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Targeting neutrophil elastase in cystic fibrosis

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Abstract

Background: Cystic fibrosis (CF) is a lethal hereditary disease characterised by neutrophil-dominated lung inflammation. These abundant neutrophils produce neutrophil elastase (NE), a destructive serine protease that has direct actions on extracellular matrix proteins and has a role in the host response to inflammation and infection.

Objective: In this review we examine the prospect of developing novel therapies for CF by targeting NE. We explore the functions of NE and of naturally occurring and synthetic NE inhibitors.

Conclusions: Targeting NE in CF offers therapeutic potential but optimal inhibitors that can be delivered safely and effectively to the lung are still under development.

Keywords

α 1-antitrypsin, cystic fibrosis, elafin, neutrophil elastase, secretory leukoprotease inhibitor

Introduction

Cystic fibrosis (CF) is a lethal hereditary disorder found mainly in Europe and North America. It is caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, a 27-exon, 250 kilobase segment of chromosome 7 [1-3]. This is a relatively common disorder with 1 in 20 Caucasians being heterozygotic carriers of a mutated CFTR gene and an incidence of CF of 1 in 3000 live births. Hispanics, Blacks, and Asians are also affected, but in smaller numbers [4]. CF affects the liver, pancreas and intestinal tract but the major causes of morbidity and mortality are the lung disease [5]. This generally begins in childhood with the production of thick mucus, chronic airway infections, and inflammation beginning in the early years and leading to progressive decrement in lung function and death from respiratory failure [5, 6]. Other lung complications of CF include haemoptysis, pneumothorax, pulmonary hypertension, and cor pulmonale [5, 6].

Mutations in the CFTR gene cause CFTR protein abnormalities. The mechanism by which this abnormal protein leads to the respiratory abnormalities associated with CF is not fully understood but one of the most striking features of the lung disease associated with CF is the sustained, intense neutrophil-dominated inflammation present from very early in life [7-9]. In the normal lung neutrophils account for approximately 1% of the inflammatory cell population in epithelial lining fluid (ELF) but in the CF lung neutrophils represent 70% of the ELF inflammatory cells [7-9]. These

neutrophils are ineffective in the clearance of bacteria, and play a significant role in the pathogenesis of the lung derangement associated with CF. The lung derangement is mediated in large part by neutrophil proteases and oxidants that overwhelm the normal anti-protease defence of the respiratory epithelial surface [7-11]. Neutrophil elastase (NE) is the major protease released by neutrophils in the CF lung and although it is not the only culprit causing the lung damage associated with CF, it clearly plays a major role in the process. NE has a number of different effects in the lung. It directly injures epithelial cells and interferes with the defence mechanisms of the lung by reducing ciliary beat frequency [12], it deranges mucus glycoprotein secretion [13], cleaves complement components [14, 15], and immunoglobulins [16], and interferes with the ability of neutrophils to kill *Pseudomonas* [9, 14]. More recently work from this laboratory has shown that NE plays a major role in the inactivation of elafin, a major lung anti-protease and anti-inflammatory [17]. Furthermore NE upregulates a host of pro-inflammatory cytokines in lung epithelium acting through toll-like receptors [18, 19] and the epidermal growth factor receptor [20-22]. In addition to all of this, it now seems likely that NE plays a pivotal role in the upregulation of other proteases including metalloproteases and cysteinyl cathepsins [23].

Given this background NE represents a real target for novel therapies in the treatment of CF.

