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Annexin V decreases PS-mediated macrophage efferocytosis and deteriorates elastase-induced pulmonary emphysema in mice


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Yoshida S, Minematsu N, Chubachi S, Nakamura H, Miyazaki M, Tsuduki K, Takahashi S, Miyasho T, Iwabuchi T, Takamiya R, Tateno H, Mouded M, Shapiro SD, Asano K, Betsuyaku T. Annexin V decreases PS-mediated macrophage efferocytosis and deteriorates elastase-induced pulmonary emphysema in mice. Am J Physiol Lung Cell Mol Physiol 303: L852–L860, 2012. First published September 7, 2012; doi:10.1152/ajplung.00066.2012.—Efferocytosis is believed to be a key regulator for lung inflammation in chronic obstructive pulmonary disease. In this study we pharmacologically inhibited efferocytosis with annexin V and attempted to determine its impact on the progression of pulmonary emphysema in mouse. We first demonstrated in vitro and in vivo efferocytosis experiments using annexin V, an inhibitor for phosphatidylethanolamine-mediated efferocytosis. We then inhibited efferocytosis in porcine pancreatic elastase (PPE)-treated mice. PPE-treated mice were instilled annexin V intranasally starting from day 8 until day 20. Mean linear intercept (Lm) was measured, and cell apoptosis was assessed in lung specimen obtained on day 21. Cell profile, apoptosis, and mRNA expression of matrix metalloproteinases (MMPs) and growth factors were evaluated in bronchoalveolar lavage (BAL) cells on day 15. Annexin V attenuated macrophage efferocytosis both in vitro and in vivo. PPE-treated mice had a significant higher Lm, and annexin V further increased that by 32%. More number of macrophages was found in BAL fluid in this group. Interestingly, cell apoptosis was not increased by annexin V treatment both in lung specimens and BAL fluid, but macrophages from mice treated with both PPE and annexin V expressed higher MMP-2 mRNA levels and had a trend for higher MMP-12 mRNA expression. mRNA expression of keratinocyte growth factor tended to be downregulated. We showed that inhibited efferocytosis with annexin V worsened elastase-induced pulmonary emphysema in mice, which was, at least partly, attributed to a lack of phenotypic change in macrophages toward anti-inflammatory one.

Annexin V, an intracellular protein that is ubiquitously expressed in a wide range of cell types, is known to inhibit PS-mediated efferocytosis. This protein has a specific binding affinity for PS and interferes with the recognition of apoptotic cells by macrophages, leading to attenuated efferocytosis (3, 19, 20). Also annexin V was shown to inhibit the phagocytosis of membrane-symmetric live red blood cells but not the Fcγ-
mediated efferocytosis, which is another pathway of efferocytosis (20). Furthermore, mutant annexin V, which lost binding affinity to PS, did not affect macrophage efferocytosis (19), clearly showing that an inhibitory effect of annexin V was PS-mediated. Notably, an inhibitory effect of annexin V on efferocytosis was concentration-dependent and a relatively higher concentration was needed to exert the effect in these studies, compared with a concentration present in human serum (4, 23, 36). The extracellular concentration of endogenous annexin V is presumably too low to affect macrophage efferocytosis. Thus, we chose to use exogenous administration of annexin V to inhibit PS-mediated efferocytosis.

In the present study, we first tested an inhibitory effect of annexin V on efferocytosis in vitro and in vivo. We also pharmacologically inhibited PS-mediated efferocytosis in PPE-treated mice with annexin V to determine its impact on the progression of pulmonary emphysema.

MATERIALS AND METHODS

Animals. C57BL/6 female mice (8–10 wk old) were purchased from Oriental Yeast (Tokyo, Japan). Animals were humanely cared for, and the animal protocol was approved by the Keio University Panel on Laboratory Animal Care.

