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https://doi.org/10.15017/1441069
Cigarette smoke impairs phagocytosis of apoptotic neutrophils by alveolar macrophages via inhibition of the histone deacetylase/Rac/CD9 pathways

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Received 17 May 2013, accepted 11 June 2013

Abstract
Efferocytosis, which is the homeostatic phagocytosis of apoptotic cells, prevents the release of toxic intracellular contents and subsequent tissue damage. Impairment of efferocytosis was reported in alveolar macrophages (AMs) of patients with chronic obstructive pulmonary disease (COPD), a common disease caused by smoking. In COPD, histone deacetylase (HDAC) activity is reduced in AMs. We investigated whether the reduction of HDAC activity is associated with the impairment of efferocytosis. Murine AMs were collected by bronchoalveolar lavage and their ability to efferocytose apoptotic human polymorphonuclear leukocytes was assessed. Pre-treatment of AMs with cigarette smoke extract (CSE) or trichostatin A (TSA), an HDAC inhibitor, suppressed efferocytosis and CSE reduced HDAC activity. TSA inhibited the activity of Rac, a key mediator of efferocytosis. These TSA-induced impairments were restored by treatment of AMs with aminophylline, a potent activator of HDAC. To further elucidate the underlying mechanism, we explored a role of CD9 in TSA-induced impairment of efferocytosis. CD9 is a transmembrane protein of the tetraspanin family that facilitates the uptake of several pathogens and other material. TSA profoundly down-regulated the expression of CD9 on AMs. The expression of CD9 was partly down-regulated by the Rac inhibitor. Pretreatment with an anti-CD9 mAb or CD9 small interfering RNA inhibited efferocytosis, which was attributable to the reduced binding of AMs to apoptotic cells. These results suggest that smoking impairs efferocytosis via inhibition of HDAC/Rac/CD9 pathways. Aminophylline/theophylline is effective in restoring the impairment of efferocytosis and might have benefit for the treatment of patients with COPD.

Keywords: aminophylline, chronic obstructive pulmonary disease, efferocytosis, tethering

Introduction
Apoptosis, a form of programmed cell death, is a mechanism for cell deletion to maintain normal tissue homeostasis as well as for pathophysiologic processes throughout the body (1). Apoptotic cells are removed by both professional phagocytes (macrophages, other members of the mononuclear phagocyte system and dendritic cells) and non-professional phagocytes (most tissue cells, including epithelium, endothelium, smooth muscle cells, stromal cells and fibroblasts) (2, 3). Phagocytosis of apoptotic cells and necrotic cells, namely efferocytosis, is the physiologic removal of dead cells before membrane permeability sets in, thus preventing the release of potentially toxic intracellular contents (3). The regulation of efferocytosis is tightly controlled by Rho-family GTPases; thus, RhoA inhibits and Rac promotes the process (4, 5). Efferocytosis may be particularly important in the removal of apoptotic neutrophils because of their large numbers during inflammation, their short lifespan and their large stores of proteases, inflammatory mediators and oxidants.

Chronic obstructive pulmonary disease (COPD), one of the most common chronic diseases associated with
cigarette smoking, is characterized by neutrophil-dominant inflammation. Clinicians continue to be challenged by evidence that its prevalence has considerably increased. To establish a relevant therapy, the elucidation of pathogenic mechanisms is warranted. The oxidants in the smoke damage a variety of cell membranes and provoke cellular dysfunction, resulting in non-physiological cell death. If the removal of dead cells is delayed, an array of intracellular danger-associated molecular patterns leaks, causing pro-inflammatory responses. Indeed, it has been reported that the efferocytosis of alveolar macrophages (AMs) is impaired in patients with COPD (6). It is reasonable to speculate that impairment of efferocytosis may contribute to the development of COPD.

Importantly, the inflammation in COPD is unresponsive to corticosteroids. Even high doses of inhaled and oral glucocorticoids have no effect on the inflammatory cells and cytokine profiles and fail to reverse the protease–anti-protease imbalance (7). The anti-inflammatory property of corticosteroids partly depends on epigenetic regulation via recruitment of histone deacetylase (HDAC). Thus, HDAC regulates the balance of acetylation/deacetylation in the chromatin structure and subsequently attenuates pro-inflammatory gene expression. In COPD, HDAC activity is reduced in AMs (8). The inhibition of HDAC activity enhances the production of CXCL8/IL-8 and matrix metalloproteinases (8, 9), leading to sustained inflammation and connective tissue destruction. However, the role of HDAC in efferocytosis has not been clarified.

