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Biochemical and Biophysical Research Communications 318 (2004) 32–37
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Macrophage phagocytosis of apoptotic neutrophils is compromised by matrix proteins modified by cigarette smoke and lipid peroxidation products

Paul A. Kirkham,[a,*] Gillian Spooner,[a] Irfan Rahman,[b] and Adriano G. Rossi[b]

[a] *Novartis Institute for Biomedical Research (Horsham), West Sussex, UK*
[b] *Respiratory Medicine Unit, MRC Centre for Inflammation Research, University of Edinburgh Medical School, Edinburgh, UK*

Received 30 March 2004

Abstract

Clearance of apoptotic cells by phagocytosis plays an important role in the resolution of an inflammatory response. Macrophages interacting with extracellular matrix (ECM) proteins upregulate their phagocytic capacity. Cigarette smoke contains highly reactive carbonyls that modify proteins which directly/indirectly affects cellular function. We observed, in vitro, that human macrophages interacting with carbonyl or cigarette smoke modified ECM proteins dramatically down regulated their ability to phagocytose apoptotic neutrophils. We also show that this interaction with carbonyl-adduct modified ECM proteins led to increased macrophage adhesion in vitro. We hypothesise that changes in the ECM environment as a result of cigarette smoking affect the ability of macrophages to remove apoptotic cells. Moreover, we postulate that this decreased phagocytic activity was as a result of sequestration of receptors involved in the uptake of apoptotic cells towards that of recognition of carbonyl adducts on the modified ECM proteins leading to increased macrophage adhesion.

Keywords: Macrophages; Neutrophils; Phagocytosis; Inflammation

Phagocytosis of apoptotic cells plays a major role in the resolution of an inflammatory response [1]. An impairment of this process can therefore lead to chronic inflammatory conditions. The presence of such a chronic inflammatory state within the airways is one major characteristic of chronic obstructive pulmonary disease (COPD) [2]. There is a pronounced general increase in inflammatory cells, with profound increases in neutrophils [3], macrophages [4], and $CD8^+$ T cells [5] being observed. Although their lifespan and recruitment dynamics may differ, they nonetheless all play a role in the ongoing inflammatory response within COPD. Moreover, it is the severity of the inflammatory response that can lead to airway obstruction and emphysema in COPD [6]. The inflammatory response can also be viewed as part of an innate tissue repair process following an insult, such as invading pathogens or damaged cells as a result of cigarette smoking. The ultimate fate of many recruited inflammatory cells is death, be it apoptotic or necrotic. It is the balance between inflammatory cell recruitment and the removal of apoptotic cells by phagocytosis that ultimately determines whether the inflammatory response will resolve [1,7]. Failure to remove inflammatory cells, such as apoptotic neutrophils, can result in secondary necrosis, releasing their toxic granule contents causing further tissue damage thereby exacerbating the inflammatory response [1,7] and ultimately leading to a chronic inflammatory state [8]. In contrast, phagocytic removal of apoptotic cells by macrophages is an immunologically silent process that does not provoke release of pro-inflammatory mediators [9]. Consequently, this limits further tissue damage promoting a resolution of the inflammatory response.

Regulating the clearance of apoptotic cells can be viewed from two perspectives, first influencing the rate at which cells enter apoptosis and second controlling the

* Corresponding author. Fax: +44-1403-323837.
E-mail address: paul.kirkham@pharma.novartis.com (P.A. Kirkham).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.04.003

phagocytic removal of apoptotic cells [7,10,11]. The former is known to be affected by inflammatory mediators and growth factors [12,13]. In addition, phagocytosis can also be controlled by a number of factors such as pro-inflammatory cytokines [14], soluble factors such as cAMP [10], glucocorticoids [15,16], extracellular matrix proteins, for example fibronectin [11] and collagen [17], or cell surface molecules such as CD44 [18]. In a disease such as COPD, where cigarette smoking can cause rises in both neutrophil and macrophage numbers in lung tissues, any factor or mechanism that can upset the balance between the number of cells entering apoptosis and the ability of macrophages to remove them could profoundly affect the outcome of the inflammatory response. Indeed, it has been shown that in COPD alveolar macrophages have a decreased propensity for phagocytosis [19] that could potentially exacerbate the inflammatory response.

