Human neutrophil elastase regulates the expression and secretion of elafin (elastase-specific inhibitor) in type II alveolar epithelial cells

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Received 19 July 1999

Abstract Elafin is a low molecular weight antiproteinase believed to be important in the regulation of elastase mediated tissue damage. The expression of elafin is known to be regulated by proinflammatory cytokines such as interleukin-1β and tumour necrosis factor but little was known regarding the effect of human neutrophil elastase (HNE). Employing a chloramphenicol acetyltransferase reporter construct of the human elafin gene, reverse transcription PCR from total cellular RNA and ELISA techniques, we have examined the effect of human neutrophil elastase on the transcription and secretion of human elafin in the pulmonary epithelial A549 cell line. Stimulation with HNE at concentrations of 10^{-10} and 10^{-11} M resulted in a significant upregulation of elafin promoter activity. Similarly, transcription of the endogenous human elafin gene was upregulated with HNE concentrations ranging from 10^{-10} to 10^{-12} M. In addition, we demonstrate that stimulation with HNE at concentrations ranging from 10^{-9} and 10^{-12} M resulted in a significant reduction in the secreted elafin protein as measured in the cell supernatant. These results provide further evidence for a role of elafin in the regulation of HNE driven proteolysis of the extracellular matrix.

Key words: Antiproteinase; Human neutrophil elastase; Elafin; Chloramphenicol acetyl transferase

1. Introduction

The excessive or unregulated release of proteolytic enzymes from inflammatory cells in the lung has been implicated in the pathogenesis of acute and chronic inflammatory lung diseases [1,2]. Amongst many potentially injurious agents, the serine protease, human neutrophil elastase (HNE), has emerged as a key effector of lung injury and its regulation is likely to be of critical importance in the maintenance of lung defence. The lung is known to contain at least three antiproteinases with activity against HNE: α-1 antiproteinase (A1Pi), secretory leukocyte protease inhibitor (SLPI) and elastase specific inhibitor (ESI), also called elafin or SKALP (skin derived antileukoprotease) [3,4].

Elafin was simultaneously described in psoriatic skin and bronchial secretions [5–8] but is present in many tissues [9]. In common with SLPI, elafin inhibits HNE, but in addition, is also a potent inhibitor of a second neutrophil derived protease: proteinase-3 (PR-3) [5,6]. The gene encoding elafin is found on chromosome 20 [10] and has been sequenced [11,12]. The 5′ flanking region has the characteristics of a gene whose regulation is governed at a tissue level. Consensus sequences may be found for 'TATA' and 'CAAT' boxes together with numerous putative binding sites for transcription factors known to regulate the transcriptional activity of tissue regulated genes [13]. Furthermore the differential expression of elafin in normal mammary cells and breast carcinoma cells appears to be regulated at a transcriptional level and mediated through the transcription factor activating protein-1 (AP-1) [14].

Although the expression of elafin has been shown to be regulated at the transcriptional level by interleukin β-1 (ILβ-1) and tumour necrosis factor (TNF) [15,16], the effect of HNE on the expression and secretion of elafin has not been described. In this study we used a reporter construct of the human elafin gene promoter, reverse transcription polymerase chain reaction (RT-PCR) and an enzyme linked immunosorbent assay (ELISA) to investigate the potential of HNE to regulate the secretion of elafin.

2. Materials and methods

2.1. Cell lines and media

The pulmonary epithelial A549 cell line derived from a human lung bronchioloalveolar carcinoma and representative of the pulmonary type II pneumocyte was employed in this study [17]. Cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% foetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere.

2.2. pCAT reporter system

Using placental genomic DNA as a template [11], PCR was employed to amplify a segment of the elafin human gene promoter from −505 bp to +28 bp [13]. Primers were obtained from MWG Biotech (Ebersberg, Germany) and primer sequences were as follows: +28 (5′-CAT ACC AAT CTT TAT GCA GTC CAG-3′) and −505 (5′-CGT CTC TGA AAC AGC AAA GTG CAA-3′). The PCR fragment was cloned, using standard techniques, into the PGEM-T cloning vector (Promega, Southampton, UK). A *Pst*I and *Sph*I restriction fragment was subcloned into the corresponding sites in the pBasic CAT vector (Promega, UK), 5′ of the CAT reporter gene. Sense and antisense strands of the DNA product were sequenced by Appligene Oncor (Illkirch, France) and were identical to those previously published except for the presence of an additional thymidine residue between positions 36 and 41. This construct was denoted 505-elafin.