Cell isolation and preparation. Human polymorphonuclear cells (hPMNs) and peripheral blood mononuclear cells were isolated from peripheral blood of a healthy Japanese volunteer (nonsmoker) by sedimentation of red blood cells with dextran and ficoll gradient as previously described (25). Briefly, heparinized blood was centrifuged (300 g, 20 min, 22°C) and plasma was replaced with PBS containing dextran (mol wt: 400,000–500,000, final concentration 0.6% wt/vol) (Sigma, St. Louis, MO). After 1 h, the leukocyte-rich supernatant was pipetted onto Histopaque1077 (Sigma) and centrifuged (700 g, 30 min, 22°C). The layer of peripheral blood mononuclear cells above the Histopaque1077 were transferred to a new tube, washed four times in PBS containing 5 mM EDTA (Sigma), and finally suspended in RPMI medium (Invitrogen, Carlsbad, CA) to plate into eight-well chamber slides at a density of 5 × 10⁶ cells/well (Electron Microscopy Sciences, Hatfield, PA). The nonadherent cells were washed out after 1 h, and the adherent cells were cultured in RPMI medium containing 20% autologous plasma-derived serum and allowed to differentiate into human monocyte-derived macrophages (hMDMs) for 8 days before use. This method allowed hMDMs to become macrophage in the wells. Following ficoll gradient separation, pelleted hPMNs were resuspended in hypotonic lysis buffer to eliminate the residual red blood cells, washed twice with PBS containing 0.25% BSA (Sigma), and used to generate the apoptotic population. Apoptosis was induced in hPMNs for efferocytosis experiments by UV irradiation (312 nm) for 10 min using a transilluminator (CosmoBio, Tokyo, Japan), and used to generate the apoptotic population. Apoptosis was induced by 0.1 M NaCl (pH 8.6). The column was run at 0.2 ml/min using the same buffer as an elution buffer. The fractions containing annexin V were concentrated to 1–2 mg of protein/ml, and finally diluted in HBSS (Sigma) at a concentration of 10 μg/40 μl before use. The concentration of LPS in the annexin V solution was just detectable at 13 pg/ml and much lower than that required to elicit lung inflammation (>10 μg/ml) (12, 30). Protein purification was verified by SDS-PAGE and Coomassie Brilliant Blue staining. Specificity was confirmed by Western blot analysis with anti-annexin V antibodies (Sigma). A competition assay using the purified annexin V and FITC-labeled annexin V from manufacture (Invitrogen) was performed to verify the affinity of purified annexin V to PS. Fresh hPMNs were fixed and permeabilized in BD Cytofix/Cytoperd (BD, Franklin Lakes, NJ) so that the annexin V can be bound to PS on the cell membrane. Permeabilized hPMNs (1.5 × 10⁹/tube) were incubated with 25 ng of FITC-labeled annexin V and different amount of purified annexin V (0, 25, 250, and 2,500 ng) in the tube for 15 min and then analyzed for fluorescein intensity by flow cytometry (Epics XL-MCL, Beckman Coulter, Brea, CA).

Efferocytosis experiment. hMDMs cultured in eight-well chamber slides were coinoculated with 1.5 × 10⁶ apoptotic hPMNs in the absence or presence of purified annexin V at a final concentration of 500 ng/ml for 1 h. Free nonengulfed hPMNs were washed out from the wells, and adherent hMDMs were fixed with 2% glutaraldehyde for 2 min. Efferocytosis was confirmed by staining hPMNs inside the hMDMs for intrinsic MPO activity using 3,3′-diaminobenzidine (Mu-toPureChemicals, Tokyo, Japan). hMDMs were confirmed not to be stained for MPO by this method. Macrophages were counterstained with hematoxylin. At least 500 macrophages were inspected efferocytosis per slide. Phagocytosing macrophages, identified as hMDMs containing MPO-positive hPMNs, were visually assessed and the percentage efferocytosis was calculated as the number of phagocytosing macrophages divided by the total number of macrophages inspected.