Neutrophil Elastase

NE is a serine protease, a group that also includes cathepsin G and proteinase 3. NE is packaged in primary granules within neutrophils [24] (**figure 1**). It is coded for by a 4-kb (5 exon, 4 intron) gene on chromosome 19 at p13.3 [25] and the sequences for the mature NE protein are included in exons II-V. The exon structure predicts a primary translation product of 267 amino acid residues including a 29-residue N-terminal precursor containing (a) a 27-residue “pre” signal peptide followed by a “pro_n” di-peptide and (b) a 20-residue C-terminal peptide. Following synthesis, the inactive precursor molecular form is trimmed, glycosylated with complex carbohydrates, transported to the Golgi, and ultimately carried to the azurophilic granules. The cleavage of the short N-terminal propeptide is typical for the granule-associated serine proteases of cells of the hemopoietic lineage where a cysteine proteinase, dipeptidylpeptidase I (also known as cathepsin C), functions as the principal posttranslational processing enzyme [26, 27]. In common with other serine proteases, NE is stored in a fully processed and activated form [28-30]. NE is a very basic protein; because of its high content of arginine residues, it has an isoelectric point of 9.4.

The mechanism by which NE cleaves a target protein is shown in **figure 2**. The NE molecule is globular and is comprised of two complex carbohydrate side chains attached to Asn⁹⁵ and Asn¹⁴⁴. The catalytic site of the NE molecule is an indentation of the molecule and is composed of the molecule and the triad His⁴¹-Asp⁹⁹-Ser¹⁷³, in which the γ -oxygen of serine becomes a

powerful nucleophile able to attack a suitably located carbonyl group on the target substrate. The bond to be cleaved must fit into the active site pocket of the NE held there by charge interactions and mediated by the residues forming the pocket. The peptide bond under attack is between two amino acid residues recognized by their side chains. An acyl-enzyme intermediate molecule is formed between serine and the carbonyl group on the target protein. To complete the process, the acyl-enzyme complex is hydrolysed with subsequent regeneration of active NE and cleavage of the protein.

Neutrophil elastase functions (figure 3)

NE is a powerful degrading enzyme and much research has been carried out to investigate this. Millimolar concentrations of NE, when released from single azurophilic granules of activated neutrophils, lead to evanescent quantum bursts of proteolytic activity before catalysis is halted by pericellular inhibitors. The manner in which NE degrades extracellular matrix despite the presence of functioning anti-protease molecules is still not fully understood. One theory holds that neutrophils can release oxidants which locally inactivate antiproteases thereby permitting NE to act on the local tissues. Work from Campbell's group looked at the function of NE as regards quantum proteolysis [31]. Calculations from first principles indicate that approximately 67,000 molecules of NE are stored in each azurophilic granule at a mean concentration of 5.33 mM, which exceeds pericellular inhibitor concentrations *in vivo* by nearly three orders of magnitude. Diffusion analysis predicts obligate catalytic activity (excess of local enzyme over inhibitor concentration)

that extends to 1.33 microns from the site of granule extrusion (7.8-fold larger than the mean radius of the granule), with a duration of 12.4 ms, when the pericellular concentration of α 1AT equals that of normal plasma. In contrast, when polymorphonuclear cells are bathed in α 1AT concentrations found in plasma from individuals with α 1AT deficiency, the radius and duration of obligate catalytic activity are increased 2.5-fold and 6.2-fold, respectively. These simulations agree remarkably well with direct observations and provide a novel, nonoxidative mechanism by which quantum bursts of extracellular proteolytic activity occur despite proteinase inhibitors in the bathing medium. Titration of local enzyme-inhibitor concentration is the dominant determinant of the size and duration of such events. Work with α 1AT deficiency serum samples showed that quantum proteolytic events are abnormally large and prolonged in α 1AT deficiency, leading directly to an increased risk of tissue injury in the immediate vicinity of activated neutrophils [32]. This may give an insight into the destructive activity of NE in patients with CF, where the problem of protease antiprotease balance is not one of inhibitor deficiency but rather enzyme excess.