Cigarette smoke, a complex mixture of chemicals and oxidants, is a major aetiological factor in the development of COPD [2]. Examples of such chemicals include the highly reactive aldehydes acrolein and 4-hydroxynonenal (4-HNE) [20], which can also be formed in vivo as a result of lipid peroxidation. These highly reactive water-soluble carbonyls can in turn, by attacking residues such as lysine, arginine, cysteine or histidine result in covalent post-translational protein modifications. Within this context, both 4-HNE and acrolein derived protein modifications have been shown to be markers for oxidative stress derived tissue damage in chronic diseases [20,21]. Moreover, 4-HNE modifications have been shown to inversely correlate with a loss of lung function including a decline in the forced expiratory volume in one second (FEV_1) in COPD [22]. It has previously been shown that macrophages can adhere to these carbonyl modified proteins through the class A macrophage scavenger receptor (SR-A) [23], a receptor that also plays a functional role in the phagocytosis and uptake of apoptotic cells [24]. However, the impact of ECM modifications by specific carbonyls found in cigarette smoke, such as 4-HNE and acrolein, on macrophage phagocytosis has not been studied. We therefore determined what effect modification of ECM proteins by reactive carbonyls could have on the clearance of apoptotic neutrophils by macrophages in vitro. Moreover, we also show that the macrophage interaction with modified ECM proteins is not solely through SR-A.

Materials and methods

Preparation and culture of human monocyte derived macrophages from healthy non-smokers. Human peripheral blood cells were separated into mononuclear cells and granulocytes from fresh, citrated blood of healthy volunteers by dextran sedimentation and discontinuous PBS-Percoll density gradient centrifugation as described [13]. Human monocytes were isolated from the mononuclear cells by plating out the mononuclear cell fraction (PBS-Percoll interphase fraction) into 48-well tissue culture plates as described previously [23]. After removing non-adherent cells (essentially the lymphocytes), the remaining adherent monocytes were cultured for six days in Iscove's MDM containing 10% autologous serum with fresh medium being added on the third day. The resulting monocyte derived macrophages were harvested by leaving in calcium/magnesium free PBS containing 2 mM EDTA for 45 min on ice followed by vigorous aspiration. The macrophages were then resuspended in fresh Iscove's MDM in a polypropylene falcon tube at a cell density of 1×10^6/ml.

Isolation and preparation of fluorescently labelled apoptotic neutrophils. Human peripheral blood neutrophils isolated as described above were then resuspended at 20×10^6/ml in Iscove's MDM and fluorescently labelled as described [25,26] with the following modifications. The neutrophils were incubated for 30 min at 37 °C in a 40 ml falcon tube with 10 μl CM-Orange (Catalog No. C2927; Molecular Probes, Leiden, The Netherlands).

The cell suspension was then diluted with Iscove's MDM to give a cell density of 4×10^6 neutrophils/ml and the cells aged (20 h, 37 °C) in tissue culture flasks in the presence of autologous donor serum (10%). Prior to use in the phagocytosis assay the neutrophils were washed (2×) in Iscove's MDM and the percentage of neutrophils that had undergone apoptosis was determined by typical morphology [13,27]. Only aged neutrophils with a viability (assessed by trypan blue dye exclusion) >98% were used.

Adhesion assay. Tissue culture plates (96 well) were coated with collagen IV by drying 100 μl of 100 μg/ml protein solution onto the surface or each well. The wells were washed with distilled water and then left untreated or modified with 100 mM acrolein or 5 mM 4-HNE in PBS for 24 h at 37 °C, after which the plates were extensively washed with PBS. PMA treated U937 cells [23] were preloaded with BCECF-AM at 10 μg/10^7 cells/ml for 30 min in PBS supplemented with 1% BSA, and then washed with Ca^{2+}/Mg^{2+} free PBS supplemented with 0.1% BSA and 5 mM EDTA. The cells were then left untreated or pretreated for 30 min with 100 μg/ml fucoidan before addition to each well at 2×10^5 cells/well. The cells were left to adhere for 60 min at 37 °C and then non-adherent cells were washed off by gentle washing with PBS supplemented with 0.1% BSA, 5 mM EDTA and the level of adherent cells remaining quantified by measuring fluorescence (Ex 485 nm; Em 538 nm).