2.3. Cell transfection

Pulmonary A549 cells were transfected using the cationic liposome TFX-20 (Promega, UK). Cells were grown to approximately 80% confluence in 60 mm cell culture dishes (Corning Costar, High Wycombe, UK). The medium was removed and cells washed with sterile phosphate buffered saline (PBS), pH 7.4 (Sigma, Poole, UK) and 10 μg of 505-elafin DNA was combined in a 1:2 charge ratio with TFX-20 in serum free MEM. 5 μg of the β-galactosidase expression plasmid pPSV-GAL (Promega, UK) was included as an internal transfection

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Abbreviations: HNE, human neutrophil elastase; A1Pi, α-1 antiproteinase; SLPI, secretory leukocyte protease inhibitor; CAT, chloramphenicol acetyltransferase; CF, cystic fibrosis

PII: S0014-5793(99)01004-2

control. pCAT basic (lacking a promoter) and pCAT control (in which the CAT gene is under the control of the SV40 promoter and enhancer sequences) were used as negative and positive controls respectively. Cells were transferred to a 37°C incubator with a 5% CO_2 atmosphere for 1 h following which any excess transfection medium was removed, the cells washed with sterile PBS and HNE applied as described below.

2.4. Cell stimulation and CAT ELISA

HNE was provided by Elastin Products (Owensville, MO, USA) and diluted in serum free MEM. Cells transfected with pCAT constructs (see above) were stimulated with HNE, applied in concentrations ranging from 10^{-9} to 10^{-12} M and serum free medium was harvested after 48 h. The protein content of the lysate was determined using a BCA reagent (Pierce, Rockford, IL, USA) and 100 μg protein added to each well of the CAT ELISA (Boehringer Mannheim, Lewes, East Sussex, UK). A further aliquot was analysed for expression of β-galactosidase (Promega, UK). Cell viability was confirmed by trypan blue exclusion on A549 cells treated as above, prior to the cell lysis step.

2.5. Reverse-transcription PCR (RT-PCR)

To assess expression of elafin mRNA in A549 cells following HNE stimulation, total cellular RNA was isolated using the SV Total RNA isolation system (Promega, UK). The purity and yield of the RNA were determined spectrophotometrically by measuring the absorbance of an aliquot at 260 and 280 nm. RNA integrity was checked by electrophoresis on a 1.2% agarose gel. The resulting RNA was stored at −80°C until RT-PCR was performed. 1 μg of total cellular RNA from A549 cells was reverse transcribed using the Access RT-PCR system (Promega, UK) prior to performing PCR to amplify the cDNA of elafin and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). As the number of PCR cycles required to detect elafin cDNA (30 cycles) was beyond the linear range for GAPDH (25 cycles), aliquots were amplified in a separate reaction tube. All reactions were carried out in duplicate. For elafin RNA amplification, a reaction tube consisting of AMV reverse transcriptase (0.1 U/μl), AMV/T*fl* 5× reaction buffer (1×), dNTP mix containing 0.2 mM of each nucleotide, 2.5 mM $MgSO_4$, T*fl* DNA polymerase (0.1 U/μl) and elafin primers 5′-GCA GCT TCT TGA TCG TGG TG-3′ (downstream) and 5′-GCC GTG GGC ATC CTG AAT GGG-3′ (upstream) to a final concentration of 1 μM each. Reaction tubes were made up to a final volume with 50 μl of nuclease free water. Elafin primers were chosen to span an intron to facilitate detection of DNA contamination. After completion of reverse transcription for 48°C for 45 min elafin cDNA was generated after 30 cycles of PCR (94°C for 1 min, 55°C for 1 min and 72°C for 1 min). The expected product was 480 bp in length. RT-PCR for GAPDH was performed in an identical manner using the downstream primer 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′ and the upstream primer 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′. The expected product was 600 bp in length. Amplification products were then analysed on 1.2% agarose gel followed by densitometry (GelPlate, UVP, Cambridge, UK). The intensity of the elafin cDNA band was normalised to the intensity of the constitutive GAPDH cDNA product.