The term neutrophil elastase is partly a misnomer as not only does NE degrade elastin but also degrades almost all extracellular matrix and key plasma proteins. NE has broad substrate specificity and is capable of degrading a wide range of extracellular matrix proteins including elastin, collagen (types I-IV), fibronectin, laminin, and proteoglycans [33-37]. Other extracellular matrix proteins degraded include platelet IIb/IIIa receptor, complement receptor, thrombomodulin, lung surfactant, and cadherins [38-

42]. In addition, NE can cleave coagulation factors (fibrinogen and factors V, VII, XII, and XIII), plasminogen, IgG, IgA, and IgM, complement factors C3 and C5, complement receptors, and gp120, the coat protein of the human immunodeficiency virus [43-45]. It may also cleave other proteases found within neutrophil granules [46] and also other protease inhibitors [47, 48] leading to their activation or their loss of function. **(Table: 1)**

NE has also been shown to upregulate pro-inflammatory cytokines. For example, NE upregulates IL8 expression and secretion from bronchial epithelial cells by signalling through toll-like receptor 4 (TLR4) [49, 18]. NE-induced upregulation of IL8 expression in human bronchial epithelial cells depends on the proteolytic activity of NE, as inhibition with the serine-protease inhibitor phenylmethylsulphonyl fluoride (PMSF) abolishes this specific activation. Similarly, Tsujimoto et al. showed that pre-treatment with an inhibitor of NE decreased lipopolysaccharide (LPS)-induced CXCL2 production by the human macrophage cell line RAW 264.7 in a dose-dependent manner [50]. NE also upregulates leukotriene B4 (LTB4) secretion from macrophages [51].

NE contributes to chronic inflammatory airway diseases by inducing mucin production in airway epithelial cells [20]. The exact mechanism by which NE mediates this effect is unclear but has been shown to involve activation of protein kinase C (PKC) and production of reactive oxygen species (ROS). ROS in turn activate TNF- α (tumour necrosis factor- α)-converting enzyme (TACE), leading to TACE-dependent release of soluble transforming growth

factor- α (TGF α) and TGF α stimulation of epidermal-growth-factor receptor (EGFR), which induces the production of mucin [21]. However other metalloproteases may also be involved in these processes including meprin α [22].

NE and other serine proteases have also been shown to have an effect at the cell surface. NE can cleave cell-surface adhesion molecules, such as intercellular adhesion molecule 1 (ICAM1), vascular cell-adhesion molecule 1 (VCAM1) and epithelial (E)-cadherin [52-54]. As cell migration involves a series of attachment and detachment events, the shedding of adhesion molecules from the cell surface by proteases might provide a mechanism to regulate and terminate cell-cell or cell-extracellular matrix interactions. It is postulated that serine proteases are involved in activation of the pro-form of tumour necrosis factor and interleukin-1 β [55, 56] but interestingly NE and cathepsin G both degrade mature TNF [57] and all three serine proteases can inactivate IL-6 [58].

Although much research has centred on the destructive nature of NE in degrading a multitude of proteins, recent evidence has emphasised the role of NE in the host immune response to infection. For example, while neutrophils may migrate normally to sites of infection in the absence of NE, NE is required for maximal intracellular killing of Gram-negative bacteria by neutrophils [59]. In addition to the intracellular activities of the neutrophil serine proteases, it has been shown that serine proteases released from neutrophils form neutrophil extracellular traps (NETs) with chromatin and that NETs bind

Gram-positive and Gram-negative bacteria [60]. These NETs allow neutrophils to deliver high concentrations of serine proteases that degrade virulence factors and kill bacteria extracellularly. A proportion of NE remains bound to the cell surface of neutrophils when azurophilic granules fuse with the plasma membrane during exocytosis. The concentrated expression of the neutrophil serine proteases on the outer surface of the plasma membrane [61, 62] allows neutrophils to modulate the inflammatory response. In support of this idea, inhibition of neutrophil serine proteases has been shown to reduce neutrophil infiltration and neutrophil-mediated injury in various modes of inflammation, including ischaemia and reperfusion injury, endotoxin-induced acute lung injury and collagen-induced arthritis [63-65].

These data show that there is a fine balance between the physiologic and deleterious effects of NE. When this balance is disturbed, as in the case of α 1AT deficiency where there is too little NE-inhibition or in CF where there is dysregulated NE release, lung damage results. While it is generally accepted that large quantities of active extracellular NE in the lung is potentially deleterious and should be inhibited to prevent further lung damage, intracellular and locally expressed NE may be important for host defence and should not be inhibited. This has important implications for the type of inhibitors which should be used.