Efferocytosis was also tested in vivo using C57BL/6 female mice. Mice were anesthetized intraperitoneally with ketamine (120 mg/kg) and xylazine (12 mg/kg) followed by intranasal delivery of either 10 μg of annexin V in 40 μl HBSS or saline alone. One hour after pretreatment with annexin V, 1 × 10⁶ apoptotic hPMNs in 40 μl HBSS were endotracheally instilled into the mouse lungs through a 22-gauge intravenous catheter, and efferocytosis was allowed to proceed for 2 h. Cells were collected in BAL fluid, and cytospin slides were prepared. Efferocytosis was identified in cytoplasmic inclusions of the same method described for the in vitro assay. Mouse alveolar macrophages were confirmed not to be stained for MPO by this method. The accuracy of this visual method had been verified previously by testing the correlation with flow cytometrical analysis (25). The assessments were done in a blind manner. The representative cytological images for in vitro and in vivo assays were taken using a Biorepo BZ-8100 (Keyence, Osaka, Japan).

Animal procedure. Mice were anesthetized intraperitoneally using ketamine (120 mg/kg) and xylazine (12 mg/kg). Mouse lungs were endotracheally instilled with PBS alone [PPE(−) group] or 1.5 units of PPE (Elastin Products, Owensville, MO) diluted in 40 μl of PBS via a 22-gauge intravenous catheter on day 1 [PPE(+) group]. PPE was chosen as an inducer in this study because previous studies have shown that it induced pulmonary emphysema, at least partly, via apoptosis. Apoptotic cells have been found in various phases following PPE administration (days 3–28) (22, 32). To determine a phase to start inhibiting efferocytosis, we first analyzed the cell profiles in BAL fluid following PPE endotracheal instillation. The initial phase of PPE-treated mice showed strong neutrophilic inflammation, and it then became a macrophage predominant, progressive process more similar to human disease after day 8 (data not shown). Also, we showed previously that neutrophil-derived inflammatory cytokines, keratinocyte-derived cytokine, and TNF-α, as well as extracellular matrix-degrading products in BAL fluid, were returned to a low grade.
at day 8 in PPE-treated mice (35). We therefore chose day 8 to start inhibiting efferocytosis and PPE-treated and untreated groups were subdivided into two arms on day 8. Mice were either given purified annexin V (10 μg in 40 μl HBSS) intranasally on a daily basis from day 8 until day 20 [Ann(+) or the same volume of HBSS alone [Ann(−)]. Treatment groups are expressed as PPE(+/−)Ann(+/−) below. Mice were killed by CO2 inhalation on day 21 for histological analysis, because day 21 is the commonly selected time point to see pulmonary emphysema in PPE-treated mouse. We also chose day 15 to evaluate the cellular profiles and other ex vivo experiments as a middle day in the phase of annexin V treatment to clarify an effect of annexin V on the progression of pulmonary emphysema.

Lung histology. On day 21, both lungs and heart were resected en bloc and the lungs were inflated with 10% neutral buffered formalin solution (Sigma) at a constant pressure of 25 cm H2O for 10 min. Following fixation in formalin for 24 h at room temperature, tissues were paraffin-embedded and transverse 3-μm sections of lung were cut (along with lobar bronchi) and stained with hematoxylin and eosin for morphological analysis. We evaluated the alveolar size by quantifying the mean linear intercept (Lm) in 10 randomly selected fields.

