FEBS Letters 574 (2004) 80–84 FEBS 28747

Gene delivery of the elastase inhibitor elafin protects macrophages from neutrophil elastase-mediated impairment of apoptotic cell recognition

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Received 14 July 2004; accepted 2 August 2004

Available online 14 August 2004

Edited by Barry Halliwell

Abstract The resolution of inflammation is dependent on recognition and phagocytic removal of apoptotic cells by macrophages. Receptors for apoptotic cells are sensitive to degradation by human neutrophil elastase (HNE). We show in the present study that HNE cleaves macrophage cell surface CD14 and in so doing, reduces phagocytic recognition of apoptotic lymphocytic cells (Mutu 1). Using an improved method of adenovirus-mediated transfection of macrophages with the HNE inhibitor elafin, we demonstrate that elafin overexpression prevents CD14 cleavage and restores apoptotic cell recognition by macrophages. This approach of genetic modification of macrophages could be used to restore apoptotic cell recognition in inflammatory conditions.

Keywords: Neutrophil elastase; Elafin; Macrophage; Apoptosis; Adenovirus; Gene therapy

1. Introduction

The removal of cells undergoing apoptosis or programmed cell death is an essential step in the resolution phase of the inflammatory response. Macrophages are particularly important in the processes of recognition and engulfment of apoptotic cells [1,2] and inflammatory mediators may impair these processes prolonging inflammation and tissue injury. Human neutrophil elastase (HNE) is a potent protease released by neutrophils during acute inflammation and mediates both direct tissue damage and the upregulation of pro-inflammatory cytokines such as interleukin (IL-8) [3–5]. HNE has also recently been demonstrated to impair apoptotic cell recognition by cleaving the phosphatidylserine receptor (PSR) on the surface of macrophages [6]. A variety of macrophage receptors have been implicated in apoptotic cell recognition, such as the PSR and CD14 [6,7] and from these the former has been shown to be inactivated by HNE in patients with pulmonary inflammation [6]. Similarly, HNE has been shown to degrade CD14 on the surface of human peripheral blood monocytes and fibroblasts [8,9].

We have chosen to focus the present study on CD14 and reasoned that a strategy aiming at protecting CD14 from cleavage by HNE would be beneficial, favouring apoptotic cell removal in an inflammatory environment. For that purpose, we used an adenovirus (Ad) gene-transfer methodology involving complexing a recombinant Ad with the cationic liposome lipofectamine. Using this technique, we show that the HNE-mediated cleavage of CD14 and ensuing impairment of apoptotic cell recognition are inhibited by Ad-mediated overexpression of human elafin, a potent HNE inhibitor. Inhibitors of HNE are thought to comprise part of the human innate immune system. Elafin [10,11] is a potent inhibitor of HNE and proteinase 3 produced in the skin and airways, which is upregulated in response to early inflammatory cytokines such as tumour necrosis factor (TNF) and IL-1 [4]. Our group has recently demonstrated elafin as a multi-faceted molecule exhibiting direct antimicrobial activity [12] and an inhibitory effect on NF-κB mediated inflammatory cytokine production [13].

We demonstrate here for the first time that human macrophages can be genetically reprogrammed to overexpress elafin, from an HNE-mediated inflammatory phenotype, associated with apoptotic cell recognition deficiency, to a phagocytic competent phenotype. These findings have implications for the use of adenovirus in ex vivo applications of macrophages in inflammatory disorders.

2. Materials and methods

2.1. Reagents

HNE was obtained from Elastin Products (Owensville, MO). Recombinant elafin was a gift from Dr. J. Fitton, Astra-Zeneca (Macclesfield, UK). The monoclonal blocking antibody against CD14 (mAb 61D3) hybridoma (IgGl mouse anti-human) was originally obtained as a gift from J.D. Capra [14]. Penicillin G, streptomycin sulfate, and Iscove's modified DMEM were obtained from Life Technologies (Paisley, UK). X-Vivo 10 serum free culture medium was obtained from Cambrex Bio Science (Wokingham, UK). Falcon tissue culture material was from A.&J. Beveridge (Edinburgh, UK). 6-well, 48-well and 96-well tissue culture plates were obtained from Corning Costar

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Abbreviations: HNE, human neutrophil elastase; 61D3, monoclonal blocking antibody against CD14; IL, interleukin; Ad, adenovirus; Add1703, control (empty) adenoviral vector; PBS, phosphate-buffered saline; PSR, phosphatidylserine receptor; TNFα, tumour necrosis factor-α; GFP, green fluorescent protein

0014-5793/$22.00
doi:10.1016/j.febslet.2004.08.008

(High Wycombe, UK). All other chemicals were purchased from Sigma Chemicals (Poole, UK).