Anti-NE Defences of the lung

In normal lungs the anti-NE defences include, α 1AT, secretory leukoprotease inhibitor (SLPI) and elafin. Individuals with CF have normal serum and ELF levels of all three [7, 8]. α 1AT is produced in the liver and from there circulates to the lung where it provides the major anti-NE protection to the lower respiratory tract [66]. The importance of α 1AT and NE in lung protection and destruction respectively is illustrated by α 1AT deficiency, a hereditary disorder associated with polymerisation of the mutant α 1AT in the liver, decreased secretion of α 1AT into the circulation and a markedly diminished anti-NE protective defence in the lung [67]. This condition is associated with premature aggressive emphysema, a condition which is aggravated greatly by the effects of cigarette smoke in oxidising and further inactivating α 1AT leading to an unopposed action of NE [68].

The roles of SLPI and elafin in protecting the lung from NE are less well defined. Both of these molecules are produced locally by epithelial cells and it has been suggested that SLPI may function as an anti-NE molecule mostly in the upper airways. More recently research has focussed on the anti-inflammatory effects of these molecules and by extension on the deleterious effects of NE in inhibiting these activities. SLPI and elafin have been shown to inhibit nuclear factor-kappa B (NF κ B) activation, and IL-8 and monocyte chemoattractant protein-1 (MCP-1) production by epithelial cells and monocytes. However, whereas elafin is inactivated by NE in CF ELF [17], SLPI is relatively immune to the direct proteolytic effects of NE but is readily proteolysed by cysteinyl cathepsins [69] and metalloproteases both of which

are upregulated by NE [23]. CysteinyI proteases also cleave and inactivate defensins [70] and lactoferrin, two locally produced anti-microbials with direct relevance to CF as defensins are key mediators of *Pseudomonas* killing and lactoferrin has a major role in inhibiting biofilm formation [71].

Individuals with CF have normal to high serum and ELF levels of all three of the major anti-NE compounds [7, 8]. However, α 1AT, elafin and SLPI in CF ELF are completely inactive, being complexed with or cleaved by NE and other proteases or rendered impotent by oxidants produced by inflammatory cells [7, 8]. The presence of large amount of neutrophils on the respiratory epithelial surface, brought there by abundant neutrophil chemoattractants (including IL8, Leukotriene B4, C5A) and α 1AT-NE complexes results in large quantities of unopposed NE on the respiratory epithelial surface of individuals with CF. The levels of NE in CF ELF vary markedly, but can be as high as 100 μ M. These levels correlate inversely with pulmonary function in individuals with CF. This illustrates one of the enduring difficulties in dealing with the neutrophil dominated inflammation in CF, namely the recurring cycle of NE induced inflammation. NE upregulates IL8 from epithelial cells and LTB4 from macrophages creating a gradient of neutrophil chemoattraction bringing more neutrophils onto the epithelial surface where they are readily activated to produce more NE by cytokines such as IL8 and TNF. Adding to this complexity the neutrophil in individuals with CF is also intrinsically abnormal and secretes more NE than non-CF neutrophils in response to IL8, TNF and LPS all of which are found in abundance in the CF lung [72].

Targeting neutrophil elastase in cystic fibrosis

(figure 4)

Strategies to prevent NE-mediated lung derangement in CF are based on the theory of increasing the level of antiproteases available to inhibit NE or lowering the burden of NE in the lung. In CF, the levels of NE in ELF vary dramatically from individual to individual, and even in the same individual over time, so that it is difficult to determine the level of augmentation required.

Approaches to augment the anti-NE defenses of the lung in CF include (a) augmentation with plasma purified α 1AT, (b) augmentation using recombinant antiproteases or other naturally occurring antiproteases, (c) use of chemical antiproteases, and (d) gene therapy. The inhibition of NE in CF, should not only directly protect the lung but also lower the neutrophil burden, and enhance host defence by protecting complement, complement receptors, and locally produced antimicrobials and antiinflammatories such as SLPI, elafin, defensins and lactoferrin.