In the current study, we aimed to investigate possible roles of HDAC in efferocytosis by murine AMs. The study demonstrated that the inhibition of HDAC activity impaired efferocytosis through the inhibition of Rac activity, which was prominently restored by treatment with an HDAC activator, aminophylline. To further investigate the underlying mechanisms, we focused on CD9, a transmembrane protein from the tetraspanin family, that facilitates the uptake of several pathogens (10–12), monocyte fusion (13) and is associated with endocytosis of exosomes by dendritic cell (14). The results of our investigation revealed that the status of HDAC activity and downstream CD9 expression might be linked to efferocytosis.

Methods

Animals

Eight- to 10-week-old C57BL/6N mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All experimental procedures were approved by the animal research ethics committee of Kyushu University.

Reagents

Mouse anti-human CD45 mAb (HI30) and rat anti-mouse CD9 mAb (KMC8) were purchased from BD Biosciences (San Diego, CA, USA). PE-conjugated rat anti-mouse CD9 mAb (KMC8) was purchased from eBioscience (San Diego, CA, USA). FITC-conjugated rat anti-mouse F4/80 mAb was purchased from AbD Serotec (Kidlington, Oxford, UK). Trichostatin A (TSA) was purchased from Wako (Osaka, Japan). Aminophylline was purchased from Abbott Japan (Tokyo, Japan). The PI3Kδ inhibitor (IC87114) was purchased from Biozol (German, Italy). The Rac inhibitor (NSC23766) and the Rho kinase inhibitor (Y27632) were purchased from Calbiochem (San Diego, CA, USA). pHrodo™ succinimidyl ester (SE) was purchased from Life Technologies (Carlsbad, CA, USA).

Cigarette smoke extract

Cigarette smoke extract (CSE) was prepared as described previously (15). Commercial cigarettes (Marlboro brand) were smoked and the mainstream smoke was drawn by application of a vacuum to the vessel containing PBS. Each cigarette was smoked for 5 min. Three cigarettes were used to generate 30 ml of CSE solution. The CSE was diluted with X-vivo (Lonza, Walkersville, MD, USA). Final concentrations of these solutions were expressed as percent values, which were calculated with the following equation: (ml of CSE solution / total ml) × 100. The “total ml” in this equation is the sum of ml of CSE solution and ml of X-vivo. Solutions ranging from 1 to 5% were used in the present studies; these values approximately corresponded to exposures associated with smoking slightly fewer than 0.25 packs per day to slightly fewer than 1 pack per day of cigarettes (15).

Cell isolation

Human PMNs were isolated from peripheral blood of healthy volunteers using gradient centrifugation as previously described (16). Briefly, blood was obtained by puncture of a peripheral vein and was drawn into vacuum tubes containing EDTA-2Na. Anticoagulated whole blood was layered over Mono-Poly resolving medium (DS Pharma Biomedical, Osaka, Japan) in a polystyrene tube. The tube was centrifuged at 400 g for 30 min. Approval for experiments with human samples was provided by the Human Ethics Committees of the Kyushu University. Mouse AMs were obtained by bronchoalveolar lavage. Mice were killed with a lethal dose of pentobarbital, and their lungs were gently lavaged with PBS via a tracheal cannula.

Induction of apoptosis

Human PMNs were exposed to ultraviolet irradiation at 302 nm for 10 min and cultured in RPMI 1640 for 2.5 h at 37°C. This method yielded the highest rate of apoptosis (>80%), assessed by flow cytometric analysis using Annexin V binding in combination with propidium iodine staining as previously reported (17, 18).

Opsonization of human PMNs

Human PMNs isolated from peripheral blood were re-suspended in DMEM at 5 × 10^6 cells/ml. Mouse anti-human CD45 mAb was added at 1 μg/10^6 cells and incubated for 30 min at 4°C while rotating.