2.2. Adenovirus (Ad) constructs

The generation of El, E3 deleted Ad constructs Ad-elafin and Ad-dl703 ("empty" Ad control) has been described previously [15,16].

2.3. Macrophage isolation and FACS analysis

Mononuclear cells were isolated from peripheral blood as follows: freshly citrated blood was centrifuged at $400 \times g$ for 20 min and the platelet-rich plasma supernatant was used to prepare autologous serum by addition of $CaCl_2$ (10 mM final concentration). Leukocytes were isolated after removal of erythrocytes by sedimentation using 6% (w/v) dextran T500 in saline by fractionation on a discontinuous gradient of isotonic Percoll solutions made in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) (CMF-PBS). Percoll concentrations of 55%, 68% and 79% were used and the leukocytes were centrifuged at $700 \times g$ for 20 min. Mononuclear cells were aspirated from the 55%/68% interface and washed three times in CMF-PBS prior to culture. Monocytes were enriched from the mononuclear fraction by selectively attaching them to 6-well plates for 40 min at 37 °C. Non-adherent lymphocytes were removed and adherent monocytes were washed twice in PBS. Monocytes were then cultured in Iscove's modified DMEM containing 10% autologous serum, penicillin G (final concentration 100 U/ml) and streptomycin sulfate (final concentration 100 μg/ml) at 37 °C in a 5% CO_2 atmosphere. Maturation into macrophages with this culture protocol has previously been demonstrated using myeloid-specific markers, including CD16 and CD51/CD61 [17]. On culture day 4, macrophages were removed with cold CMF-PBS and plated at 70 000 cells/well on 48-well plates in X-Vivo 10 serum free culture medium. On culture day 6, Ad vectors were preincubated for 15 min at room temperature in X-vivo 10 medium with lipofectamine (Invitrogen, Paisley, UK) at a ratio of 5×10^4 lipofectamine molecules to each Ad particle (Ad particle concentration was determined from absorbance at 260 nm for each Ad construct according to established protocols) and added to day 6 macrophages at a multiplicity of infection (MOI) of 100. Macrophages were incubated in the virus-containing medium for 2 h before replenishment with X-vivo 10 growth medium.

Where indicated, HNE (1 μM) was added directly the following day and incubated for 1 h prior to removal. Following HNE treatments, cells were washed with ice cold CMF-PBS and suspended in 2% goat serum (Serotec Ltd, Oxford, UK). Cells were incubated with 61D3, a CD14 blocking monoclonal antibody [7], for 30 min, washed twice and then incubated with phycoerythrin-conjugated goat anti-mouse IgG secondary antibody for 15 min. Washed macrophages were then analysed on a FACScan cytometer (Becton Dickinson and Co.).

2.4. HNE inhibition assay

The HNE activity of macrophage supernatants, following incubation with HNE, was examined to determine any inhibitory effect conferred by infection with Ad-elafin or incubation with r-elafin. The HNE activity assay has been described in detail elsewhere [18]. Briefly, all dilutions were performed in assay buffer (50 mM Tris, 0.5 M NaCl and 0.1% Triton X-100, pH 8.0). Cell culture supernatant (90 μl) was incubated in a 96-well microtitre plate for 30 min at 37 °C before addition of the chromogeneic substrate *N*-methoxysuccinyl–Ala–Ala–Pro–Val–*p*-nitroanilide. The change in absorbance measured spectrophotometrically at 405 nm (MR5000 Plate Reader, Dynatech, Dynex, Billinghurst, UK) was expressed as a function of time. In a separate experiment to measure HNE activity within apoptotic Mutu cells, two independent batches of apoptotic Mutu cells (3 million cells) were pelleted and washed with PBS and lysed in 0.4 ml of HNE buffer assay. Cell lysate (50 μl) was added to a microtitre plate and HNE activity recorded according to the protocol described above.

