N-acetylcysteine and the human serum components that inhibit bacterial invasion of gingival epithelial cells prevent experimental periodontitis in mice

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Purpose: We previously reported that human serum significantly reduces the invasion of various oral bacterial species into gingival epithelial cells *in vitro*. The aims of the present study were to characterize the serum component(s) responsible for the inhibition of bacterial invasion of epithelial cells and to examine their effect on periodontitis induced in mice. **Methods:** Immortalized human gingival epithelial (HOK-16B) cells were infected with various 5- (and 6-) carboxy-fluorescein diacetate succinimidyl ester-labeled oral bacteria, including *Fusobacterium nucleatum, Provetella intermedia, Porphyromonas gingivalis*, and *Treponiema denticola*, in the absence or presence of three major serum components (human serum albumin [HSA], pooled human IgG [phIgG] and α1-antitrypsin). Bacterial adhesion and invasion were determined by flow cytometry. The levels of intracellular reactive oxygen species (ROS) and activation of small GTPases were examined. Experimental periodontitis was induced by oral inoculation of *P. gingivalis* and *T. denticola* in Balb/c mice. **Results:** HSA and phIgG, but not α1-antitrypsin, efficiently inhibited the invasion of various oral bacterial species into HOK-16B cells. HSA but not phIgG decreased the adhesion of *F. nucleatum* onto host cells and the levels of intracellular ROS in HOK-16B cells. N-acetylcysteine (NAC), a ROS scavenger, decreased both the levels of intracellular ROS and invasion of *F. nucleatum* into HOK-16B cells, confirming the role of ROS in bacterial invasion. Infection with *F. nucleatum* activated Rac1, a regulator of actin cytoskeleton dynamics. Not only HSA and NAC but also phIgG decreased the *F. nucleatum*-induced activation of Rac1. Furthermore, both HSA plus phIgG and NAC significantly reduced the alveolar bone loss in the experimental periodontitis induced by *P. gingivalis* and *T. denticola* in mice. **Conclusions:** NAC and the serum components HSA and phIgG, which inhibit bacterial invasion of oral epithelial cells *in vitro*, can successfully prevent experimental periodontitis.

Keywords: Albumins, Bacteria, Epithelial cells, Immunoglobulin G, Periodontitis.

INTRODUCTION

The gingival sulcus harbors one of the most complex bacterial communities in the human body, called dental plaque. The gingival epithelium provides strong physical and chemical barriers between plaque bacteria and gingival tissue. The continuous shedding and turnover of the gingival epithelial cells prevents persistent bacterial colonization [1]. In addition, gingival epithelial cells are adjoined by tight junction-related structures that may provide a barrier to bacterial invasion [2]. Gingival epithelial cells also secrete antimicrobial peptides

that can actively kill bacteria. Chemokines secreted by gingival epithelial cells guide neutrophils to the gingival sulcus, and the transmigrated neutrophils play a pivotal role in the maintenance of periodontal health [3].

The host maintains homeostasis with the plaque bacteria in periodontal health. However, the accumulation of dental plaque accompanied by the dysbiosis of the bacterial community results in periodontitis, that is, chronic inflammation of periodontal tissue that leads to alveolar bone loss. Bacterial invasion has been suggested as a potential primary cause of inflammation and tissue destruction in periodontitis [4]. Increased amounts of bacteria have been detected within gingival tissues from periodontal lesions compared with those from healthy sites [2]. Well-characterized periodontal pathogens such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, and *Treponema denticola* commonly have the ability to invade epithelial cells and gingival tissues [5,6]. Therefore, we hypothesized that the bacterial invasion of gingival epithelial cells could be a possible target for the prevention of periodontitis.

Gingival epithelial cells for *in vitro* studies are usually cultured in serum-free medium. We previously observed that the addition of human serum to the culture medium substantially decreases the invasion of various oral bacterial species into gingival epithelial cells [7]. The subgingival plaque and junctional/sulcular epithelium are bathed in gingival crevicular fluid (GCF), which is the transudate of gingival tissue interstitial fluid. GCF derives approximately 30% of its protein composition from serum [8]. Although Guentsch et al. [9] reported that human serum albumin (HSA) inhibits the adhesion and invasion of *P. gingivalis* and *A. actinomycetemcomitans* to oral epithelial cells, the effect of other serum components on bacterial invasion has not been studied. The aims of this study were to characterize the serum component(s) responsible for the inhibition of bacterial invasion and to examine their effect on the prevention of experimental periodontitis in mice.