Cell labeling with pHrodo-SE

Apoptotic human PMNs or opsonized human PMNs were suspended in PBS at 10^6 cells/ml. One microliter of 1 mg ml^-1 pHrodo-SE was added per 50 ml of cell suspension. After
incubation for 30 min at room temperature, cells were washed twice with PBS and re-suspended.

**Phagocytosis assay**

Two hundred thousand AMs per well were plated in six-well plates in X-vivo medium. After 24 h or 48 h of culture, non-adherent cells were gently washed, and pHrodo-labeled apoptotic human PMNs or opsonized human PMNs were added at a 5:1 ratio (apoptotic cells: AM) and incubated at 37°C for 2 h in X-vivo medium. AMs were gently washed to remove uningested apoptotic cells. AMs were detached from the plate by a cell lifter. Thus, collected cells were transferred to a FACS tube and analyzed by a flow cytometer. FITC-labeled anti-mouse F4/80 mAb stained macrophages and pHrodo-labeled apoptotic cells alone served to determine cut-offs for phagocytosis. For CSE studies and HDAC inhibitor studies, AMs were pre-treated with CSE or TSA for 48 h before administration of pHrodo-labeled cells. In several experiments, AMs were pre-treated with anti-CD9 mAb for 24 h before pHrodo-labeled cell administration. For Rho family GTPase studies, AMs were pre-treated with a Rac inhibitor or a Rho kinase inhibitor for 24 h before pHrodo-labeled cell administration.

**HDAC activity assay**

HDAC activity of AMs was assessed by the HDAC fluorimetric cellular activity assay kit following the manufacturer’s protocol (BIOMOL Research Laboratories, Plymouth Meeting, PA, USA). AMs were seeded at 5 x 10⁴ to 1 x 10⁵ cells/well in a 96-well plate in 100 μl X-vivo medium and incubated at 37°C for 24 h. After 24 h incubation, the medium was replaced, and a fluorogenic, cell-permeable substrate was added. Incubation was continued at 37°C for 2 h. Next, a developer solution B containing the HDAC fluorimetric assay reagent was added to the wells. The mixture of solution A and B was incubated for 5 min at room temperature and mixed with solution B. The mixture of solution A and B was incubated for another 5 min at room temperature and added to the plated cells. AMs transfected with CD9 siRNA were cultured for 48 h and analyzed. The gene-silencing effect was assessed by flow cytometry.

**RhoA and Rac activity assay**

Half a million AMs per well were plated in 24-well plates in X-vivo medium. After 24 h of culture, active RhoA or Rac was evaluated by ELISA and colorimetric approaches (G-LISA assays; Cytoskeleton, Denver, CO, USA). Cells were washed with ice-cold PBS and rocked at 4°C with an ice-cold lysis buffer. The lysates were cleared by centrifugation (10,600 x g, 2 min, 4°C) and incubated in a RhoA or Rac-GTP affinity plate for 30 min at 4°C on a shaker (200–400 r.p.m.). After 30 min of incubation, the wells were washed and incubated with a primary antibody for 45 min at room temperature on a shaker (200–400 r.p.m.). After 45 min of incubation, the wells were washed and incubated with secondary HRP-labeled antibody for 45 min at room temperature on a shaker (200–400 r.p.m.). Then the wells were washed, the signal was developed with HRP detection reagents, and the absorbance was measured at 490 nm.

**Analysis of surface expression on AMs**

Two hundred thousand AMs per well were plated in 6-well plates in X-vivo medium. After 48 h of culture, cells were washed using a flow buffer (PBS with 4% BSA, 0.2% azide, 0.2% EDTA). AMs were detached from the plate by a cell lifter. Thus, collected cells were transferred to a FACS tube. To block non-specific binding via Fc receptors, rat anti-mouse CD16/CD32 mAb (BD Biosciences, San Diego, CA, USA) was added at 0.5 μg/10⁶ cells for 20 min. Cells were further incubated with fluorescence-conjugated mAbs for 30 min in the dark. The cells were washed, re-suspended in flow buffer and processed for flow cytometric analysis by using the FACS caliber flow cytometer and Cell Quest software (BD Biosciences, San Jose, CA, USA).