Augmentation with purified plasma α 1AT

Much of the work on intravenous administration of purified α 1AT has been carried out in the context of α 1AT deficiency. The seminal paper by Wewers et al [73] showed that once weekly intravenous administrations of plasma purified α 1AT led to levels of serum α 1AT above a putative protective threshold of 11 μ M throughout the duration of therapy. This serum data was complemented by data showing that ELF levels of α 1AT and anti-NE capacity were also raised by this intravenous administration to the normal α 1AT ELF levels. As a next step from this it was deemed logical that the enormous NE

burden on the epithelial surface in CF could be inhibited by intravenous augmentation with α 1AT [8]. Purified plasma α 1AT was administered at dosages ranging from 60 to 120 mg/kg once weekly. However, only dosages in the 120-mg/kg range were able to elevate α 1AT levels sufficiently to inhibit the NE burden in these individuals. Unfortunately, the inhibition was brief, with reappearance of active NE on the respiratory epithelial surface within 1 week after administration, suggesting that this approach, although effective in the short term was not feasible as it would require frequent intravenous administration of large doses of α 1AT to have the desired effect. Recombinant forms of α 1AT have not been effective when administered intravenously as those raised in yeast are non-glycosylated with a resultant very short plasma half life while those produced from transgenic animals had different glycosylation to plasma purified α 1AT also leading to alterations in half life. [74-76]. Animal studies evaluating intravenous infusion of SLPI showed an unacceptably high level of proteinuria [77]. Slowing the infusion decreased the levels of protein in urine but this approach has not been pursued. Because of these results and because CF is mainly a bronchial disease it was decided that aerosolization of α 1AT to the lungs of individuals with CF would offer benefits.

Aerosol Augmentation with α 1AT

This was first attempted in 1991 [66]. 1.5-3mg/kg of purified plasma α 1AT was administered twice daily to individuals with CF. This resulted in inhibition of NE on the respiratory epithelial surface and re-establishment of the ELF anti-

NE capacity, but only if the levels of α 1AT in ELF exceeded $8\mu\text{M}$. However this study also highlighted another potentially useful effect of inhibiting NE in CF. After suppression of the NE burden in CF ELF with aerosolized α 1AT, the ability of CF ELF to inhibit neutrophil killing of *Pseudomonas* was abolished. This was due in part to the inhibition of NE in CF ELF preventing it from cleaving complement receptors on the neutrophils. Furthermore suppression of NE in CF is associated with decreased IL-8 in CF ELF and decreased neutrophil numbers on the epithelial surface [8]. These findings have been replicated by others. Berger et al showed that aerosolised plasma purified α 1AT inhibited NE in ELF and that this inhibition was related to the doses of α 1AT delivered [78]. They did not show a significant effect on IL-8 or neutrophil count. Interestingly there was no correlation between sputum and ELF NE levels. A recent study from Griese et al, examined the effect of 4 weeks of plasma purified α 1AT inhalation on lung function, protease-antiprotease balance and airway inflammation in CF patients [79]. They found that this therapy increased α 1AT levels and decreased the levels of NE activity, neutrophils, pro-inflammatory cytokines and the numbers of *P. aeruginosa*. However, as might be expected, given the short duration of this study, there was no effect on lung function. This study mainly evaluated sputum based parameters and speculated on the reasons for some of the differences noted between parameters measured in sputum and ELF.