Fig. 1. Recombinant annexin V (Ann) binds to phosphatidylserine (PS) and impairs phagocytosis of apoptotic polymorphonuclear cells (PMNs) in vitro and in vivo. A: permeabilized human PMN cells (hPMNs) were incubated with 25 ng of FITC-Ann and various concentrations (0, 25, 250, and 2,500 ng) of purified recombinant Ann. Fluorescein intensity was analyzed by flow cytometry. B: human monocyte-derived macrophages (hMDMs) were coincubated with apoptotic hPMNs in the presence (Ann) or absence (Ctrl) of recombinant Ann. The percentage of phagocytosing hMDMs was assessed after 1 h of incubation. Representative pictures of both groups were shown (n = 4 in each group). C: mouse was anesthetized and given recombinant Ann in HBSS (Ann) or HBSS alone (Ctrl) intranasally and then instilled apoptotic hPMNs into the lungs 1 h later. After 2 h, bronchoalveolar lavage (BAL) fluid was collected and macrophage efferocytosis was evaluated. Representative pictures of both groups are shown (n = 4-5). Data are shown as means ± SE. *P < 0.05, **P < 0.01.
per lung specimen for each mouse. Lm was manually counted from images taken using a Biozero BZ-8100. Immunohistochemical staining for single-stranded DNA (ssDNA) was also carried out to detect cell apoptosis in the lung specimens. The slides were deparaffinized in xylene and rehydrated with ethanol. Antigen retrieval was performed with 0.01 M citrate buffer solution (pH 6.4) at 60°C for 10 min. Endogenous peroxidase activity was neutralized by incubating sections in 3% hydrogen peroxide for 15 min. Sections were washed and rehydrated in Blocking One (Nacalai Tesque, Kyoto, Japan) to block the non-specific binding sites for 10 min. Anti-ssDNA rabbit polyclonal antibody (DAKO Cytomation, Kyoto, Japan) was used as the primary antibody and incubated overnight at 4°C. Apoptotic cells were finally visualized in the lung specimens using Dako Envision+ HRP kit (Dako, Tokyo, Japan) according to the manufacturer’s recommendations. Hematoxylin was used for counterstaining. The number of ssDNA-positive cells per alveolar epithelial cells was manually counted from three randomly selected fields in the blinded manner.

**Bronchoalveolar lavage.** The whole lungs were lavaged four times with 1 ml of PBS and stored in a tube on ice on days 8 and 15. Bronchoalveolar lavage (BAL) fluid was centrifuged at 800 g for 5 min at 4°C, and the supernatant was stored at −80°C until required. Cell pellets were resuspended in 1 ml of PBS, and cell counts were performed using a hemocytometer. Cytospin slides were prepared by centrifugation at 50 g for 5 min, and cells were stained with Diff-Quick stain (International Reagents, Kobe, Japan) for differential cell centrifugation at 50°C performed using a hemocytometer. Cytospin slides were prepared by centrifugation at 50 g for 5 min, and cells were stained with Diff-Quick stain (International Reagents, Kobe, Japan) for differential cell counts. In some experiments, the whole cell pellet was used either for RNA extraction or to quantify cell apoptosis. Endogenous concentrations in 0.3% hydrogen peroxide for 15 min. Sections were washed and rehydrated with ethanol. Antigen retrieval was performed using 0.01 M citrate buffer solution (pH 6.4) at 60°C for 10 min.

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**Protein analysis in bronchoalveolar lavage fluid.** Bio-Plex suspension array system (Bio-Rad, Hercules, CA) was used to measure the protein concentration of VEGF and TNF-α in BAL fluid. The assay was performed according to the manufacturer’s instructions.

**Detection of cell death in BAL fluid.** Since apoptosis and subsequent efferocytosis are very dynamic events and evidence is hardly found in the lung specimens, we took a sensitive method to quantify the apoptosis in BAL cell pellet. Histone-binding fragmented DNA was measured by enzyme-linked immunosorbent assay (Cell Death Detection ELISA kit, Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions.

**Statistics.** All data were expressed as means ± SE. Difference among groups was analyzed by ANOVA followed by Bonferroni’s comparison. For all analyses, a P value < 0.05 was considered statistically significant. All statistical analyses were performed using StatView software (SAS Institute, Cary, NC).

**RESULTS**

**Inhibition of efferocytosis by annexin V.** We first tested binding affinity of purified annexin V to PS located inside and outside of plasma membrane by using permeabilized PMNs. In the absence of purified annexin V, FITC-labeled annexin V from manufacture bound to PS on permeabilized hPMNs; thus all hPMNs showed higher FITC fluorescein intensity. When purified annexin V was added, hPMNs reduced fluorescein intensity in a dose-dependent manner (Fig. 1A), showing that purified annexin V competed with FITC-labeled annexin V.
bound to PS on permeabilized PMNs (Fig. 1A). In vitro efferocytosis experiment using hMDMs confirmed the decrease in efferocytosis by 25% with annexin V (Fig. 1B, \( P < 0.01 \)). Similarly, in vivo efferocytosis by mouse alveolar macrophages was inhibited with preinstillation of intranasal annexin V by 22% (Fig. 1C, \( P < 0.05 \)).