2.5. Apoptotic cells recognition assays

Macrophages were prepared and Ad infections performed in X-vivo 10 serum free medium as above in 48-well plates. Where indicated, HNE (1 μM) and r-elafin (15 μg/ml = 2.5 μM) were added directly for 1 h prior to the interaction assay with apoptotic cells. The group I Burkitt lymphoma cell line Mutu I, induced into apoptosis by 16 h incubation with 1 μg/ml of the calcium ionophore ionomycin (Calbiochem, Nottingham, UK), was used as a source of apoptotic cells. Typically, around 70% of cells were apoptotic following this treatment as determined by FACS analysis of annexin V staining. For some experiments, the 61D3 CD14-blocking monoclonal antibody was mixed with the apoptotic Mutu cells. The culture medium was removed and 750 000 apoptotic Mutu cells were added to each 48-well for 1 h at 37 °C before being washed extensively with ice cold CMF-PBS to remove unbound apoptotic cells. Cells were then fixed in 1% paraformaldehyde and stained with Diff-quick for counting. Cultures were scored by light microscopy according to the proportion of macrophages that had internalised or bound apoptotic Mutu cells by established criteria [7,19]. At least 300 macrophages were assessed per sample.

2.6. Statistics

Results are presented as means ± S.D. and differences between treatments were tested using the Student's *t* test.

3. Results and discussion

Macrophages are important phagocytic cells involved in the recognition and removal of apoptotic cells, integral processes in the resolution of the inflammatory response [1,2]. Recent data have suggested that HNE, a pro-inflammatory enzyme released during uncontrolled inflammation [3], may be responsible for the impairment of apoptotic cell recognition by macrophages, by cleaving key receptors on these cells [6]. The

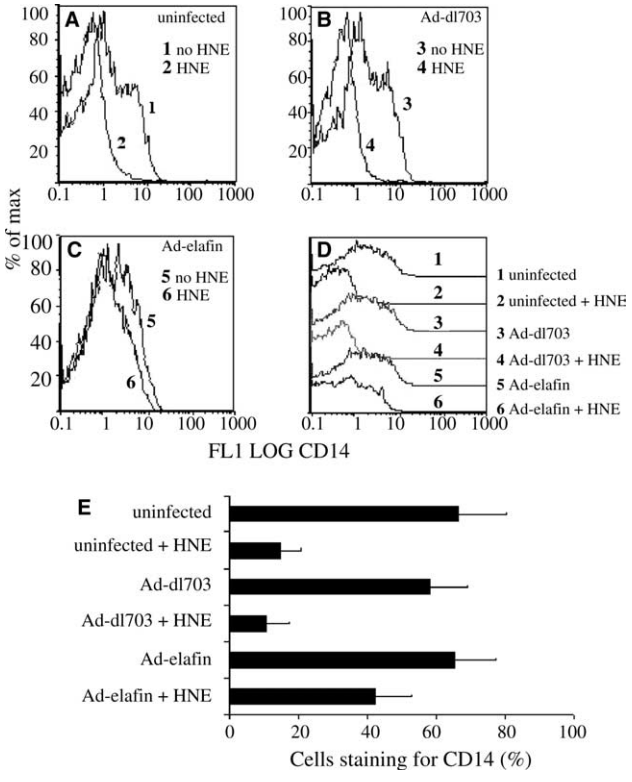

Fig. 1. The effect of HNE on surface expression of CD14 on human macrophages. Where indicated, macrophages were incubated with HNE (1 μM) for 1 h prior to removal. Binding of the 61D3 (anti-CD14) antibody was then analysed by flow cytometry. Adenovirus infections were performed at an MOI of 100 using the protocol with lipofectamine described in Section 2. (A) Uninfected cells. (B) Ad-dl703-infected cells (MOI 100). (C) Ad-elafin infected cells (MOI 100). (D) Composite figure illustrating 61D3 binding in the different conditions from a separate donor. (E) Mean percent of macrophages staining for CD14 ± S.D. Data are from experiments performed on two separate donors.