MATERIALS AND METHODS

Bacteria and cell culture

F. nucleatum ATCC 10953, *P. gingivalis* ATCC 33277, *P. gingivalis* clinical isolate KUMC-P4 [10], *Prevotella intermedia* ATCC 25611, and *T. denticola* ATCC 33521 were grown and counted as previously described [6]. For the fluorescence studies, bacteria were stained with 5μM of 5-(and-6)-carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Carlsbad, CA, USA) before being used. Immortalized human gingival keratinocyte HOK-16B cells were maintained in keratinocyte growth culture medium (Clonetics, San Diego, CA, USA) containing supplementary growth factors. For bacterial infection, HOK-16B cells were plated one day before infection. At 80% confluence, the cells were infected with live bacteria at a multiplicity of infection of 1000 in the medium without antibiotics. The infected cells were cultured at 37℃ in a water-saturated atmosphere of 95% air and 5% CO_2.

Reagents

HSA and human α-1 antitrypsin isolated from human plasma were purchased from Sigma (Saint Louis, MO, USA). Intravenous immunoglobulin (IVIG from Green Cross, Yongin, Korea) was used as pooled human immunoglobulin G (phIgG). Sterile human serum purchased from Sigma was used after heat inactivation.

Flow cytometric adhesion and invasion assay

The flow cytometric adhesion and invasion assay was performed as previously described [6]. Briefly, HOK-16B cells were plated at 6×10^4 cells/well in triplicate in 24-well plates. For the adhesion assay, HOK-16B cells were incubated with CFSE-labeled bacteria in the absence or presence of various serum components for 1 hour on ice. The cells were then washed, detached with trypsin-ethylenediaminetetraacetic acid and immediately analyzed by flow cytometry. For the invasion assay, HOK-16B cells were infected with CFSE-labeled bacteria in the absence or presence of various serum components or N-acetylcysteine (NAC from Sigma) for 24 hours at 37℃. The cells were detached, washed, and then analyzed by flow cytometry after quenching the fluorescence of the bacteria bound on the surface with 300 μL of 0.4% trypan blue. Cells exposed to the same amount of CFSE-labeled bacteria after fixing with 3.7% formaldehyde served as a negative control. The viability of the HOK-16B cells was determined based on their forward scatter and the FL-3 fluorescence of the trypan blue staining, and bacterial invasion was analyzed only for the live cells. It was confirmed that bacterial infection or serum components did not affect the viability of HOK-16B cells.

Measurement of reactive oxygen species in HOK-16B cells

HOK-16B cells were plated at 2.5×10^4 cells/well in 96-well flat bottom black plates (NUNC, Roskilde, Denmark). The next day, the cells were stained with 10μM 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, Life Technologies Korea, Seoul, Korea) for 45 minutes, washed, and then infected with CFSE-labeled *F. nucleatum* in the absence or presence of various serum components or NAC. In our preliminary study, the basal and *F. nucleatum*-induced reactive oxygen species (ROS) levels increased continuously during the 24 hours infection period. Therefore, the DCF fluorescence was read at 24 hours using a FLUOstar OPTIMA (BMG LABTECH, Ortenberg, Germany).

Small GTPases pull-down assay

HOK-16 B cells were plated at 2×10^6 cells per 100-mm plate. At 80% confluence, the cells were infected with *F. nucleatum* in the absence or presence of HSA, phIgG, or NAC for the indicated time. The cells were lysed with ice cold 1× lysis buffer. The lysates were incubated with Rhotekin-bound agarose (Cell Biolabs, San Diego, CA, USA) to isolate Rho-GTP and with PAK-PBD-bound agarose (Cell Biolabs) to isolate both Rac-GTP and Cdc42-GTP. The GTPγs-and GDPγs-treated cell lysates were included as positive and negative controls, respectively. The agarose/protein complex was collected by

centrifugation, washed, and then subjected to western blotting using anti-Rac1, anti-RhoA, or anti-Cdc42 antibodies.

Experimental periodontitis and the measurement of alveolar bone loss

The experimental protocol and animal handling procedures were approved by the Seoul National University Animal Care and Use Committee (#SNU-130911-7) and conducted in accordance with the recommendations in the Guide from the Institute of Laboratory Animal Resources, Seoul National University, under the Laboratory Animals Act 9025 of the Republic of Korea. Six-week-old female Balb/c mice were purchased from Orient Bio (Seongnam, Korea) and housed under specific-pathogen-free conditions in the Laboratory Animal Facility at the School of Dentistry, Seoul National University. Experimental periodontitis was induced as described by Baker et al.

