serum, with a change of medium every 3 days. To avoid cell surface markers, we used Sigma’s CDS. CDS contains no protein and allows the dislodging of cells without enzymatic modification or the adsorption of foreign proteins.

Cytokine measurements. To investigate the production of inflammatory cytokines by epithelial cells, we collected the supernatant from each culture. The production of cytokines (IL-6, IL-8, MCP-1, and TNF-α) was measured using OptEIA ELISA kits (PharMingen, San Diego, CA). The concentrations of the cytokines in the supernatants were determined using the LS-PLATEmanager 2004 data analysis program (Wako Pure Chemical Industries, Osaka, Japan).

RNA interference. Transfections for targeting endogenous PAR-2, NF-κB, and laminit A/C were carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and short interfering RNA (siRNA; final concentration, 200 nM) for 24 h at 37°C, according to Invitrogen instructions. The viability of the cell cultures after transfection was more than 95%, as assessed by a 0.2% trypan blue exclusion test, and the cell’s morphological character was unchanged after transfection. siRNAs for PAR-2 and NF-κB were purchased from Santa Cruz Biotechnology, and the siRNA for laminit A/C was purchased from B-Bridge International.

RESULTS

Human oral, lung, and kidney epithelial cells do not secrete proinflammatory cytokines upon stimulation with PAMPs. We previously reported that various human epithelial cells do not secrete proinflammatory cytokines (28). As shown in Fig. 1, human oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells did not secrete IL-8 upon stimulation with the synthetic PAMPs FSL-1 (a TLR2 agonist), poly(I-C) (a TLR3 agonist), lipid A (a TLR4 agonist), poly(U) (a TLR7 agonist), CpG DNA (a TLR9 agonist), FK156 and FK565 (NOD1 agonists), and MDP (a NOD2 agonist). In contrast, human epithelial cells secreted IL-8 in response to TNF-α and IL-1α as positive controls.

Treatment with anti-PR3 Abs primed human oral, lung, and kidney epithelial cells to secrete IL-6, IL-8, MCP-1, and TNF-α upon stimulation with PAMPs. We demonstrated previously that anti-PR3 Abs enhance TLR and NOD agonist PAMP-induced secretion of proinflammatory cytokines by human monocytic THP-1 cells and human peripheral blood mononuclear cells (30). In the present study, we examined the production of inflammatory cytokines in human epithelial cells upon stimulation with PAMPs after priming with anti-PR3 Abs. When human oral, lung, and kidney epithelial cells were preincubated with 1 μg of anti-PR3 Abs/ml for 6 h and subsequently challenged with the various TLR and NOD agonist PAMPs for a further 18 h, massive production of IL-8 was observed, whereas stimulation with the anti-PR3 Abs by themselves had scarcely any effect (Fig. 2). Priming effects were also observed for the production of IL-6, MCP-1, and TNF-α (Fig. 3).

PR3 ANCA-containing sera primed human oral, lung, and kidney epithelial cells to secrete IL-8 upon stimulation with PAMPs. To further demonstrate the possible immunopathological properties of ANCA from WG patients, we examined whether the ANCA-containing sera were capable of priming human epithelial cells similarly to murine anti-PR3 Abs. We
obtained sera from patients with WG at Tohoku University Hospital and carried out experiments. The titers of PR3 ANCA and MPO ANCA in sera were determined. All sera from four patients were PR3 ANCA positive (titer, >3.0 U/ml), whereas none was MPO ANCA positive (titer, <1.3 U/ml). In this study, representative PR3 ANCA-positive sera from a WG patient (sample S9-32 from patient S9) were compared with normal serum. We examined the production of IL-8 upon stimulation with TLR and NOD agonist PAMPs. IL-8 production was significantly induced when human oral, lung, and kidney epithelial cells were incubated with the patients' sera compared with the production in cells incubated with normal serum (Fig. 4). These results clearly indicated that PR3 ANCA in the sera from WG patients exerted a priming effect similar to that of murine monoclonal anti-human PR3 Abs in vitro.

Treatment with PAR-2AP primed human oral, lung, and kidney epithelial cells to secrete IL-8 upon stimulation with PAMPs. As we found that anti-PR3 Abs primed human monocytic cells via PAR-2 (30), we examined whether the priming effects of PAR-2AP also occurred in human epithelial cells.

FIG. 1. Human oral, lung, and kidney epithelial cells did not secrete IL-8 in response to synthetic PAMPs. Oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells were stimulated with FSL-1 (1 nM), poly(I-C) (10 μg/ml), lipid A (10 ng/ml), poly(U) (10 μg/ml), CpG DNA (10 nM), FK156 (100 μg/ml), FK565 (100 μg/ml), MDP (100 μg/ml), TNF-α (10 ng/ml), or IL-1α (10 ng/ml) for 24 h in triplicate. Human TNF-α and IL-1α were used as positive controls. The levels of IL-8 in the culture supernatants were determined by ELISAs. Data are expressed as mean values ± standard deviations (SD). *, P < 0.01 versus results for cells stimulated with medium alone. The results presented are representative of three different experiments demonstrating similar results.