Inhibition of efferocytosis exacerbates the pulmonary emphysema in mice. Representative pictures of lung histology in each treatment group are shown in Fig. 2A. Lm values, representative of the size in alveolar space, were higher in both PPE-treated groups [Fig. 2B, PPE(+)Ann(−)] vs. PPE(−)Ann(−), \( P < 0.05 \) and PPE(+)Ann(+) vs. PPE(−)Ann(+), \( P < 0.001 \)]. Inhibition of PS mediated efferocytosis by annexin V further increased Lm value in PPE-treated groups by 32% [Fig. 2B, PPE(+)Ann(+) vs. PPE(+)Ann(−), \( P < 0.05 \)], while no effect was seen in PPE-untreated groups. We also assessed cell apoptosis in lung specimens by immunohistochemical staining for ssDNA (Fig. 3) and found that all treatment groups had a small number of apoptotic cells which was \(< 1\%\) of alveolar epithelial cells (Table 1). The ratio of ssDNA-positive cells was 0.80% in the PPE(+)Ann(+) group, which was not statistically different from that in the PPE(+)Ann(−) group (0.31%). Similarly, the difference between the PPE(−)Ann(−) and PPE(−)Ann(+) groups was not statistically significant (Table 1; 0.21 and 0.47\%, respectively).

Cell profiles in bronchoalveolar lavage fluid. Total and differential cell counts were performed on BAL fluid obtained on day 15 and are shown in Fig. 4. The majority of cells in BAL fluid were macrophages in each group and few neutrophils or lymphocytes were seen. The numbers of total cells and macrophages had a tendency to be increased by the treatment with PPE alone (Fig. 4), but the difference did not reach a statistical significance. Both numbers were increased in the PPE(+)Ann(+) group compared with that in the PPE(−)Ann(+) group (Fig. 4, \( P < 0.01 \) and \( P < 0.01 \), respectively) or in the PPE(+)Ann(−) group (Fig. 4, \( P < 0.05 \) and \( P < 0.05 \), respectively). Annexin V showed no effect on cell numbers in PPE-untreated groups. These results were paralleled to that for the Lm values in lung histology obtained on day 21.

mRNA expression in macrophages. Semiquantitative PCR was used to analyze mRNA expression in macrophage-rich BAL cells and revealed a similar trend among the groups for expression of MMPs-2 and -12 mRNA. Expression of MMP-2 mRNA was statistically higher in the PPE(+)Ann(+) group compared with the PPE(+)Ann(−) group (Fig. 5, \( P < 0.01 \)) with expression of MMP-12 also increased. For both MMPs-2 and -12, mRNA expression was higher in the PPE(+)Ann(+) group compared with the PPE(−)Ann(+) group (Fig. 5, \( P < 0.001 \) and \( P < 0.05 \), respectively). MMP-9 expression had a distinct pattern from the other MMPs. MMP-9 mRNA levels in the PPE(−)Ann(+) group were more than 10-fold higher than those in the PPE(−)Ann(−) group (Fig. 5, \( P < 0.01 \)), and levels in the PPE(+)Ann(+) group tended to be higher than in the PPE(+)Ann(−) group (Fig. 5, \( P = 0.13 \)). Expressed mRNA levels of growth factors, VEGF and KGF, did not differ significantly; however, KGF mRNA expression in the PPE(+)Ann(+) group tended to be lower than that in the PPE(+)Ann(−) group (Fig. 5, \( P =