[11] with slight modification. The mice (n = 40) were given sulphamethoxazole/trimethoprim (Samil, Ansan, Korea) in their drinking water for 10 days. Following a four-day resting period, mice were randomly divided into four groups: Sham, periodontitis (PD), PD_HSA+phIgG, or PD_NAC. The sham group received 2% carboxymethylcellulose in phosphate buffered saline; the other groups received oral inoculation of *P. gingivalis* KUMC-P4 (10^9 cells) plus *T. denticola* (10^9 cells) in 100 μL of 2% carboxymethylcellulose six times on alternative days. During the inoculation period of two weeks, the PD_HSA+phIgG and PD_NAC groups were fed with 6.6-mg/mL HSA plus 1-mg/mL phIgG and 10mM NAC in their drinking water, respectively. All mice were euthanized four weeks after the last inoculation. The right half of the maxilla was defleshed, immersed overnight in 3% hydrogen peroxide and stained with 1% methylene blue. The images of the lingual sites were taken under a
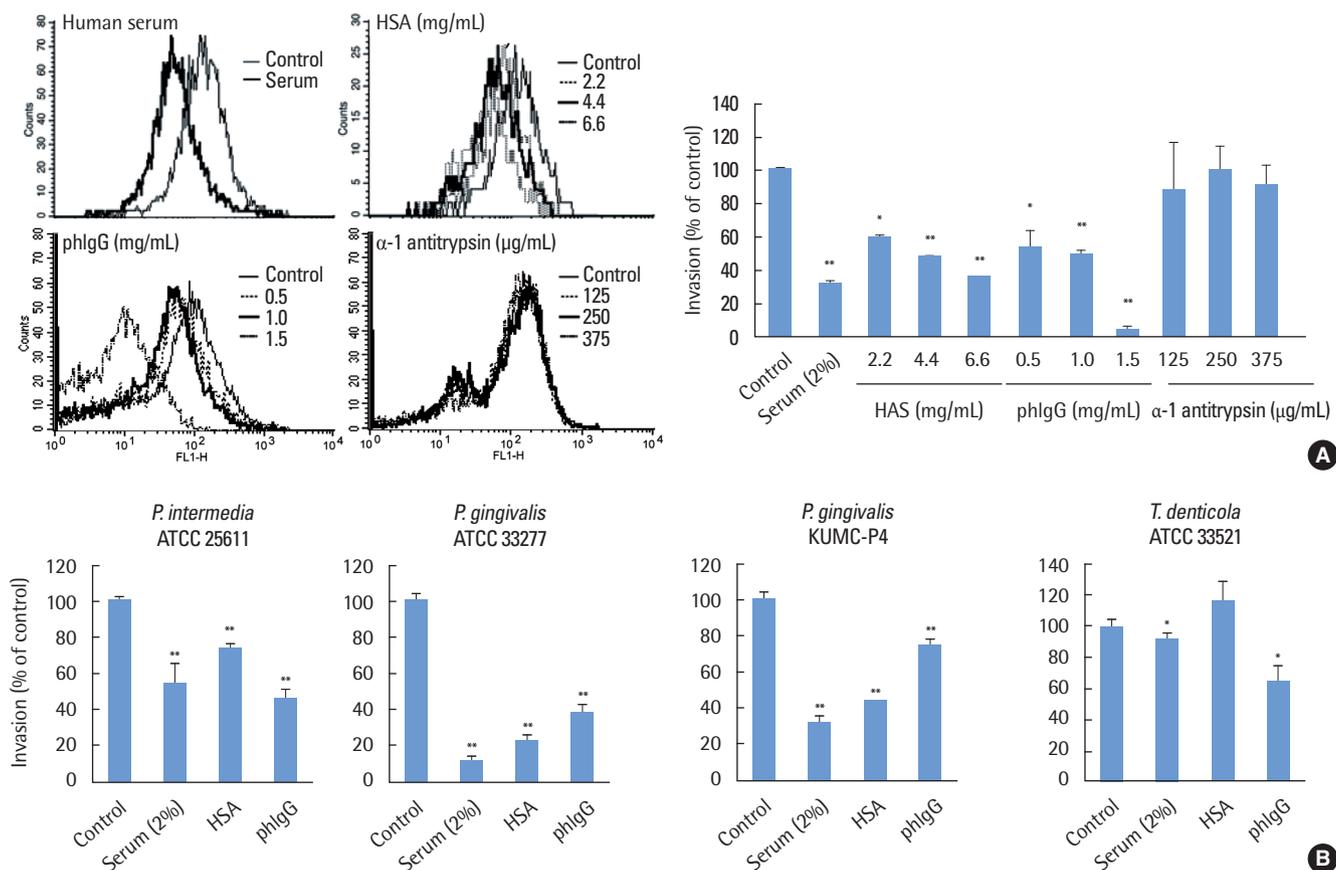