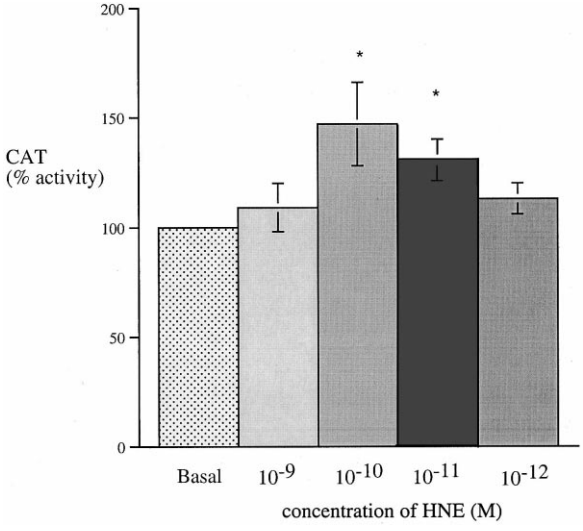

Fig. 1. Elafin promoter activity after HNE stimulation. A549 cells were co-transfected with the 505-elafin and β-galactosidase constructs. Following transfection excess medium was removed and cells were incubated in serum free medium containing HNE in the above concentrations for a 48 h period. Cells were then lysed and CAT activity determined relative to the expression of β-galactosidase. Asterisks indicate statistical significance ($n = 3$) from basal conditions (cells with no HNE stimulation).

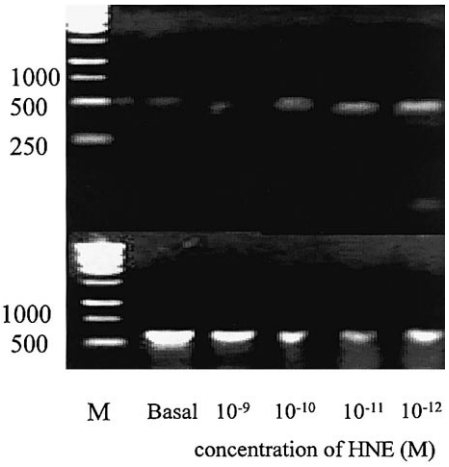

Fig. 2. Elafin mRNA stimulation with HNE in A549 cells. Top: elafin mRNA upregulation as assessed by RT-PCR. Basal: cells with no HNE stimulation. Bottom: GAPDH expression as assessed by RT-PCR; M: molecular weight markers (bp).

2.6. Elafin ELISA

Supernatants from unstimulated and HNE stimulated A549 cells were assayed for elafin protein content by ELISA. We used a modification of our original assay [15]. Briefly, 96 well plates (Linbro, Flow Labs, McLean, VA, USA) were coated with a 1 in 500 dilution of anti-elafin IgG in carbonate buffer, pH 9.6 overnight at 4°C. After blocking, samples and standards (diluted in PBS-gelatin) were applied for 2 h at room temperature followed by a further 2 h incubation with biotinylated anti-elafin IgG at 2 mg/ml. After addition of streptavidin-horseradish peroxidase (Sigma Chemical Co., St. Louis, MO, USA) for 2 h at room temperature, plates were washed and 100 μl of chromogenic substrate (2,2′-azino-bis-3-ethyl benz-thiazoline-6-sulphonic acid; Sigma Chemical Co., St. Louis, MO, USA) containing 0.001% H_2O_2 was applied to each well and absorbance was read at 490 nm with a microplate reader (Dynatech, Guernsey, UK).

2.7. Statistics

Results are expressed as mean ± S.E.M. and represent the mean of three experiments. Results were analysed using ANOVA following rank transformation of data. $P < 0.05$ was accepted as significant.

3. Results

3.1. Effect of HNE on expression of the elafin promoter (505-elafin construct)

Fig. 1 demonstrates the effect of HNE treatment on the CAT activity of the 505-elafin construct following transfection into A549 cells. Results are expressed as a percentage of the basal CAT activity derived from the same construct in the absence of HNE stimulation (arbitrarily assigned the value of 100%). HNE at a concentration of 10^{-9} M increased CAT activity to 109 ± 11%, 10^{-10} M to 147 ± 19%, 10^{-11} M to 131 ± 9% and 10^{-12} M to 113 ± 7% demonstrating a significant upregulation of the elafin promoter stimulation with

10^{-10} and 10^{-11} M HNE ($P < 0.05$). Cell viability as assessed by trypan blue demonstrated no significant difference between different treatments.

3.2. Effect of HNE on expression of elafin mRNA

The effect of HNE on steady-state elafin mRNA levels in A549 cells, relative to the expression of GAPDH mRNA, is shown in Fig. 2 (a representative experiment) and in Fig. 3 (quantification from three different experiments). As above, the amount of elafin mRNA levels in cells receiving medium without HNE was arbitrarily assigned a value of 100%. Stimulation with HNE at a concentration of 10^{-9} M increased expression to 184.3 ± 8.3%, 10^{-10} M to 663 ± 186%, 10^{-11} M to 470 ± 101% and 10^{-12} M to 301 ± 42%. The observed upregulation was significant for concentrations between 10^{-10} and 10^{-12} M ($P < 0.05$) but did not attain significance for 10^{-9} M. Cell viability assessed by trypan blue exclusion demonstrated no significant difference between different treatments.