Phagocytosis assay. Tissue culture plates (24 well) were precoated with 10 μg/ml fibronectin in PBS and left to incubate for 16 h at 37 °C. Collagen IV precoated 24-well plates were obtained from Becton–Dickinson. Plate wells were then left untreated or treated with either cigarette smoke condensate [28], 100 mM acrolein, 5 mM 4-HNE or 10 mM HOCl as described above. Human monocyte derived macrophages were then plated out at 1×10^6/well and left to incubate for 45 min at 37 °C. As positive and negative controls for regulation of phagocytic uptake anti-CD44 (approx. 10 μg/ml of Ab from 5A4 hybridoma) and 2 mM dibutyryl-cAMP, respectively, were added to macrophages plated on either unmodified collagen IV or fibronectin and left for a further 15 min at 37 °C. Apoptotic CM-Orange loaded neutrophils (4×10^6) were then added to each well and left to incubate with the macrophages for 1 h at 37 °C. The macrophages were then harvested with trypsin/EDTA and kept on ice in the dark prior to analysis by FACS.

Flow cytometric analysis. Samples were analysed by flow cytometry in a FACSCalibur with CellQUEST software (Becton–Dickinson, UK). Neutrophil and macrophage populations were identified and separated by forward and side scatter profiles. Data acquisition was terminated when 3000 events in the macrophage gate had been collected. During analysis the macrophage population was then plotted against forward scatter and FL-2 and separated into red fluorescent positive and negative populations. The number of red fluorescent positive events was then divided by the total sum of events in the macrophage gate to determine the percentage of macrophages that had ingested apoptotic neutrophils.

Results and discussion

Many factors have been shown to upregulate the nonphlogistic clearance of apoptotic leukocytes by phagocytosis, such as lipoxins, cytokines, and corticosteroids [14,15,29]. Interaction of macrophages with ECM components such as fibronectin through the β1 integrin receptors [11] and collagen through Fc receptors can also upregulate phagocytosis. In this study we have investigated the impact of modification of ECM proteins by cigarette smoke and its constituents, namely reactive aldehydes and oxidants, on macrophage function through their ability to phagocytose apoptotic neutrophils.

Table 1
Percentage of macrophages phagocytosing apoptotic neutrophils on unmodified ECM proteins

	Mean (%)	SEM
Collagen IV	34.5	9.6
Fibronectin	32.4	8.4

Macrophages plated onto surfaces coated with either collagen IV or fibronectin were allowed to engulf dye labelled apoptotic neutrophils for a period of 1 h. Three thousand macrophages were then analysed by flow cytometry and the number of macrophages engulfing dye labelled apoptotic neutrophils in this population is represented as a percentage of the total population counted. Data are means ± SEM from three experiments, each experiment using human monocyte derived macrophages from a different donor.

Cigarette smoke and carbonyl modified ECM proteins decrease nonphlogistic phagocytosis of apoptotic PMN

Two ECM proteins, collagen IV and fibronectin, were studied for their effect on macrophage phagocytic activity when modified with a variety of agents. In Table 1, the mean basal levels of phagocytosis from three separate experiments are shown when macrophages are plated onto either collagen IV or fibonectin. Each experiment used monocyte derived macrophages from a different donor. This table also shows that both ECM proteins, collagen IV or fibronectin, triggered similar levels of phagocytic activity towards apoptotic neutrophils. Fig. 1A illustrates a representative FL-2 histogram profile for these gated macrophages engaged in phagocytosis when plated out onto unmodified collagen IV compared to that for acrolein modified collagen IV (Fig. 1B). A reduction in FL2 fluorescence for the macrophage population is observed as less dye labelled apoptotic neutrophils are engulfed. In contrast, Fig. 1C demonstrates an increase in FL-2 fluorescence as anti-CD44 stimulation of macrophages upregulates phagocytic uptake of dye labelled apoptotic neutrophils.