Figure 1. Human serum albumin (HAS) and pooled human IgG (phIgG) but not α1-antitrypsin inhibited the invasion of oral bacteria into HOK-16B cells. (A) HOK-16B cells were infected with carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labeled *Fusobacterium nucleatum* for 24 hours in the absence or presence of human serum, HSA, phIgG or α-1 antitrypsin at the indicated concentrations. After quenching the fluorescence of the bacteria bound on the surface, the cells were analyzed by flow cytometry. The FL-1 intensities of the infected cells in the presence of human serum or serum components are overlaid on those of the control infected cells in the absence of an additive (left panel). Bacterial invasion was calculated by subtracting the mean fluorescence intensity of the fixed cell control from that of the infected cells and is expressed as the percentage of the control condition without serum additives (right panel). (B) HOK-16B cells were infected with various CFSE-labeled oral bacterial species in the absence or presence of human serum, HSA (4.4 mg/mL) or phIgG (1 mg/mL) for 24 hours, and the bacterial invasion was analyzed by flow cytometry. Bacterial invasion is expressed as the percentage of the control condition without serum additives. The mean ± standard error of the mean of two experiments in triplicate is presented. *P. intermedia*: *Provetella intermedia*, *P. gingivalis*: *Porphyromonas gingivalis*, *T. denticola*: *Treponiema denticola*. *$P<0.05$ and **$P<0.0001$ compared with control.
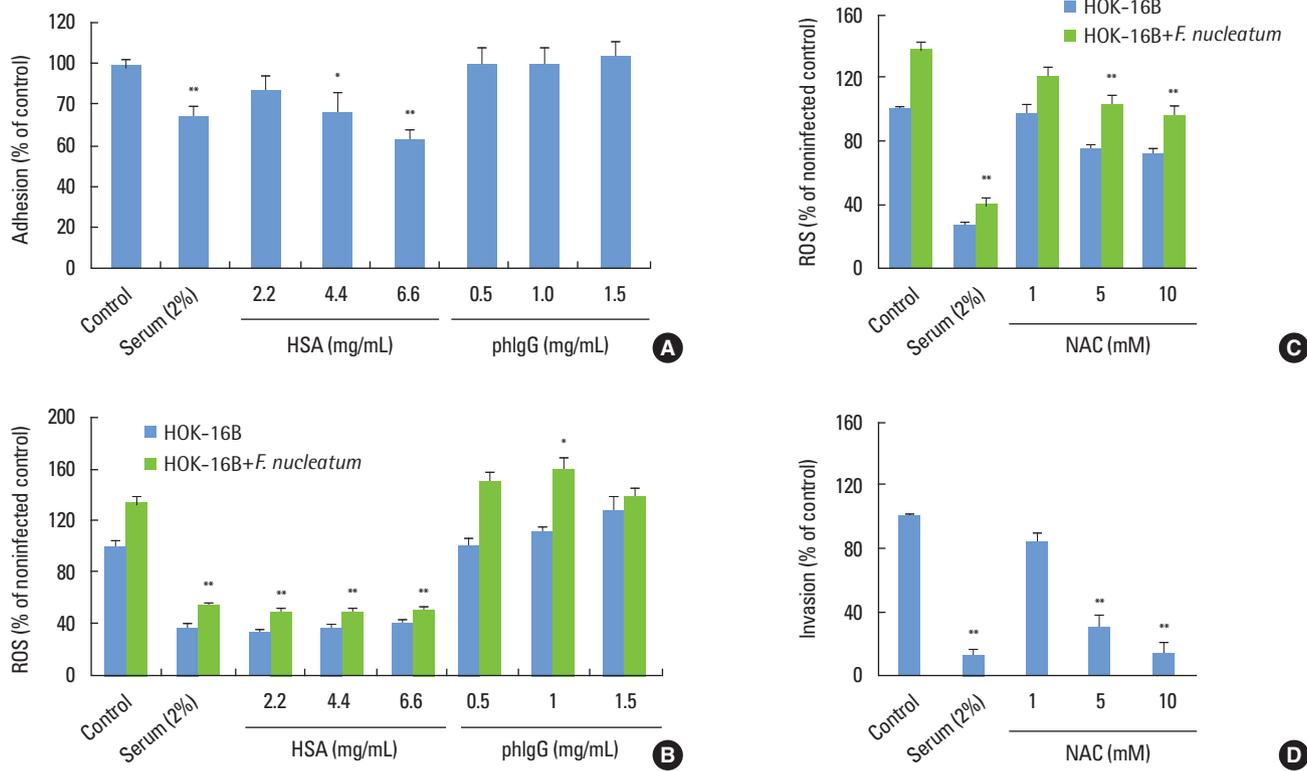
Figure 2. Human serum albumin (HAS) but not pooled human IgG (phIgG) reduced bacterial adhesion and the levels of intracellular reactive oxygen species (ROS) in HOK-16B cells. (A) HOK-16B cells were incubated with carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labeled *Fusobacterium nucleatum* on ice for 1 hour in the absence or presence of human serum, HAS, or phIgG. After washing, the cells were analyzed by flow cytometry without quenching. Bacterial adhesion was calculated by subtracting the mean fluorescence intensity of the HOK-16B cells alone from that of the bacteria-infected cells and is expressed as the percentage of the control adhesion. (B) HOK-16B cells prestained with 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) were incubated with various concentrations of HSA and phIgG in the absence or presence of *F. nucleatum* infection for 24 hours. The levels of intracellular ROS were measured by DCF fluorescence. (C) HOK-16B cells prestained with carboxy-H_2DCFDA were incubated with various concentrations of N-acetylcysteine (NAC) in the absence or presence of *F. nucleatum* infection for 24 hours. (D) HOK-16B cells were infected with CFSE-labeled *F. nucleatum* in the absence or presence of various concentrations of NAC, and the invasion of *F. nucleatum* was analyzed by flow cytometry. The mean ± standard error of the mean of two experiments in triplicate is presented. *$P < 0.05$ and **$P < 0.0001$ compared with control.