PSR was susceptible to HNE cleavage although the expression of other apoptotic cell recognition receptors, CD36 and CD32, was relatively preserved. We have demonstrated that CD14, a receptor with susceptibility to HNE, participates in the resolution phase of the inflammatory response as a cell surface receptor for apoptotic cells [7] in addition to acting as a receptor for bacterial lipopolysaccharide [20]. To test whether HNE could cleave macrophage CD14, we treated this cell type with purified HNE and analysed CD14 cell surface expression by FACS analysis. Compared to untreated cells, HNE-treatment drastically reduced the level of cell surface CD14 (Fig. 1A).

We decided to target HNE because of its deleterious action in inflammatory conditions and its central role in cleaving a variety of apoptotic cell receptors (CD14 and phosphatidylserine [6] being two out of a list likely to grow). We designed a gene therapy strategy to protect macrophages from the HNE-mediated cleavage of surface receptors. Traditional gene transfer vectors have proven disappointing for the transfection of macrophages [21] and we have previously developed a hybrid method, involving pre-complexing of adenovirus with cationic liposomes (lipofectamine) [13]. This method greatly enhances transfection in other cell types both in vitro and in vivo [22,23]. We have adapted this method for peripheral blood monocyte-derived macrophages and show here an impressive increase in transfection levels using green fluorescent protein (GFP) as a gene reporter (Fig. 2). Using this technique, 92% of macrophages exhibited GFP expression 24 h after infection with Ad-GFP (counting in a low power field). Similarly, when macrophages were infected with Ad-elafin/lipofectamine, elafin supernatant levels were increased from 10.4 (±4.11) ng/ml with Ad-elafin alone to 232 (±13.1) ng/ml (values are means and S.D. derived from a representative experiment performed in triplicate). Elafin was not detected in the supernatant from uninfected cells. Having established the disruptive activity of HNE on macrophage CD14 in our System (see above, Fig. 1A), we demonstrated that Ad-elafin inhibited HNE-mediated shedding of CD14 compared to macrophages infected with Ad-dl70/3, an Ad vector with no transgene cassette (Fig. 1B and C). When serum-free supernatants were tested for HNE activity, most HNE was inhibited when a saturating concentration of r-elafin was added extracellularly (Fig. 3). Although Ad-elafin was efficient in preserving CD14 expression, it conferred by contrast a relatively modest, albeit significant, inhibition of HNE when compared to Ad-dl70/3 (Fig. 3). This result was predicted from the elafin concentrations in Ad-elafin infected cell supernatants that were theoretically not high enough to neutralise the quantity of HNE added.

Finally, we investigated the effect of HNE on macrophage apoptotic cell recognition and whether Ad-elafin had a protective role. A variety of cells, such as lymphocytes and neutrophils, are routinely used in experimental systems as apoptotic target cells. We chose Group 1 Burkitt lymphoma cells (Mutu I) over neutrophils because of the absence of endogenous HNE in the former (HNE levels were below the levels of detection from apoptotic mutu cell lysates, see Section 2), which could have interfered with our assays. Previous work has demonstrated that binding of viable Mutu cells is negligible in this system and any interaction that does occur is with spontaneously apoptotic Mutu cells [19]. Macrophages were either untreated/uninfected (control) or infected with Ad-elafin (Ad-dl70/3 as a control) and incubated where indicated with HNE. In a variation of these experiments, the CD14 blocking antibody 61D3 [7] was used in order to provide a positive control of CD14 blockade. Fig. 4 shows that HNE treatment of untransfected cells inhibited Mutu cell recognition by 54%, compared to untransfected cells alone. This level of inhibition was comparable to that of 61D3 treatment. The prominent role of CD14 (the 61D3 antigen) for interaction and initial tethering of apoptotic Mutu cells with human macrophages has previously been characterised. Blocking antibodies and