**Small interfering RNA (siRNA) transfection**

AMs cultured in a 6-well plate were transfected with CD9 siRNA (SMF27B-0251) using GeneSilencer® (Genlantis, San Diego, CA, USA) according to the manufacturer’s instructions. 1000 ng siRNA was diluted per well. Two transfection solutions (solution A, containing the siRNA and solution B, containing the transfection reagent) were prepared. Solution A was incubated for 5 min at room temperature and mixed with solution B. The mixture of solution A and B was incubated for another 5 min at room temperature and added to the plated cells. AMs transfected with CD9 siRNA were cultured for 48 h and analyzed. The gene-silencing effect was assessed by flow cytometry.

**Binding reaction of AMs and apoptotic cells**

AMs cultured on multi-well glass slides with or without anti-CD9 mAb (20 μg ml⁻¹) were coincubated with apoptotic PMNs at a 5:1 ratio (apoptotic cells: AM). After 1 h of incubation at 16–20°C, AMs were gently washed to remove unbound apoptotic cells, fixed and stained for myeloperoxidase (MPO) using the DAB staining kit (Muto Pure Chemicals, Tokyo, Japan). The binding index was calculated by dividing the number of bound apoptotic cells by the total number of macrophages counted, multiplied by 100 (17).

**Statistical analysis**

Statistical analysis and P value calculations were conducted using the JMP statistical program (SAS Institute). For multiple comparisons, data were analyzed by ANOVA. When ANOVA indicated significance, the Dunnett’s and Tukey-Kramer parametric tests were used for single and multiple comparisons. Non-parametric data were analyzed using the Wilcoxon signed-rank test. P values of <0.05 were considered significant.

**Results**

**Efferocytosis is regulated by HDAC**

Freshly isolated AMs were used to examine the effect of CSE on efferocytosis. Apoptosis of human PMNs was effectively induced by UV irradiation (Fig. 1A). After pre-treatment with CSE, AMs were incubated with apoptotic human PMNs for 2 h. Typical efferocytosis was shown under microscopic evaluation (Fig. 1B). In a preliminary study, we assessed efferocytosis by a phagocytic index. The phagocytic index was calculated by dividing the number of ingested apoptotic cells by the total number of macrophages counted, multiplied by 100 (2, 19,
Next, we assessed efferocytosis by flow cytometric analysis. We used pHrodo™ dyes as an indicator for phagocytosis. pH-sensitive pHrodo™ dyes are almost non-fluorescent at neutral pH and fluoresce brightly in acidic environments. Thus, labeled cells that are non-phagocytosed or attached to macrophages are dim. Once these cells are engulfed, the signal becomes brighter because of the acidic environment in the phagosomes. Approximately 20–30% of untreated AMs phagocytosed apoptotic cells (Fig. 1C). The percentage of phagocytic AMs was similar when the amount of apoptotic cells and/or incubation time were increased. Efferocytosis was significantly inhibited by CSE pre-treatment in a dose-dependent manner (Fig. 1D). Concentrations >5% CSE were toxic to the AMs. HDAC activity was significantly inhibited by CSE pre-treatment at concentrations >2.5% (Fig. 1E). When AMs were pre-treated with TSA, an HDAC inhibitor, and then incubated with apoptotic PMNs, efferocytosis was inhibited by TSA in a dose-dependent manner (Fig. 1F). In cell cultures, TSA at 30 nM did not affect cell viability while TSA at 100 nM decreased cell viability. In a previous study, reduced HDAC activity by cigarette smoke was reported to be mediated through PI3Kδ activation (21). Therefore, we examined the effect of IC87114, a PI3Kδ-specific inhibitor, on efferocytosis and HDAC activity. The CSE-induced inhibition of efferocytosis and of HDAC activity was prevented by the incubation of AMs with CSE in the presence of IC87114 (Fig. 1G). These results suggest that HDAC positively regulates efferocytosis and that cigarette smoke-induced PI3Kδ activation impairs efferocytosis through the inhibition of HDAC activity.