dissecting microscope (40×). The distance from the cementoenamel junction to the alveolar bone crest at six predetermined lingual sites below each cusp were blindly measured with CellSens digital imaging software (Olympus Korea, Seoul, Korea) on coded images.

Statistical analysis

The differences between the control and experimental groups in the *in vitro* studies were determined using a two-tailed Student *t*-test. All animal data groups passed a normality test. Thus, the one-way analysis of variance and *post hoc* tests were applied to determine the between-group differences. $P < 0.05$ was adopted as the significance level.

RESULTS

HSA and phIgG but not α1-antitrypsin inhibit the invasion of oral bacteria into gingival epithelial cells

To identify the responsible component(s) of human serum that

inhibit the bacterial invasion of gingival epithelial cells, three major components of human serum, including albumin, IgG, and α1-antitrypsin, were chosen and their effect on the invasion of CFSE-labeled *F. nucleatum* into immortalized human gingival epithelial HOK-16B cells was examined by flow cytometric invasion assay. *F. nucleatum* was chosen because of its high invasive ability among the several oral species with known invasive capacity [6]. The selected concentrations of each serum component are equivalent to those present in 5%, 10%, and 15% of human serum, respectively [12]. Both HSA and phIgG inhibited the invasion of *F. nucleatum* in a dose-dependent manner, while α1-antitrypsin had no effect (Fig. 1A left panel). In repeated experiments, the HSA significantly inhibited the invasion of *F. nucleatum* but less effectively than 2% human serum at all three concentrations. The phIgG was more effective than the HSA, inhibiting the invasion of *F. nucleatum* almost completely at 1.5 mg/mL (Fig. 1A right panel).

We next investigated whether the inhibitory effect of HSA and phIgG on bacterial invasion could be extended to other bacterial

species. *P. intermedia*, *P. gingivalis*, and *T. denticola* are increased in periodontal lesions and have substantial invasive capacities [6,13]. HSA significantly decreased the invasion of *P. intermedia* and *P. gingivalis* but not that of *T. denticola*, while phIgG was effective for all three species. In addition, both HSA and phIgG were also effective for the *P. gingivalis* clinical isolate KUMC-P4 (Fig. 1B).