FIG. 2. Human oral, lung, and kidney epithelial cells preincubated with anti-PR3 Abs secreted IL-8 in response to synthetic PAMPs. Oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells were preincubated for 6 h with anti-PR3 Abs (1 μg/ml) or with an equal amount of an isotype-matched immunoglobulin G (IgG) Ab. Subsequently, the cells were stimulated with FSL-1 (1 nM), poly(I-C) (10 μg/ml), lipid A (10 ng/ml), poly(U) (10 μg/ml), CpG DNA (10 nM), FK156 (100 μg/ml), FK565 (100 μg/ml), or MDP (100 μg/ml) for 24 h in triplicate. The levels of IL-8 in the culture supernatants were determined by ELISAs. Data are expressed as mean values ± SD. *, significantly different from results for cells in the respective cultures incubated with control IgG (P < 0.01). The results presented are representative of three different experiments demonstrating similar results.
Clear priming effects of PAR-2AP on IL-8 production by epithelial cells in response to TLR and NOD agonist PAMPs were observed (Fig. 5).

The priming effect of anti-PR3 Abs occurred in a PAR-2- and NF-κB-dependent manner. To clarify the signaling pathway of the priming effects of anti-PR3 Abs upon stimulation with TLR and NOD agonist PAMPs, we utilized RNA interference assays targeting PAR-2 and NF-κB p65. PAR-2 and NF-κB p65 protein levels determined by flow cytometry were suppressed by ca. 80% by specific siRNAs in the cells for up to 72 h (28, 30). As shown in Fig. 6, the priming effects induced by anti-PR3 Abs were almost completely inhibited in cells in which PAR-2 and NF-κB were suppressed but not in cells in which lamin was suppressed. These results demonstrated that the priming effects of anti-PR3 Abs occurred in a PAR-2- and NF-κB-dependent manner.

**DISCUSSION**

Among ANCA, those targeting PR3 (PR3 ANCA) have a strong and specific association with WG (27, 32). Besides their significance as seromarkers, a pathogenic role has been proposed for these auto-Abs in relation to their capacity to activate leukocytes in vitro (4, 10, 13, 15, 22, 23, 25, 26). We previously reported that incubation with anti-PR3 Abs significantly upregulates the production of proinflammatory cytokines upon stimulation with various PAMPs (30). The first signs and symptoms in WG patients are in the oral cavity, lungs, and kidneys. In the present study, an alternative approach was chosen to define the priming effects of PR3 ANCA from WG patients on human oral, lung, and kidney epithelial cells; the epithelial cells were preincubated with substimulatory concentrations of human PR3 ANCA or murine anti-PR3 Abs, and the possible activation of the cells by various TLR and NOD agonist PAMPs was examined. Without preincubation, the epithelial cells generally did not secrete proinflammatory cytokines (Fig. 1). Surprisingly, preincubation with murine anti-PR3 Abs primed them to secrete proinflammatory cytokines (Fig. 2 and 3). In addition, similar to murine anti-PR3 Abs, PR3 ANCA-containing sera from WG patients, but not control serum, primed the cells to secrete proinflammatory cytokines (Fig. 4). These results indicated that PR3 ANCA in the sera from WG patients exerted a clear priming effect similar to that of murine anti-PR3 Abs in vitro and strengthen our
conclusion and the relevance of our study to the human situation.

Concerning signaling pathways, anti-PR3 Abs activated human cells via PAR-2 and NF-κB in a TLR- and NOD-dependent manner (30, 31). PAR-2AP had a priming effect with potency similar to that of anti-PR3 Abs (Fig. 5), and the effects of anti-PR3 Abs also occurred through PAR-2 and NF-κB. We used solely chemically synthesized PAMPs, because natural microbial components are inevitably contaminated with minor bioactive components that might have affected the results. Therefore, these results clearly indicated that anti-PR3 Abs primed human epithelial cells for TLR- and NOD-dependent cell activation.

It is conceivable that microbial components (PAMPs) exhibit powerful immunoadjuvant activities against various antigens, including autoantigens, through TLR and NOD pathways, which in turn may induce severe autoimmune diseases. In the important roles of PR3 ANCA in the regulation of inflammatory leukocyte functions, the ANCA, being only weak direct activators of monocytes and neutrophils to release cytokines per se, exert a definite priming effect on these leukocytes, enhancing their responsiveness to secondary stimulation with PAMPs (30). In this study, we first reported that oral, lung, and kidney epithelial cells were primed to secrete proinflammatory cytokines by anti-PR3 Abs upon stimulation with PAMPs whereas these epithelial cells normally did not produce proinflammatory cytokines in response to PAMPs. The first signs and symptoms in WG patients are severe inflammation in the
oral cavity, lungs, and kidneys. Such cooperation between PR3 ANCA and PAMPs may well trigger exacerbations of WG during infections and contribute to the persistence of inflammatory lesions, which may be a novel model for the pathogenesis of WG.

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