3.3. Effect of HNE on secretion of the elafin protein

The effect of HNE on the secretion of elafin protein from A549 cells is shown in Fig. 4. As above, the level of elafin protein in the supernatant harvested from the untreated cells was assigned a value of 100%. Stimulation with HNE resulted in a decrease in elafin supernatant content as follows: 10^{-9} M decreased secretion to 70 ± 5%, 10^{-10} M to 75 ± 12%, 10^{-11} M to 72 ± 5% and 10^{-12} M to 71 ± 11%. Thus at all concentrations, stimulation of the A549 cells with HNE resulted in a statistically significant reduction in the level of secreted elafin in the cell supernatant ($P < 0.05$).

4. Discussion

The biological function of elafin (also called ESI or SKALP) remains unknown but in view of its specificity for HNE and PR-3, it has been ascribed a role in the proteolytic regulation of extracellular matrix components [4–8,10]. The molecular weight of elafin is sufficiently low to permit access to the protected neutrophil-substrate microenvironment and the presence of a repetitive sequence of valine, lysine, glycine and glutamine residues in the precursor protein (known to facilitate transglutaminase crosslinking between proteins and the extracellular matrix) is likely to be important in anchoring the protein within the interstitial environment of the lung [9–11].

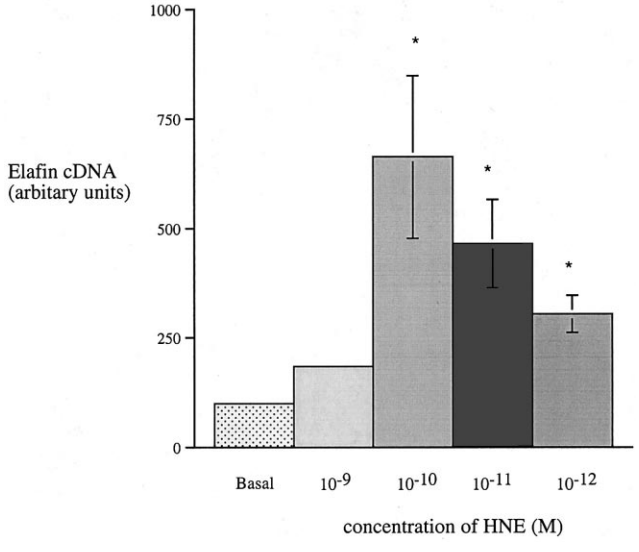

Fig. 3. Quantification of elafin mRNA stimulation by HNE. Elafin cDNA band intensities were determined by densitometry and were normalised with those of GAPDH (see Fig. 2 for a representative experiment). Asterisks indicate statistical significance from basal conditions ($n = 3$).

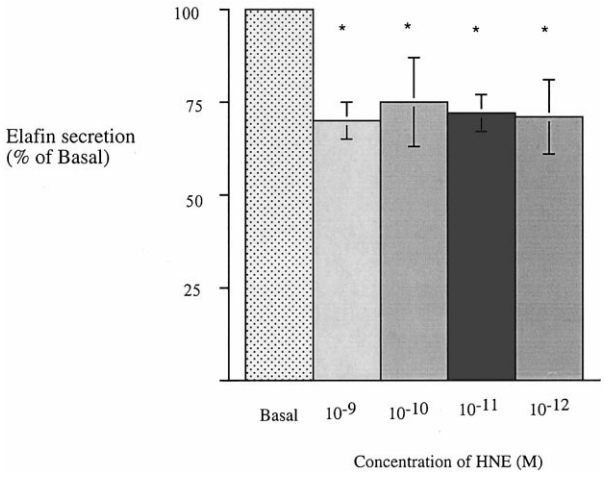

Fig. 4. Elafin secretion in A549 cells. A549 cells were stimulated with HNE in serum free medium at the above concentrations for 48 h. Cell supernatants were then harvested and concentration of elafin protein determined by ELISA. Asterisks indicate statistical significance ($n = 3$) from basal conditions (cells with no HNE stimulation).