Table 1. Immunohistochemical staining for ssDNA

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>% ssDNA-Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPE(−)Ann(−)</td>
<td>2</td>
<td>0.21 (0.00–0.42)</td>
</tr>
<tr>
<td>PPE(−)Ann(+)</td>
<td>3</td>
<td>0.47 (0.42–0.53)</td>
</tr>
<tr>
<td>PPE(+)Ann(−)</td>
<td>4</td>
<td>0.31 (1.00–1.08)</td>
</tr>
<tr>
<td>PPE(+)Ann(+)</td>
<td>4</td>
<td>0.80 (0.05–1.12)</td>
</tr>
</tbody>
</table>

Ranges are in parentheses. PPE, porcine pancreatic elastase, Ann, annexin V, ssDNA, single-stranded DNA.
0.06). TNF-α mRNA expression levels were similar for all groups.

**Protein levels of VEGF and TNF-α in BAL fluid.** The concentration of VEGF protein in BAL fluid was significantly lower in PPE(+)Ann(+) group compared with the PPE(+)Ann(-) group (Fig. 6, \(P < 0.05\)). There was no difference in TNF-α protein concentrations in BAL fluid between the groups (Fig. 6).

Cell death detection. Apoptotic cells were identified using histone-fragmented DNA and were found to be significantly higher in the PPE-treated group (Fig. 7, \(P < 0.001\) on day 8. By day 15 there was no significant difference in apoptosis among the groups (Fig. 7).

**DISCUSSION**

We showed in the present study that annexin V attenuated macrophage efferocytosis in vitro and in vivo, and worsened pulmonary emphysema in PPE-treated mice. Inhibition of PS-mediated efferocytosis with annexin V altered the macrophage phenotype toward a more inflammatory one and resulted in an increase in the amount of emphysema. In line with previous observations in other mouse models (24, 34, 38), our observations supported the concept that PS-mediated efferocytosis is a key regulator for lung inflammation. Defective efferocytosis has been observed in COPD but its relevance to the pathophysiology remained unclear. We provide the first evidence that an inhibition of PS-mediated efferocytosis actually worsens pulmonary emphysema in mice.

Annexin V can bind to PS externalized on surface of apoptotic cells with high affinity and specificity. Consistent with previous reports (3, 19, 20), we showed that annexin V decreased macrophage efferocytosis in vitro (Fig. 1B). We also demonstrated an in vivo experiment and showed that intranasal...
administration of annexin V attenuated macrophage efferocytosis in vivo, too (Fig. 1C). We applied same delivery system of annexin V to PPE-treated mice and found that annexin V treatment worsened pulmonary emphysema. An inhibitory effect conferred with annexin V was previously shown to be PS-mediated and concentration-dependent (19, 20). Hundreds of nanograms per milliliter or a higher concentration of annexin V was required to exert the efferocytosis inhibition (3, 19, 20). We delivered a daily dose of 10 μg that would achieve the required concentration. In contrast, an endogenous concentration of annexin V in BAL fluid obtained on day 15 was much lower and similarly detectable among treatment groups at 61–143 pg/ml (determined by ELISA, data not shown). Annexin V is an intracellular protein and its serum concentration was reported to range between 1.8 and 2.5 ng/ml in healthy subjects and 5.7 and 11 ng/ml in subjects with various diseases (4, 23, 36). No data is currently available about endogenous level of annexin V in human COPD. Even though we could not deny the possibility that endogenous annexin V plays some roles in our study, it is more reasonable to consider exogenous administration of annexin V did cause the change in our model.