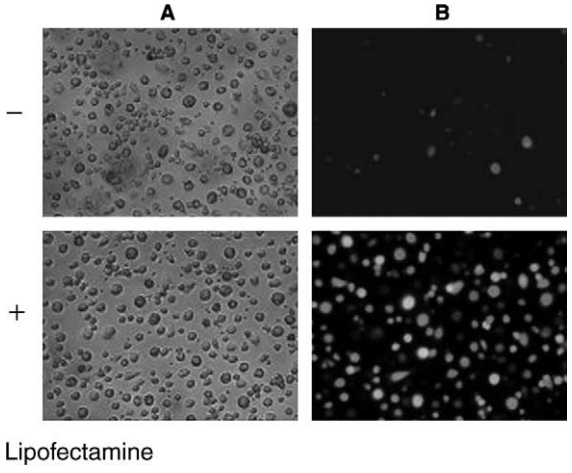

Fig. 2. Lipofectamine facilitates Ad-GFP infection of macrophages. Peripheral blood monocyte-derived macrophages were infected on culture day 6 with Ad-GFP (100 PFU/cell). Where indicated, virus was precomplexed with lipofectamine (+) according to protocols in Section 2. Photomicrographs were taken 24 h after Ad infection and GFP fluorescence was observed. (A) No filter and (B) green filter.

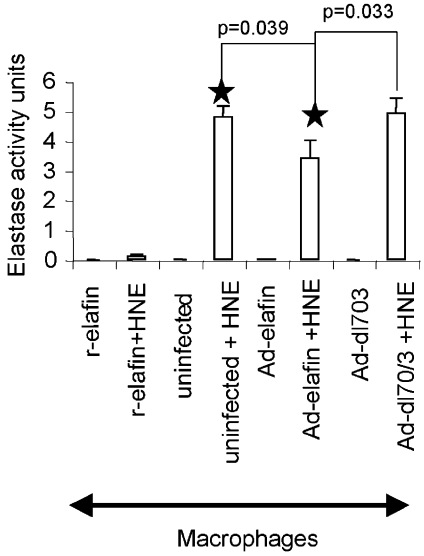

Fig. 3. Comparison of HNE inhibitory activity of macrophage culture supernatants. Macrophages were prepared and infected with Ad-vectors using the protocols described in Fig. 1. Following incubation with HNE, culture supernatants were removed for determination of residual HNE activity. Supernatants were transferred to a microtitre plate and cleavage of a chromogenic HNE substrate was measured spectrophotometrically over time. *P* values are given for the comparisons of Ad-dl703 (control adenovirus) and uninfected cells to Ad-elafin. Data are means ± S.D. from experiments performed in triplicate on three separate donors ($n = 3$).

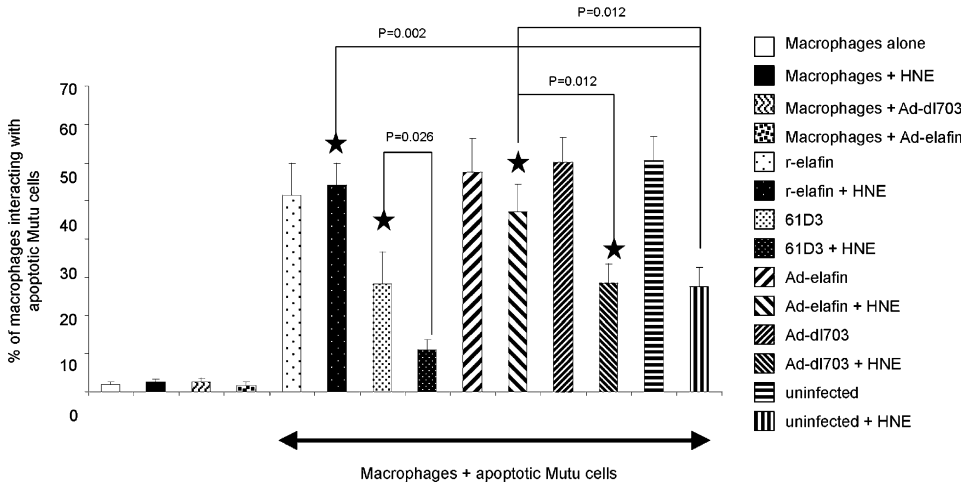

Fig. 4. Elafin protects macrophages from HNE-mediated impairment of Mutu cell recognition. Macrophages were cultured and infected with Ad vectors using the same protocol from Fig. 1. Where indicated, HNE (1 μM) and r-elafin (2.5 μM) were added in serum free X-vivo 10 medium for 1 h prior to adding the apoptotic Mutu target cells with or without 61D3 (CD14 blocking monoclonal antibody). After fixing and staining, the percentage of macrophages interacting with apoptotic Mutu cells was calculated. Interaction counts performed on macrophages that had not been exposed to Mutu cells are also shown to exclude any significant contribution from apoptotic cells originating from within the culture. *P* values are given for each comparison. Data are means ± S.D. from experiments performed in quadruplicate on three separate donors ($n = 3$).