**HDAC regulates efferocytosis via Rac activation**

Efferocytosis is tightly controlled by Rho-family small GTPases. Thus, Rac induces efferocytosis whereas RhoA inhibits efferocytosis via Rho kinase activation (4, 5). Consistently, efferocytosis was significantly inhibited by the treatment of AMs with NSC23776, a Rac inhibitor, whereas it was significantly enhanced by treatment with Y27632, a Rho kinase inhibitor (Fig. 2A, 2B).

**Aminophylline enhances HDAC activity, Rac activity and efferocytosis**

CSE and/or TSA significantly inhibited efferocytosis, HDAC activity and Rac activity, but not RhoA activity (Fig. 3). Previous studies have shown that theophylline is a potent HDAC activator (8, 22). Pre-treatment of AMs with aminophylline, a more soluble derivative of theophylline, significantly enhanced efferocytosis, HDAC activity and Rac activity, but not RhoA activity (Fig. 3). Notably, these enhancements were evident even in the presence of CSE and/or TSA. These results suggest that the potency of aminophylline as an HDAC activator may overcome the concurrent burden of HDAC inhibition induced by pharmacological compounds and cigarette smoking.

**HDAC regulates efferocytosis partly via induction of CD9-mediated tethering**

CD9 expression on AMs was down-regulated by CSE, TSA and the Rac inhibitor (Fig. 4A, 4C). Given that CSE inhibited the expression of recognition molecules on AMs (6, 23), we examined the expression of CD14, CD31 and CD91 on AMs in the presence of TSA. TSA inhibited the expression of CD91 on AMs.
AMs. The expression of CD14 and CD31 was not affected by TSA (Fig. 4B). TSA-induced inhibition of CD9 expression was not restored by pre-treatment with aminophylline (Fig. 4D). To elucidate the role of CD9 in efferocytosis, AMs were cultured for 24 h in the presence or absence of anti-CD9 mAb and then incubated with apoptotic human PMNs (Fig. 4E). Efferocytosis, but not HDAC activity, was significantly inhibited by anti-CD9 mAb treatment. AMs transfected with CD9 siRNA also showed impaired efferocytosis. We further examined in which phase CD9 affected efferocytosis. Efferocytosis is composed of two steps, tethering and subsequent phagocytosis. To investigate the tethering step independently of phagocytosis, interactions between AMs and apoptotic cells were conducted at 16–20°C, which permitted binding, but not phagocytic, events to occur (Fig. 4F) (17). Tethering events were evaluated by the binding index. Tethering of apoptotic cells to AMs was significantly reduced by anti-CD9 mAb treatment compared with untreated AMs (Fig. 4G). In contrast, phagocytosis of IgG-opsonized cells was equivalent for both untreated AMs and anti-CD9 mAb-treated AMs (Fig. 4H). In previous studies, the inhibition of apoptotic cell recognition molecules did not affect phagocytic events (17, 24). These results suggest that HDAC regulates efferocytosis at least partly via the induction of the CD9-mediated tethering of apoptotic cells and that Rac is partly responsible for the regulation of CD9 expression. Finally, we examined the effect of double or triple use of inhibitors (Fig. 4I). Using two or three inhibitors had greater effects than using a single inhibitor. Single administration of each drug (TSA, Rac inhibitor, or anti-CD9 mAb) showed a similar magnitude of suppression of efferocytosis. However, TSA in combination with a Rac inhibitor or anti-CD9 mAb showed stronger suppression than a Rac inhibitor in combination with anti-CD9 mAb. Triple administration did not lead to further suppression. These results suggest that the
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HDAC/Rac/CD9 pathway may be substantially divergent but partly involve convergent sub-pathways.

Discussion

The present study demonstrated that treatment of AMs with TSA, an HDAC inhibitor, impairs efferocytosis. The Rac family of small GTPases has been implicated in many cellular functions (25). Rac is required for membrane ruffling and efferocytosis whereas RhoA activation negatively regulates efferocytosis through its downstream effector Rho kinase (3). In the present study, a Rac inhibitor impaired efferocytosis whereas a Rho kinase inhibitor enhanced efferocytosis. TSA decreased activities of Rac but not RhoA. Previous studies revealed that the positive regulator Rac and the negative regulator Rho act in balance to enhance or inhibit the uptake of apoptotic cells (2, 26). Our findings suggest that TSA impairs efferocytosis by altering the balance of Rac and RhoA activity toward negative regulator dominance.