HSA but not phIgG reduces bacterial adhesion and the levels of intracellular ROS in HOK–16B cells

Bacterial adhesion is a critical step prior to invasion into host cells. The effect of HSA and phIgG on bacterial adhesion was thus examined. The HSA significantly reduced the adhesion of *F. nucleatum* onto HOK-16B cells in a dose-dependent manner. In contrast to our expectations, phIgG did not inhibit bacterial adhesion (Fig. 2A).

The antioxidant function of HSA is well-characterized [14]. ROS

are now recognized to play essential roles in signal transduction throughout various physiological processes, including the actin cytoskeleton reorganization that is required for bacterial invasion [15,16]. Thus, the effect of HSA and phIgG on the levels of intracellular ROS in HOK-16B cells was determined by DCF fluorescence. Infection with *F. nucleatum* up-regulated ROS in HOK-16B cells. All three doses of HSA and 2% human serum equivalently reduced more than 50% of both the basal and *F. nucleatum*-induced ROS. In contrast, phIgG slightly increased the basal levels of ROS in a dose dependent manner (Fig. 2B). To confirm the role of ROS in bacterial invasion, HOK-16B cells were infected with *F. nucleatum* in the presence of NAC, a free radical scavenger. NAC inhibited both the levels of ROS and the invasion of *F. nucleatum* in a dose-dependent manner (Fig. 2C, D).
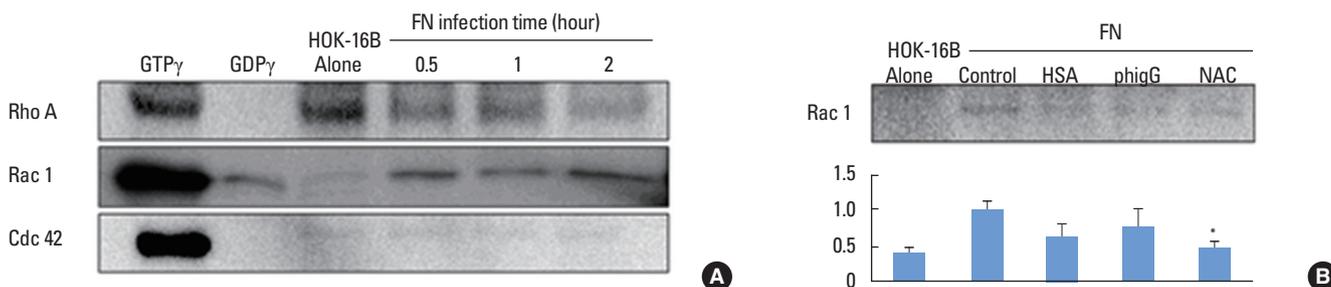
Figure 3. Human serum albumin (HAS), pooled human IgG (phIgG), and N-acetylcysteine (NAC) inhibited the *Fusobacterium nucleatum*-induced activation of Rac1. (A) The lysates of the HOK-16B cells infected with *F. nucleatum* (FN) for 0.5, 1, or 2 hours were subjected to pull-down assays for RhoA, Rac1, and Cdc42. As a positive and a negative control, the lysates of the HOK-16B cells alone were incubated with GTPγ and GDPγ, respectively, prior to being subjected to the pull-down assays. (B) The lysates of the HOK-16B cells infected with *F. nucleatum* for 2 hours in the absence or presence of HSA (4.4 mg/mL), phIgG (1 mg/mL), or NAC (5mM) were subjected to a pull-down assay for Rac1. The band intensities in the gel images from three experiments were measured and are expressed as the mean ± standard error of the mean. *$P < 0.05$ compared with control.
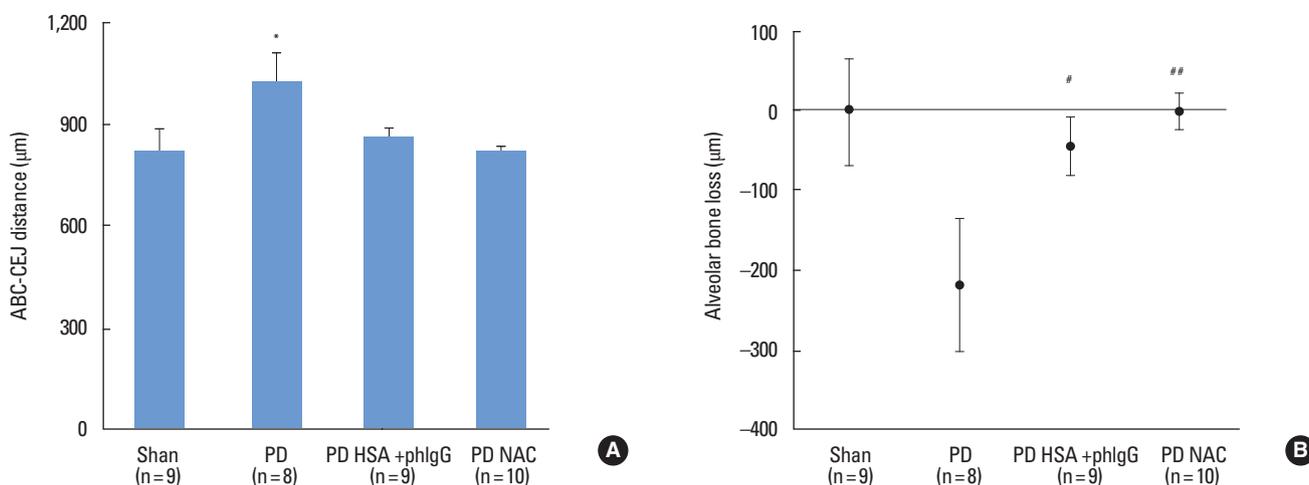
Figure 4. Human serum albumin (HAS)+pooled human IgG (phIgG) and N-acetylcysteine (NAC) prevented experimental periodontitis in mice. Experimental periodontitis (PD) was induced via the oral inoculation of *Porphyromonas gingivalis* and *Treponiema denticola*. During the inoculation period of two weeks, the PD_HSA+phIgG and PD_NAC groups were fed with 6.6-mg/mL HSA plus 1-mg/mL phIgG and 10mM NAC in their drinking water, respectively. Distances between the cementoenamel junction to the alveolar bone crest (ABC–CEJ) at six lingual sites per mouse were measured blindly (A), and the alveolar bone loss in experimental periodontitis was calculated (B). *$P < 0.01$ compared with sham. #$P < 0.05$ and ##$P < 0.01$ compared with PD.