peptides against the $\alpha_v\beta_3$, integrin (Vitronectin receptor) and CD36 produced little if any reduction of apoptotic Mutu cell interaction in this system [19]. Interestingly, when 61D3 and HNE were used in combination, a further decrease in recognition occurred, suggesting that the sites of action of 61D-3 and HNE are not identical. It is likely that in addition to cleaving CD14 (Fig. 1), HNE is interfering with additional (unidentified) surface receptors in our system and notably the degree of inhibition never approached 100% even with 61D3 and HNE together. HNE cleavage of the PSR was associated with reduced ingestion of apoptotic Jurkat cells by human macrophages [6] and the role of PSR cleavage in reducing interaction in our system is unknown.

When r-elafin was added extracellularly with HNE, macrophage recognition was restored to normal values. Similarly, when macrophages were transfected with Ad-elafin, prior to HNE challenge, a very significant increase in macrophage recognition of apoptotic cells was obtained, compared to untransfected cells and cells transfected with Ad-dl70/3, demonstrating the elafin transgene specific effect (Fig. 4). In addition, Ad-transfection on its own (Ad-dl70/3 and Ad-elafin) did not influence apoptotic cell recognition. HNE, Ad-elafin and Ad-dl703 had no effect alone on the background rate of apoptotic cell recognition in the absence of apoptotic Mutu cells (Fig. 4; 2nd, 3rd and 4th bars from the left).

These results illustrate the potential of elafin gene therapy to rescue the capacity of macrophages to recognise apoptotic cells in the presence of HNE. Although expression of CD14 was relatively preserved in Ad-elafin infected macrophages (Fig. 1C), the effect on residual supernatant HNE activity was less marked (Fig. 3) indicating the possibility that elafin may facilitate apoptotic cell recognition through additional mechanisms. Indeed, we have shown that elafin has broad ranging anti-inflammatory properties, inhibiting the action of the transcription factor NF-κB and the release of pro-inflammatory cytokines such as TNF and IL-8 [13]. Since HNE has been shown to have pro-inflammatory signalling effects [4,5], it is likely that the Ad-elafin transfection of macrophages provides a further anti-inflammatory signal beneficial to apoptotic cell recognition. This signal would reinforce the endogenous anti-inflammatory phenotype conferred by binding of apoptotic cells to macrophages [24] that may otherwise be diminished by HNE. Interestingly, the endogenous synthesis of another neutrophil elastase inhibitor, secretory leukocyte protease inhibitor (SLPI), has recently been shown to be up-regulated following macrophage recognition of apoptotic cells [25]. However, as pointed out by Odaka et al. [25], the slow kinetics of production of SLPI after the apoptotic cell recognition event suggests that SLPI is unlikely to act in vivo at an autocrine level during the onset of the interaction but rather in a paracrine fashion, during phagocytosis of incoming cells.

In summary, we show here that using adenovirus as a gene transfer vector in a hybrid method with lipofectamine as a facilitating agent, it is possible to very efficiently transfect monocyte-derived macrophages ex vivo with elafin, a potent elastase inhibitor. This rescues these cells from an HNE-induced pro-inflammatory to an anti-inflammatory phenotype favouring apoptotic cell recognition and clearance. The potential for systemic delivery of macrophages transfected ex vivo with antinflammatory genes has been demonstrated in murine glomerulonephritis [26,27] and this methodology may prove useful in inflammatory conditions where direct use of adenovirus gene vectors is precluded, because of their intrinsic immunogenicity. Cystic fibrosis, characterised by an excessive load of unchecked HNE and a recognised "paralysis" of innate immune mechanisms [28], provides a fascinating model in which to test this paradigm.

Acknowledgements: The authors thank the Edinburgh University Wellcome Trust Cardiovascular Research Initiative for providing a Clinical Training Fellowship to P.A.H.

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