Secretory Leukoprotease Inhibitor

Another candidate as an anti-NE molecule for CF is recombinant SLPI, a 12-kDa-nonglycosylated single-chain polypeptide identical to the naturally

occurring SLPI [80-83]. Advantages of SLPI as a potential therapeutic option are that it is acid stable which may allow it to retain function in the acidic pH of the metabolically active neutrophils [84, 85], its isoelectric point of 9 is close to that of NE which may allow it to track and bind to tissue sites favoured by NE [86, 87], its relative imperviousness to degradation by NE and its possible enhanced antioxidant function by increasing glutathione levels in the local milieu [88]. Despite the postulated advantages, delivery of SLPI to the lungs has proven unimpressive [89]. Aerosolization of 100 mg of recombinant SLPI twice daily to individuals with CF was associated with inhibition of NE on the respiratory epithelial surface, as well as decreases in the levels of IL-8 and neutrophil numbers in ELF [8]. However, relatively more recombinant SLPI than α 1AT is required to suppress ELF NE levels in individuals with CF. Furthermore, recombinant SLPI does not accumulate on the respiratory epithelial surface either in controls or in individuals with CF, and most of the anti-NE effects are gone within 12 hours of administration. The reasons for this are not fully elucidated but may be due to compartmentalization of SLPI to the epithelial surface, absorption of recombinant SLPI into epithelial cells and macrophages, and/or binding of recombinant SLPI to molecules in the interstitium after passing through the epithelium [89, 7]. Also more recently it has been shown that SLPI is easily degraded by cysteinyl cathepsins which are present in very high quantities in CF ELF [69]. Thus, although SLPI has several theoretical advantages to augment anti-NE in the lung, the successful use of the molecule as an effective therapy for CF presents considerable challenges.

Other naturally occurring antiproteases

Elafin is a cationic protease inhibitor of human NE, proteinase 3, and porcine pancreatic elastase. It was previously shown that elafin and its precursor trappin-2 retain the ability to inhibit neutrophil serine proteinases when they are covalently bound to extracellular matrix proteins by tissue transglutaminase [90]. More recent work in U937 cells showed that elafin inhibits LPS-induced production of MCP-1 by preventing the LPS-induced activation of AP-1 and NF κ B [91]. This anti-inflammatory effect appears to be dependent upon an effect of elafin on the ubiquitin-proteasome pathway. Due to the selective expression of elafin at mucosal surfaces, as well as in alveolar macrophages, monocytes and neutrophils, the ability of elafin to inhibit LPS signalling may be important in CF and may represent another therapeutic option. Unfortunately in areas of NE excess elafin is degraded and inactivated by the NE [17].

Human monocyte/neutrophil elastase inhibitor (MNEI), a 42-kDa serine protease inhibitor (serpin) protein, is an effective inhibitor of NE, cathepsin G, and proteinase-3 [92]. Recombinant MNEI has been given daily by aerosol to rats, previously infected with *P. aeruginosa*. The treatment with MNEI significantly decreased the extent of inflammatory injury and improved clearance of bacteria from the infected rat lungs. Similar results have been obtained for aerosolized α 1AT in a rat model of CF lung inflammation [93]. This suggests another potential parameter to evaluate the efficacy of anti-protease therapy in CF.

Other naturally occurring antiproteases include eglin-c, a single-chain polypeptide purified from the medicinal leech *Hirudo medicinalis*.

Recombinant eglin-c inhibits NE and cathepsin G, as well as several other proteases, and intratracheal instillation of eglin-c has been shown to attenuate the severity of NE induced emphysema in hamsters [94-96]. However, as might be expected of a protein from a nonhuman source, eglin-c is allergenic to humans and cannot be used clinically in its present form.

NE is markedly inhibited by *cis*-unsaturated fatty acids, such as oleic acid. A family of serine protease inhibitors has been identified in the seeds of squash (*Curcubita maxima*), several of which might be potentially useful [97]. A number of NE inhibitors have been isolated from microorganisms, including elasnin, elasninal, chymostatin, leupeptin, valinal, and antipain [98]. As for eglin-c the clinical application of these substances is limited by the possibility of immune reactions.

Three naturally occurring dithiol reducing systems have been examined for their effects on elastase activity: 1) *Escherichia coli* thioredoxin (trx) system, 2) recombinant human thioredoxin (trx) system, and 3) dihydrolipoic acid (DHLLA) [99]. The ability of Trx and DHLLA to limit elastase activity combined with their mucolytic effects makes these compounds potential therapies for CF.