The expression of elafin has been shown to be upregulated in lung epithelial cells in response to IL-1β and TNF suggesting that it is likely to be a key early component in the lung defence to inflammation and injury [15,16]. Further evidence for the role of elafin in the regulation of elastase mediated tissue damage is provided by this study in which we demonstrate that the regulation of elafin expression in the lung may be controlled by HNE, one of its main target enzymes. In a type II pulmonary epithelial cell line, stimulation with HNE at concentrations of 10^{-10} and 10^{-11} M results in significant upregulation of the human elafin promoter and transcription of the elafin gene product. The magnitude of upregulation observed using the promoter construct (maximum 147%, Fig. 1), although significant, is less than that observed by analysis by RT-PCR in which HNE at a concentration of 10^{-10} M resulted in a 6-fold upregulation of the elafin mRNA (Fig. 3). Whilst this may be due to different sensitivities between the two experimental techniques it is possible that this promoter construct does not include other important regulatory regions necessary for the response elicited by HNE. Our choice of promoter was based on previous work by Zhang and colleagues who analysed the effect of serial deletions on the expression of the elafin promoter in human mammary cells, finding that maximal expression required the inclusion of a region between −505 and −386 bp [13]. However, it is currently not known whether pulmonary cells require the presence of other regions to provide maximal expression as is the case for the other antiproteinases found in the lung. Indeed, the differential expression of SLPI transcripts in the hepatocellular carcinoma cell line (Hep G2), human uterocer-

vical carcinoma cell line (HeLa) and the pulmonary epithelial cell lines A549 and HS-24 may be dependent upon the density of hepatocyte nuclear factor 3/forkhead (HNF-3/fkh) binding domains [18]. Additionally, it may favour the existence of other enhancer regions not included in this construct. The regulation of A1Pi in response to IL-6 is known to be influenced by the inclusion of an Oct1 site in the 3′ enhancer region which co-operates with the NF-IL-6 site in the 5′ enhancer through which IL-6 classically mediates its effect [19]. Alternatively, HNE induced upregulation of steady-state elafin mRNA levels detected by RT-PCR could reflect increased mRNA stability, independent of promoter activity. Indeed, PMA induced SLPI mRNA transcripts have been shown to be more stable in the pulmonary epithelial HS-24 cell line than in the HeLa cell line [20].

In both the elafin promoter (Fig. 1) and the RT-PCR analysis (Figs. 2 and 3), we found that a higher concentration of HNE (10^{-9} M) only produced minimal upregulation of elafin. To rule out a possible toxic effect we assessed cell viability by trypan blue exclusion and found no significant difference between treatments. Significantly, similar results have been observed in relation to SLPI by other investigators: Saitoh and colleagues have reported that whilst HNE at concentrations between 10^{-10} and 10^{-12} M upregulated the expression of SLPI mRNA in human airway submucosal gland tissue, higher HNE concentrations (10^{-7}–10^{-8} M) did not [21]. The expression of SLPI mRNA has also been shown by us and others to be induced by HNE but has been demonstrated to be reduced in epithelium from inflamed pulmonary airway epithelium [15,22,23]. This observation is worthy of further studies as the airways of patients with inflammatory lung diseases such as cystic fibrosis (CF) and acute respiratory distress syndrome demonstrate high levels of unbound HNE [24,25] which may impair the local expression of small molecular weight inhibitors contributing to an uncontrolled inflammatory response. Inhibition of the expression of elafin by high elastase concentrations will also impair protection against PR-3 which has recently been shown to be present in high levels within the CF airway and can be correlated with markers of airway inflammation and levels of lung function [26]. Interestingly, we found recently in a CF tracheal cell line that HNE also downregulated the elafin promoter activity at higher concentrations whereas it upregulated its activity at low concentrations [27].

The apparent dissociation between the upregulation of the transcriptional product and the downregulation of the secreted protein is intriguing (Fig. 4). Similar observations have been made by both our group and others with respect to SLPI in which HNE stimulation resulted in a significant upregulation of SLPI mRNA but an apparent downregulation of protein secretion [15,28,29]. As our ELISA recognises HNE-elafin complexes within the ranges reported in these experiments, this downregulation cannot be accounted for by an artefact of our ELISA. One possible explanation could be that a proportion of the secreted protein remains bound to the cell surface. It has recently been shown that the HNE mediated downregulation of SLPI protein in primary bronchial epithelial cells may be prevented by incubating the cells with A1Pi [29]. HNE treatment may thus sequester inhibitors such as elafin and SLPI on the cell surface which may then be released when A1Pi becomes available and facilitates the transfer of HNE to this larger inhibitor [30]. As a further possibility we may also consider that elafin may be synthesised for intracellular use. The expression of elafin is known to be subject to cell cycle regulation, being downregulated during the S phase, suggesting that it may possess an intracellular protease target involved in cell cycle regulation [13].

In summary, our data provide the first demonstration, as far as we are aware, using three independent techniques, that the expression and secretion of elafin in the human lung is regulated by HNE. The genesis of serial reporter deletion constructs should enable the dissection of the key regulatory regions involved and is the subject of further work by our group.

Acknowledgements: This work was funded by the Scottish Hospitals Endowment Research Trust and the Norman Salvesen Emphysema Research Trust.

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