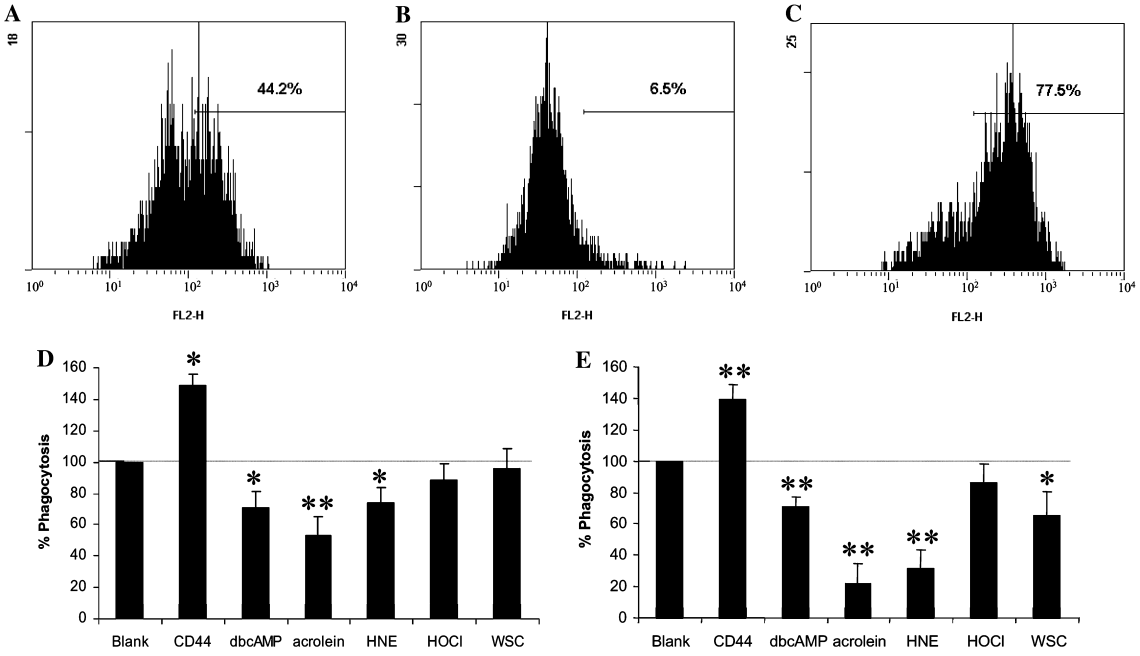

Fig. 1. Extracellular matrix proteins modified by 4-HNE, acrolein or cigarette smoke down regulate macrophage phagocytosis of apoptotic neutrophils. (A–C) Representative laser flow cytometry profiles for FL2 versus cell number demonstrating phagocytic potential of gated macrophages when plated onto unmodified collagen IV (A), acrolein modified collagen IV (B) or pre-treatment with anti-CD44 prior to plating onto unmodified collagen IV (C). Tissue culture plates precoated with either (D) Fibronectin or (E) collagen IV were left untreated or treated with either acrolein, 4-HNE, HOCl or cigarette smoke condensate (WSC). Gated macrophages were analysed by flow cytometry for FL-2 versus cell number as described in Materials and methods. The number of FL-2 positive events under each condition is then normalised and expressed as % phagocytosis, relative to macrophage phagocytosis on unmodified matrix protein. Data are means ± SEM from three experiments, $*p < 0.05$, $**p < 0.01$ as determined by a two-tailed Student's t test.