More recently work has been done on greglin, a novel locust serine protease inhibitor with a high affinity for NE [100]. This is a fast acting and tight binding

inhibitor of human NE, which also binds neutrophil cathepsin G, pancreatic elastase and chymotrypsin with lower affinity. This has been shown to inhibit NE activity in sputum supernatants from CF patients.

Low-molecular weight inhibitors of NE

A number of synthetic NE inhibitors have been developed as potential therapeutic agents for α 1AT deficiency. These include irreversible inhibitors such as the peptide chloromethyl ketones [101] and reversible inhibitors such as peptide boronic acids [102], peptide aldehydes [103], substituted tripeptide ketones [104], or β -lactams that have been modified to inhibit NE [105]. One of the problems with the low-molecular-weight reversible inhibitors is that they can release NE, allowing it to destroy tissue. Although the irreversible inhibitors such as chloromethyl ketone have been shown to function effectively *in vivo* in hamsters to reduce many of the effects of intratracheally administered NE, the toxicity of chloromethyl ketones prevents clinical use.

β -lactam-based compounds have been identified as specific potent inhibitors of NE [105]. The fact that these molecules clear rapidly from circulation suggests that they may be limited to aerosol therapy. From kinetic data, Gorrini et al concluded that the macrolide antibiotic erythromycin acts as an alternate substrate NE inhibitor and that flurythromycin acts as an inactivator [106]. L-658,758, a cephalosporin-based drug, is another antibiotic which has inhibitory effects on NE, proteinase-3 and CF sputum soluble elastase [107].

Small molecules can gain access to tissue sites inaccessible to larger molecules. This is a potential advantage and a potential disadvantage. While these inhibitors may prevent NE from attacking connective tissue in confined areas such as beneath the surface of neutrophils they may also inhibit proteases at sites where they perform a physiological function, such as intracellularly or in their role as facilitators of cell movement through tissue [108].

Initial work *in vivo* and *in vitro*, in rats, on a new, rapidly acting, potent and specific NE inhibitor, EPI-HNE-4 demonstrated an effective inhibition *in vitro* of the high levels of active NE present in a medium as complex as sputum from children with CF by almost complete block of N-formyl-methionine-leucine-phenylalanine-induced migration of purified human neutrophils across a Matrigel basement membrane [109]. Intratracheal administration of EPI-HNE-4 and intravenous administration brought about inhibition of NE and of neutrophil influx in the rat. This suggested a potential for associated aerosol and systemic administration of EPI-HNE-4 in the treatment of CF. Further work on this agent showed that it can be nebulised efficiently without decrease in its activity and that mixing the inhibitor with (99m)Tc human serum albumin tracer should allow quantification of its deposition in CF patients [110]. EPI-HNE4 is also resistant to hydrolysis by neutrophils in inflammatory lung secretions, including metalloproteases (MMPs) 7, 8 and 9, and the elastase-related protease 3 and cathepsin G [111]. While these initial results are encouraging this agent is still some distance from demonstration of clinical efficacy.

Gene therapy

Whilst various viral vectors continue to be evaluated as a method of transfer of CFTR in CF [112], the delivery of the α 1AT cDNA via different vectors to increase the antiprotease activity in CF is another possibility. Plasmid cationic liposome mediated α 1AT gene transfer to a CF bronchial epithelial cell line was first performed in 1996 [113]. This protected the cells from NE damage. To date there have been a number of animal studies evaluating the potential of transferring the α 1AT cDNA to muscle liver and lung in the hope of producing therapeutic levels of α 1AT in plasma. These studies used an adeno-associated virus vector delivery system and achieved significant levels of α 1AT over prolonged periods of time, up to months, and a subsequent phase I trial revealed no serious vector-related adverse events [114]. However to date no human trials have been forthcoming probably because of the availability of an alternative in plasma purified augmentation therapy.