Treatment of AMs with an inhibitor of PI3Kα prevented the CSE-induced impairment of efferocytosis, suggesting the contribution of oxidative stress and subsequent activation of PI3Kα on the impairment of efferocytosis. Aminophylline reportedly activates HDACs through the inhibition of PI3Kα (27). In the present study, aminophylline prominently enhanced HDAC activity and efferocytosis even in the presence of TSA and/or CSE.

We explored the mechanisms underlying how HDAC activation induces efferocytosis in AMs. CD9 is associated with phagocytosis of several pathogens and other material (10–12, 14). However, the involvement of CD9 in efferocytosis has not been studied. CD9 expression on AMs was down-regulated by HDAC inhibition in the present study. The down-regulation was partly dependent on reduced Rac activity. Importantly, efferocytosis but not HDAC activity was reduced by anti-CD9 mAb. These results indicate that Rac/CD9 plays a role in HDAC-mediated efferocytosis in AMs. The HDAC inhibitor decreases CD9 expression on a mouse macrophage cell line (9) and peritoneal macrophages (28). In addition, CD9/CD81 double-knockout mice show airspace enlargement, a COPD-like phenotype (9). Lines of previous investigations therefore support our findings.

Several recognition molecules on AMs interact with apoptotic cells (3). Previous studies reported that CSE inhibits the expression of recognition molecules, including CD36, integrin αvβ3, CD31, CD44 and CD91, on AMs (6, 23). In the present study, TSA inhibited the expression of CD91, but not CD14 and CD31 on AMs. Interaction between phagocytes and apoptotic cells was assessed by the binding index. (H) AMs were cultured for 24h in the absence or presence of anti-CD9 mAb and incubated with pHrodo-labeled opsonized PMNs at 37°C for 2h. Phagocytosis was assessed by flow cytometry. (I) AMs were treated with TSA, a Rac inhibitor and/or anti-CD9 mAb. Pre-treated AMs were incubated with apoptotic PMNs for 2h, and then efferocytosis was assessed. Data represent the mean ± SEM (n = 4–6 per group). Asterisk, significantly different from untreated AMs (P < 0.05). Double asterisks, significantly different from untreated AMs (P < 0.05). Hash, significantly different from anti-CD9 mAb-treated AMs (P < 0.05). Triple asterisks, significantly different from AMs treated with a Rac inhibitor and anti-CD9 mAb (P < 0.05).
and apoptotic cells takes the following steps (3). First, tethering receptors (e.g. CD14 and/or CD31) hold the apoptotic cells in close approximation to the apoptotic cells. Second, tickling receptors (e.g. CD91 or phosphatidylserine receptor) then engage eat-me signals on the apoptotic cell surface, followed by signal transduction, resulting in membrane ruffling. Third, the apoptotic cells are ingested into a characteristic fluid-filled effecosome, where they are digested. In the present study, tethering but not phagocytosis was markedly reduced by anti-CD9 mAb, which implies that CD9 might be a presumptive tethering receptor in the process of efferocytosis.

In the present study, aminophylline restored efferocytosis, HDAC activity and Rac activity in the presence of TSA or CSE although TSA-induced inhibition of CD9 expression was not restored by aminophylline. The beneficial effect of aminophylline on HDAC/Rac-mediated down-regulation of CD9 might be nullified by currently unknown effect of aminophylline on the expression of CD9. Aminophylline may restore the impaired efferocytosis through its effect on CD9-independent pathways. Indeed, experimental results shown in Fig. 4I suggest that the HDAC/Rac/CD9 pathway is not tandem but involves parallel sub-pathways.

In summary, cigarette smoke impairs AM efferocytosis through the inhibition of HDAC/Rac/CD9 pathways. The inhibition is triggered by the activation of PI3Kδ and is markedly restored by treatment with aminophylline. The protective effect of aminophylline/theophylline may provide a novel therapeutic strategy to restore efferocytosis in patients with COPD.

Funding
Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan and the National Institute of Biomedical Innovation, Japan.

Acknowledgements
We thank Ms Makiko Kogo and Misae Awane (Kyushu University) for technical assistance.

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