Therefore, normalising basal phagocytosis levels to 100% for macrophages plated onto unmodified fibronectin (Fig. 1D) and collagen IV (Fig. 1E), the effect of various modifying agents on the ECM proteins, and the resultant impact on macrophage phagocytosis are shown. When cigarette smoke condensate was used to modify ECM proteins, only modified collagen IV (Fig. 1E) showed any significant reduction in phagocytic activity ($p < 0.05$) by as much as 40%. Highly reactive aldehydes, such as acrolein and 4-HNE, are constituents of cigarette smoke. Therefore, ECM proteins were also treated in vitro with these aldehydes in order to determine whether they could account for the effect of cigarette smoke exposed ECM proteins on reducing macrophage phagocytic activity. When collagen IV (Fig. 1E) was modified with either 4-HNE or acrolein, there was an 80% reduction ($p < 0.01$) in phagocytic activity. Fibronectin (Fig. 1D) on the other hand only gave a 50% ($p < 0.01$) and 30% ($p < 0.05$) reduction in phagocytic activity when modified with acrolein and 4-HNE, respectively. For positive and negative controls in this experiment, CD44 and dbcAMP, as has previously been shown [10,18], were able to increase and decrease macrophage phagocytic activity respectively. These results would therefore imply that the reactive aldehyde content of cigarette smoke may, in part, be responsible for the observed decrease in phagocytic activity when the macrophages were plated onto collagen IV that had been previously exposed to cigarette smoke condensate. Interestingly, unlike collagen IV, fibronectin when exposed to cigarette smoke did not affect phagocytosis. This could be explained by collagen IV having more susceptible amino acid residues exposed to attack by reactive aldehydes, leading to greater levels of post-translational modification. Indeed, when ECM proteins are exposed to modification by reactive carbonyls alone, be it acrolein or 4-HNE, there was a greater reduction in phagocytic activity of macrophages in contact with modified collagen IV as compared to modified fibronectin.

ECM proteins exposed to oxidative stress do not impact on nonphlogistic phagocytosis of apoptotic PMN

Cigarette smoke is also a direct source of oxidants with high levels being present (10^{17} oxidants/puff). Therefore in order to determine the impact of oxidative stress itself on ECM protein environment and how this could impact on macrophage function, ECM proteins were pre-treated with the powerful oxidant HOCl. This oxidant can also be produced in vivo under conditions of oxidative stress [30]. Our results showed that ECM protein pre-treatment with HOCl had no significant effect on phagocytic activity towards apoptotic neutrophils (Figs. 1D and E). Whilst HOCl has been shown to destroy or modify the arginine residues in the integrin related RGD binding motif in ECM proteins, thereby affecting cell adhesion and survival [31], removal of the RGD motif within fibronectin failed to modulate phagocytic activity [11]. Consequently, our data would agree with these previous findings.

Receptors for phagocytosis and macrophage adhesion

Numerous receptors, including type A macrophage scavenger receptor (MSR-A) and CD36, have been shown to be involved in the uptake and recognition of apoptotic cells [24,32,33]. Indeed, there have been at least 10 molecules identified so far that have been implicated in macrophage uptake of apoptotic cells [34]. Some of these receptors, such as MSR-A and CD36, have also been shown to be involved in macrophage adhesion to ECM proteins post-translationally modified by lipid peroxidation products or cigarette smoke [23,35,36]. In Fig. 2 we show that both 4-HNE and acrolein adducts on collagen IV can facilitate macrophage adhesion in a non-integrin related manner, as these experiments were performed in the presence of Ca^{2+}/Mg^{2+} free media containing 5 mM EDTA, thereby preventing any classical integrin related adhesion events. Interestingly, the adhesion to 4-HNE adducts is greater than that seen with acrolein by a factor of 2. Moreover, this increased adhesion occurred when using a 20-fold lower concentration of 4-HNE compared to acrolein for modifying the matrix protein. In a similar context, macrophage adhesion to cigarette smoke condensate modified matrix protein has also been demonstrated by others, but occurred to a much lesser extent than that observed for acrolein alone [23], and may account for the smaller impact of cigarette smoke modified matrix