HSA, phIgG, and NAC inhibit the *F. nucleatum*-induced activation of Rac1

The bacterial invasion of host cells depends on the dynamic rearrangement of the actin cytoskeleton. The Rho family GTPases, including RhoA, Rac1, and Cdc42, are important regulators of actin cytoskeleton dynamics [17]. To examine the involvement of RhoA, Rac1, and Cdc42 in *F. nucleatum* invasion, HOK-16B cells were stimulated with *F. nucleatum* for 0.5, 1, or 2 hours, and the activation of RhoA, Rac1, and Cdc42 was examined using a pull-down assay. *F. nucleatum* induced activation of Rac1 and concomitant inactivation of RhoA, while Cdc42 was not affected (Fig. 3A). Subsequently, the effect of HSA, phIgG, and NAC on the *F. nucleatum*-induced Rac1 activation was examined. All three reagents reduced the amount of activated Rac1 upon infection with *F. nucleatum* (Fig. 3B), corroborating their inhibitory effect on invasion.

Both HSA+phIgG and NAC prevent experimental periodontitis in mice

We tested whether these reagents with inhibitory effects on bacterial invasion could prevent periodontitis in mice. Periodontitis was induced by oral infection with the periodontal pathogens *P. gingivalis* and *T. denticola* and the effect of either HSA+phIgG or NAC on alveolar bone loss was examined. Because HSA was more effective than phIgG at inhibiting *P. gingivalis* invasion but was not effective on *T. denticola*, HSA and phIgG were administered together. *P. gingivalis* and *T. denticola* resulted in significant alveolar bone destruction. Both the PD_HSA+phIgG and PD_NAC groups showed significantly lower amounts of alveolar bone loss than the PD group ($P=0.03$ and $P=0.006$, respectively). In particular, NAC completely prevented the alveolar bone loss induced by the periodontal pathogens (Fig. 4).

DISCUSSION

In this study, we demonstrated that the two serum components HSA and IgG inhibit the bacterial invasion of gingival epithelial cells and reduce alveolar bone loss in experimental periodontitis. HSA substantially inhibited the invasion of various oral bacterial species except *T. denticola*. A potential underlying mechanism for this effect may be indicated by our observations that HSA decreased the adhesion of *F. nucleatum* to HOK-16B cells, which may involve the capability of HSA to bind to the surface structures of various bacteria [18]. This coincides with a previous report that described the inhibitory effect of HSA on the adhesion and invasion of *P. gingivalis* and *A. actinomycetemcomitans* to oral epithelial cells [9]. HSA is an acidic protein that increases the net negative charge on the bacterial surface and reduces hydrophobic interaction [19], one of the major forces in nonspecific bacteria-host interactions [20,21]. Following adhesion to the host cells, the bacterial invasion process involves the rearrangement of the actin cytoskeleton of host cells. *F. nucleatum* induced activation of Rac1, which is also involved in the invasion of group B *Streptococcus, Salmonella* and *Campylobacter jejuni* into