Delivering an agent to the lung

A further hurdle to the targeting of NE to achieve therapeutic potential in CF is the delivery of an agent to the lung. This is a problem that has been faced in attempting to optimise delivery of antibiotics to the lung in CF [115]. There are many advantages to aerosol delivery as opposed to systemic administration; the agent is delivered directly to the site of action, there is less systemic toxicity, it can be self administered and higher concentrations are available at the site of the lung disease. Drawbacks of this approach include difficulties in determining pharmacokinetics and sampling of ELF to quantify inhibitory

effects. Nebulisation can affect drug activity and, depending on whether administration is preferable in the upper or lower airways, optimum particle size and device type must be evaluated. Factors such as biofilm, the physical and chemical composition of mucus and the degree of parenchymal destruction and bronchiectasis in CF patients can significantly alter drug distribution and bioavailability. In the case of antibiotics two classes of antagonistic sputum components have been identified. They are small molecules which physically decrease antibiotic penetration into bacteria, and large glycoprotein molecules, which bind and sequester aminoglycosides [116]. Sputum glycoproteins such as mucin and DNA in CF patients can bind polycations such as aminoglycosides. Glycoprotein content varies amongst CF patients but can bind approximately 90% of the drug. Soluble sputum components such as monovalent and divalent cations, vary little among patients [117]. Divalent ions bind approximately 5% of aminoglycoside given and DNA binds approximately another 2%.

The desired properties of an ideal aerosolised therapeutic agent in CF patients are many. Particle sizes ranging from 1 to 5 μm appear to be optimal for reproducible drug delivery to the airways [118, 119]. The agent should be sterile and nonpyrogenic as allergic reactions to impure recombinant or plasma-purified preparations may occur [120]. To aid in patient compliance it should be reasonably palatable. To allow for the varying conditions that it will encounter in the lungs of CF patients which will alter the delivered concentration it should have a wide margin of dosage safety. The ideal

aerosolised agent should be easily nebulised and should be chemically stable.

Expert Opinion

There is no doubt that the lung disease in CF is mediated, at least in part, by the effects of NE. These effects are myriad, directly acting to degrade extracellular matrices, impairing host defence, increasing mucin and cytokine production and upregulating other potentially deleterious proteases in the lung. Whereas normally NE has an important role itself in host defence, in CF the secretion of NE is dysregulated so that there is an excess of extracellular NE and the normal anti-NE protective mechanisms are ineffective. In this context it makes sense that extracellular active NE in the lung epithelial surface should be inhibited. Given the newly discovered anti-inflammatory effects of many anti-NE molecules it becomes more important than ever to try to restore the normal NE/anti-NE balance in the CF lung. At the present time most experience is with the natural inhibitors of NE such as plasma purified α 1AT but other recombinant and synthetic molecules are being studied. The major problems with plasma-purified α 1AT have been fears of blood borne infections and lack of supply. Recombinant preparations also have potential problems; some cannot be given by intravenous route and those given by aerosol need to be rigorously tested for impurities. The future is likely to see customised combinatorial therapies designed specifically for CF patients that take into account bacterial colonisation status, NE activity, and mucus, DNA and glycoprotein levels. Aerosolisation is the most promising route of administration and aerosolisation of anti-NE molecules may be combined with

other aerosolisation therapeutics. Problems posed by drug binding and sequestration, charge factors and accessibility to the airway epithelium pose major challenges and advances in delivery technologies are needed.

The effects of successful anti-NE therapy will need to be evaluated at a variety of levels. Firstly the therapies must be shown to inhibit NE and in doing so decrease other pro-inflammatory cytokines and proteases. Conceivably this could lead to decreased bacterial load which could serve as a useful surrogate measure of clinical efficacy both in short term and long term studies. The previous gold standard in all lung studies of an improvement or decreased deterioration in pulmonary function will not be achievable in short term studies and may best be reserved for the more definitive stage 3 studies along with other measures such as quantitative CT scanning.

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