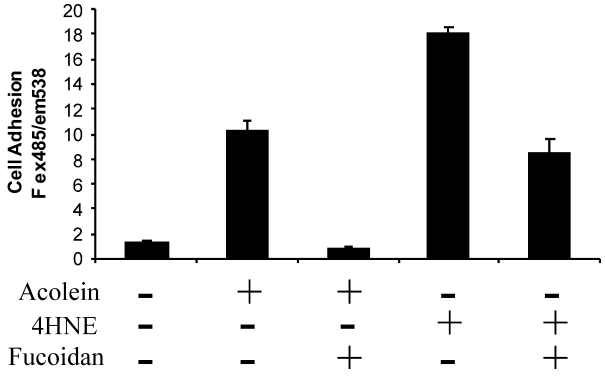

Fig. 2. 4-HNE and acrolein modified collagen IV promotes macrophage adhesion. Tissue culture plates precoated with human collagen IV were modified with either acrolein or 4-HNE. PMA differentiated U937 cells loaded with BCECF were plated out onto the modified collagen coated plates in the presence or absence of 100 μg/ml fucoidan. Adherent cells were detected by measuring fluorescence. Data are presented as means ± SEM for quadruplicate determinations. Further details are as described in Materials and methods.

proteins in down regulating phagocytosis as observed here (Fig. 1). Prevention of MSR-A mediated binding by use of fucoidan, whilst completely blocking acrolein adduct based adhesion, only blocked 4-HNE adduct related adhesion by 50%. Therefore, unlike acrolein modified protein, adhesion to 4-HNE modified protein is not completely mediated through MSR-A, as the MSR-A inhibitor fucoidan failed to block all macrophage adhesion. This would suggest that another as yet unidentified receptor is involved in recognition of 4-HNE modified ECM protein.

The number of macrophages adhering to acrolein or 4-HNE modified collagen IV has been shown to be dependent on the extent of modification present [23]. Moreover, adhesion to 4-HNE adducts could still be achieved after modifying protein with a single exposure to low micromolar concentrations of 4-HNE compared to that for acrolein [23]. However, frequency of exposure to carbonyls, as a result of cigarette smoking for example, will also have a big impact on the level of protein modifications attained [23]. Consequently, because adhesion to carbonyl modified collagen IV was not only greater but occurred using lower concentrations of 4-HNE than that for acrolein (Fig. 2), it is possible that 4-HNE modifications may be more pathophysiologically important than acrolein. Indeed, Rahman et al. [22] have recently shown that there are increasing levels of 4-HNE adducts within lung tissue with increasing severity of COPD status as defined by a worsening FEV_1.

Besides MSR-A, another receptor involved in recognition of carbonyl-modified proteins is CD36 and may well account for the fucoidan insensitive binding to 4-HNE modified ECM protein. Nevertheless, we propose that it is the sequestration of receptors towards adhesion of the macrophages to the post-translationally modified ECM proteins (seen in Fig. 2) that prevents recognition of and consequently reduces phagocytosis of apoptotic neutrophils as observed here (Fig. 1). This hypothesis is similar to that described by Maxeiner et al. [35] and El Khoury et al. [36] where MSR-A and/or CD36 receptors were shown to mediate macrophage adherence to surfaces coated with either carbonyl modified collagen IV or oxLDL, the latter being a rich source of carbonyl modified proteins. Moreover, this adhesion prevented scavenger receptor phagocytosis of modified LDL particles but not Fc mediated phagocytosis of IgG coated red blood cells. Although MSR-A and CD36 are involved in the clearance of apoptotic cells, other receptors are just as important. This was demonstrated by Teder et al. [8] using mice deficient for the hyaluron receptor CD44. These mice exhibited an impaired clearance of apoptotic neutrophils and a persistent inflammatory state following non-infectious lung injury. Consequently, the clearance of apoptotic cells most likely involves the coordinated response of numerous receptors and a dysfunction in any one can have a detrimental effect.

In conclusion, we have shown that surfaces coated with ECM proteins modified by carbonyls in cigarette smoke or products of lipid peroxidation, acrolein and 4-HNE, can trigger macrophage adhesion. We postulate this may account, in part, to the increased macrophage retention and adhesion observed by others in the lungs of both COPD and cigarette smoke exposed rats. This in turn results in a down regulation in the ability of these adherent macrophages to phagocytose apoptotic neutrophils. Our in vitro findings may therefore provide another mechanism by which post-translational alterations in the extracellular environment as seen in COPD [22] could affect cellular function. Such a sequence of events in vivo could lead to an imbalance in the effective clearance of apoptotic neutrophils resulting in secondary necrosis, exacerbating the inflammatory response [1,7] and establishment of a chronic inflammatory state.

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