nonphagocytic host cells [22-24]. Rac1 induces membrane ruffling [25], and activated Rac1 has been observed at the protruding lamellipodia [26]. Rac1 is also involved in the production of ROS as an essential component of nonphagocytic NADPH oxidase complexes [15], and the ROS then regulate actin cytoskeletal remodeling, the formation of lamellipodia and membrane ruffling [16]. HSA suppressed the levels of both basal and *F. nucleatum*-induced ROS. Together with the interference in adhesion, therefore, the suppression of ROS must underlie the inhibitory effect of HSA on bacterial invasion. The critical role of ROS in *F. nucleatum* invasion was confirmed by the use of NAC, which substantially inhibited the invasion of *F. nucleatum* to HOK-16B cells.

Treatment with phIgG effectively inhibited the invasion of all tested species, although the underlying mechanisms for this are not yet clear. phIgG inhibited the *F. nucleatum*-induced Rac1 activation without inhibiting either the bacterial adhesion or the levels of ROS in the HOK-16B cells. One potential mechanism by which phIgG inhibits bacterial invasion could be through the neutralization of key adhesins. For example, *F. nucleatum* FadA and the fimbriae of *P. gingivalis* play important roles in the attachment to and invasion of host cells [27,28]. Although the levels of antibodies specific to these adhesins in the phIgG were not determined, the presence of IgG specific to FadA or *P. gingivalis* fimbriae in the sera from both healthy individuals and patients with periodontitis has been reported [29,30]. The inhibition of the invasion of *P. gingivalis* by antibodies to beta 1 integrin, an epithelial cell receptor for the *P. gingivalis* fimbriae, has been demonstrated [31]. Similarly, the neutralization of adhesins on the bacterial surface with phIgG would impede the specific adhesin-receptor interaction, inhibiting bacterial invasion. Nonetheless, the fact that phIgG did not inhibit the adhesion of *F. nucleatum* may indicate the greater role of nonspecific interaction than specific interaction in bacterial adhesion to host cells, as it is the case in bacterial adhesion to solid surface [32].

In the current study, *F. nucleatum* was chosen to investigate the inhibitory mechanisms of HSA and phIgG on bacterial invasion of epithelial cells. However, the inhibitory effects of HSA and phIgG observed on other bacterial species may employ other mechanisms, which warrant further investigation.

The HSA+phIgG administered during the period of bacterial inoculation significantly reduced the alveolar bone loss induced by *P. gingivalis* and *T. denticola*. In the course of gingivitis and periodontitis, GCF is increased and transformed into true inflammatory exudates, deriving 70% of its protein contents from serum [7] where HSA and IgG constitute 54% and 17% of the total protein, respectively. Furthermore, gingival bleeding is the first sign of gingival inflammation. The increase of GCF has been understood to act negatively in the pathogenesis of periodontitis by providing nutrient sources to proteolytic bacteria such as periodontal pathogens. However, our results imply that gingival bleeding may have a physiologic function as a part of defense response by providing serum components to inhibit bacterial invasion.

It is interesting that NAC completely prevented the experimentally

induced alveolar bone loss in mice. NAC administered in drinking water is expected to inhibit the bacterial invasion of epithelial cells in the oral cavity. Toker et al. [33] showed that NAC administered via gastric feeding prevented ligature-induced periodontitis in rats through the inhibition of osteoclast formation. In contrast with that study, in which NAC was administered throughout the entire experiment, NAC was given only during the period of bacterial inoculation in our study. Therefore, the effect on osteoclastogenesis is expected to be minimal. However, ROS comprise the important antimicrobial machinery of phagocytes and are also involved in diverse immune signaling pathways, such as Toll-like receptors and tumor necrosis factor-α [15]. Thus, systemic application of NAC for the prevention of periodontitis warrants further investigation.

In conclusion, we have shown that NAC and the serum components HSA and phIgG inhibited the bacterial invasion of oral epithelial cells *in vitro* and successfully prevented alveolar bone loss in the murine experimental periodontitis model. This suggests that the bacterial invasion process could be a potential drug target for the prevention of periodontitis.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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