Despite the inhibition of PS-mediated efferocytosis that we and others have shown (3, 19, 20), we unexpectedly observed a similar amount of apoptotic cells in lung specimens (Fig. 3, Table 1) and in BAL fluid (Fig. 7) among all groups. Although a minimal effect of annexin V was implied on increased apoptosis by immunohistochemical assessment in the lung specimens, the difference was not significant and apoptosis was detected < 1% of structural cells in all groups (Table 1). We believe the lack in increased apoptotic cells is due to several factors. The fraction of apoptotic cells in both lung specimen and BAL cells should be very small, and trying to see a difference may be too hard. Another explanation is that there are alternate pathways for efferocytosis. In more detail, we interfered with efferocytosis by shielding PS on apoptotic cells with annexin V. It is possible that macrophages may have recognized apoptotic cells via the eat-me molecules on apoptotic cells other than PS (reviewed in Ref. 37). As we and others showed that PPE-treated mice had, unlike efferocytosis experiments, a relatively small number of apoptotic cells (Fig. 3 and 7, Table 1) (22, 27, 32), PS-independent pathways could compensate a lack of PS-dependent efferocytosis. Another possibility is that the apoptotic cells underwent secondary necrosis and subsequent lysis if they were not efferocytosed, thus decreasing the numbers of apoptotic cells present.

It is of importance that PS-mediated efferocytosis, unlike other pathways, potentially promotes the phenotypic changes in macrophage toward anti-inflammatory one (10). It has been reported previously that the pharmacological inhibition of PS-mediated efferocytosis caused augmented lung inflammation by LPS (24), higher lethality in influenza virus infection (38), and enhanced immunogenicity of apoptotic cells (34) in mice. It should be possible to speculate that PS-dependent and -independent efferocytosis may have different consequences, particularly in terms of altering macrophage phenotypes. In our observations, macrophages from the PPE(+)/Ann(+) group kept manifesting a proinflammatory phenotype: higher MMPs and a trend of lower growth factor expression. We presume that the phenotypic consequence by inhibiting PS-mediated efferocytosis could be one of the mechanisms to augment the lung destruction regardless the overall efferocytosis.

Although PPE-treated groups experienced same inflammatory process between days 1 and 7, inhibition of PS-mediated efferocytosis with annexin V starting from day 8 led to a progression of pulmonary emphysema. The accompanying increase in the number of alveolar macrophages let us focus on macrophages as the main effector to this observation. In the PPE mouse model, alveolar destruction gradually progresses over 1 mo following PPE instillation (33), but little is known about the proteases involved particularly in the later phase. Elastolytic activity of PPE itself instilled on day 1 is presumably limited, and neutrophil-derived proteases have to be responsible for the progression of pulmonary emphysema in the initial phase. Plantier et al. (31) previously showed that neutrophil-derived MMPs-2 and -9 were involved in the pro-

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**Fig. 6.** Ann treatment reduces VEGF protein expression. PPE-treated and untreated mice were given either Ann in HBSS or HBSS alone from days 8 to 14. BAL fluid was collected on day 15, and the concentration of VEGF and TNF-α protein was determined ($n = 5–6$). Data are shown as means ± SE. *$P < 0.05$.

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**Fig. 7.** Apoptosis profiles in BAL fluid. PPE-treated and untreated mice were given either Ann in HBSS or HBSS alone from days 8 to 14. Fragmented DNA, indicating the amount of apoptotic cells, in BAL cell pellets was measured by enzyme-linked immunosorbent assay on days 8 and 15 ($n = 4–6$). Data are shown as means ± SE. NS, not significant; ***$P < 0.001$.
gression of pulmonary emphysema between days 0 and 3 in PPE-treated mouse. In a later phase, after day 8, macrophage-derived proteinases would be responsible for further destruction of the lungs. Since MMP-2 mRNA expression in macrophages was significantly upregulated in parallel with an increase in alveolar size by annexin V in PPE-treated mice (Fig. 5), the proteinase suggested to effect the parenchymal destruction of the lungs. Although the difference in MMP-12 expression in macrophages from the PPE(+)/Ann(+) and the PPE(+)/Ann(−) groups did not reach a statistical difference, the PPE(+)/Ann(+) group had an increased number of macrophages. It is expected that macrophage expansion and MMP-2 and 12 upregulation must synergistically increase proteolytic activity. In mouse models of smoking-induced pulmonary emphysema, MMP-12, but not MMP-9, was found to be the key proteinase involved (5, 6). MMP-2 might also be important in other mouse models of pulmonary emphysema (40). In human studies, MMP-2 positive macrophages were increased in the lung specimens from subjects with more emphysema and impaired lung function (2). In addition Ohnishi et al. (29) showed that the increase in MMP-2 in the lungs, which was associated with increased elastolytic activity, was positively related to the parenchymal destruction in the lungs from subjects with pulmonary emphysema. In our study, a direct relevance between upregulation of MMPs and worsened pulmonary emphysema was not proved; however, gathering observation in our, as well as in previous, studies let us consider the relevance between two. Interestingly, we detected elevated MMP-9 transcriptional activity in the PPE(−)/Ann(+) group similar to that in the PPE(+)/Ann(+) group, which was not associated with the progression of pulmonary emphysema. One possible explanation is that MMP-9 was increased by insufficient PS-mediated efferocytosis even in normal cell turnover without PPE-induced inflammation, and elevated MMP-9 alone was insufficient to cause development of emphysema in mice. In fact, MMP-9 knockout mice were not protected from the development of cigarette smoke-induced pulmonary emphysema (1). Since increased TNF-α expression in both mRNA and protein levels was not observed, augmented macrophage infiltration nor MMPs upregulation did not seem to be due to a TNF-α mediated effect.

Alternatively, pulmonary emphysema might be attributed to abnormal repair mechanisms. The tissue repair system is thought to be effective (21) and exogenous administration of KGF or hepatocyte growth factor (HGF) promoted lung repair in elastase-induced pulmonary emphysema (31). In addition, defective VEGF signaling was associated with progression of pulmonary emphysema via cell apoptosis (16, 17). The recent study by Kearns et al. (18) showed VEGF increased a macrophage efferocytosis. Since macrophage efferocytosis was associated with the production of VEGF (11) and HGF (26), the efferocytosis must be important to form autocrine-like loop for growth factors and to promote the tissue repair. This might be true for our observation, and decreased KGF expression in the PPE(+)/Ann(+) group, while the difference was not statistically significant, plays some role to the progression of pulmonary emphysema. We observed a decreased VEGF protein in BAL fluid, but VEGF mRNA expression from a macrophage did not reach statistical difference, suggestively VEGF secretion from epithelial cells was important in total VEGF activity in PPE-treated mouse. Epithelial cells also have efferocytosis ability, and a dead cell is believed to be engulfed by neighboring epithelial cells, too. Therefore it could be possible that annexin V inhibited efferocytosis by epithelial cells, too, and the epithelial cells failed to gain anti-inflammatory phenotype and produced less VEGF.

A question remained to be elucidated in the present study. We attempted to inhibit PS-mediated pathway with annexin V, but the proof of inhibited efferocytosis was not shown. When a macrophage engulfed an apoptotic cell and a phagosome is formed, which in turn fuses with lysosome and forms phagolysosome to degrade the contents (7). Efferocytosis is a serial and very dynamic event that completes very quickly, and a picture taking a moment to prove the efferocytosis in situ is extremely hard to find. Similar studies inhibiting PS-mediating efferocytosis did not show a proof of inhibition, too (24, 38). Yet we could not deny the possibility that annexin V exerted unexpected effects on cellular function other than via shielding PS on apoptotic cells and inhibiting efferocytosis. We feel that this is less likely, given the lack of changes in thePBS control mice in the presence or absence of Annexin. Should annexin have a primary signaling pathway on the macrophage, we would expect phenotypic changes in all macrophages exposed to annexin V. More research is needed, however, to determine the other role of annexin V in emphysema progression.

In summary, in the present study we showed that disruption of PS-mediated efferocytosis caused progression of pulmonary emphysema in a PPE-treated mouse model. Inhibited PS-mediated efferocytosis disturbed phenotypic changes in macrophages toward anti-inflammatory one and elicited higher transcriptional activities of macrophage-derived MMPs.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.Y., N.M., S.C., M. Miyazaki, K.T., T.M., T.I., and T.B. conception and planning relating to this study.

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L860 ANNEXIN V DETERIORATES PULMONARY EMPHYSEMA IN MICE

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