

Should we Target Allergen Protease Activity to Decrease the Burden of Allergic Airway Inflammation?

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Abstract: The prevalence of allergy and asthma has been increasing steadily in the developed world. Despite this growing health problems associated with these conditions few new therapeutic options are becoming available. Relatively recent developments have identified a role for proteases in promoting allergic sensitisation and allergic airway inflammation. It is also known that most major allergens have associated protease activity that is closely related to their efficacy to induce sensitization and inflammation. Here we review the evidence for the significance of protease activity in allergic airway disease and offer suggestions as to where the field could progress.

Keywords: Asthma, allergen, serine proteases, protease-activated receptors, allergic inflammation.

INTRODUCTION

The prevalence of allergy and asthma has been increasing throughout the western world over the last 20 years. The use of inhaled and nasal corticosteroids and leukotriene receptor antagonists as preventive medications and inhaled β -agonists and anticholinergics in various combination, are highly effective and enough to control the disease in many patients with asthma and rhinitis [1]. However, a significant percentage of the patients either because of side-effects or lack of effectiveness of available medications, need other treatment options. Therefore, we still need novel and more effective therapeutic approaches. The field is very active [2] in both the biotech and the pharmaceutical industry because identifying new therapeutic options for these common diseases has the potential to address the needs of large and growing sector of the population.

It is well accepted that “environmental” changes described by the “hygiene hypothesis” [3, 4] are responsible, at least in part, for the increased prevalence of allergic disease. However, the aeroallergens associated most commonly with allergic diseases remain relatively stable. These include Grass and tree pollens, house dust mite (HDM), animal dander and mold allergens, with specific variations depending on the locale and environmental conditions. HDM [5] is a major allergen in large parts of the world and often associated with asthma, while cockroach allergens [6] have gained notoriety through their association with the devastating epidemic of inner city asthma in the United States [7, 8].

The observations that the number of allergens is limited compared to the total number of inhaled proteins indicates that in addition to genetic susceptibility and environmental factors that determine whether an individual becomes allergic, intrinsic characteristics of allergens must be important. However, these characteristics have not, so far, been

explored systematically to identify new therapeutic modalities for allergic diseases.

Many of the common allergens that induce asthmatic sensitization, such as HDM, cockroach and fungal allergens are proteases. Significantly, the protease activity of these allergens appears crucial for the development of allergic sensitization [9], and is also involved in increased IgE production [10]. Even allergens that have no proteolytic activity, such as various pollens, are packaged in particles that contain proteases [11]. Furthermore, inhaled air has a number of proteases from bacteria and other microorganisms and these proteases are seen by the respiratory mucosa at the same time as inhaled proteins. The aim of this review is to highlight the mechanism involved in the biological effects of the protease activity of major allergens and offer greater understanding that inhibition of these events may provide novel therapeutic approaches to attenuate allergic sensitization and/or inflammation.

PROTEASE ACTIVITY AND ALLERGIC SENSITIZATION

The importance of the protease activity for allergic sensitization is evident in a number of mouse models. Proteins without protease activity induce immune tolerance when they come in contact with the murine airway mucosa in the absence of any adjuvant [12, 13], while proteins with protease activity induce allergic sensitization [14, 15].

Allergens with protease activity are also able to mediate sensitization to non-protease protein when they are simultaneously exposed to the airway mucosa [16] or increase IgE to bystander allergens [10]. In addition, enzymes similar to the ones associated with occupational sensitization have been shown to be able to induce allergic sensitization after mucosal exposure using animal models [17, 18].

These observations highlight another important, as yet unanswered, question. In everyday sensitization of humans our airway mucosa is exposed to allergens in conjunction with other inhaled proteases that come from bacteria, viruses and other particles in the air. What is the role of these proteases in allergic sensitization? Animal studies again indicate

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that a protease can mediate allergic sensitization to a different protein when exposed at the same time [19, 20].

There are various theories on how the protease activity of various allergens leads to allergic sensitization. Protease activity may degrade extra cellular attachment molecules and make it easier for allergens to penetrate through the airway mucosa [21]. There are significant recent observations in this field. Der p1 induces increased epithelial permeability through breakage of tight junctions [22] and reduction of their ZO-1 content [23]. HDM serine proteases can also compromise the function of tight junctions on the airway epithelium through proteolysis of occludin [24]. In addition proteolytic enzymes from a number of tree, grass and weed pollens can degrade ZO-1 and occluding and increase the permeability of tight junctions [25]. The general consensus is that degradation of proteins that participate in tight junctions, desmosomes and adherens junctions by allergens with protease activity facilitate the development of allergic sensitization at mucosal surfaces [26].

The above effects would increase access of the allergens to the immune system, but other immunomodulatory mechanisms should also play a significant role. For example, allergens cleave cell surface membrane molecules that are important in the regulation of the allergic response, such as CD25 and CD23 [27]. While the exact mechanisms by which these proteases are involved in allergic sensitization are not clearly defined one characteristic common to many protease allergens, including HDM, cockroach and fungal allergens, is their ability to activate Protease activated Receptor 2 (PAR-2).

ALLERGENS AND PROTEASE-ACTIVATED RECEPTORS (PAR)

PAR comprise a family of G-coupled receptors activated by serine proteases [28]. Four members of the family (PAR-1 to PAR-4) have been identified. Proteases cleave within the N-terminus of the receptors and expose a tethered ligand domain that binds and activates the cleaved receptor (Fig. 1A). Synthetic peptides with sequence similarity to the receptor tethered ligand, called PAR-2 activating peptides (PAR-2 AP) bind and activate PAR-2 without protease-mediated cleavage (Fig. 1B). These peptides have been used extensively in research settings because they are very potent and specific and eliminate the problem of non-PAR-2-mediated effects that can be seen with serine proteases. Scrambled peptides with the same amino acid composition, called PAR-2 control peptides (PAR-2 CP) are used to study the specificity of the PAR-2 AP effects.

Among the receptors of this family, PAR-2 is of particular interest in airway diseases because a number of enzymes released from airway epithelium (trypsin-like protease [29] and inflammatory cells (mast cell tryptase [30] or proteases released from neutrophils [31]) have the potential to activate this receptor. PAR-2 is also activated by HDM [32] cockroach [33, 34] and fungal allergens [35] and this list is expected to grow as investigation in this area continues..

PAR-2 is expressed in the lung on structural cells [36-42] and inflammatory cells such as mast cells [43, 44], neutrophils [45] and alveolar macrophages (AM) [46], but its exact physiological roles are not clear [47]. PAR-2 activation induces leukocyte rolling, adhesion and extravasation [48] and

mediates inflammation and nitric oxide-dependent vasodilation [49-51]. Furthermore, PAR-2 is expressed in neurons of the nasal mucosa [52] and its activation on the peripheral projections of spinal afferent neurons, stimulates local release of CGRP and SP [53], and thus this receptor probably plays a central role in neurogenic inflammation associated with allergic responses.

We have shown high expression of PAR-2 on the respiratory mucosa of the upper and lower airways of the mouse [54] and others have shown its expression in murine [55] and human airways [56, 57]. PAR-2 may be of further interest in inflammatory diseases in the lung because it can be upregulated by a number of pro-inflammatory cytokines [58] that are present in the lung in allergic airway inflammation. Viral infections, such as in influenza, increase PAR-2 expression in the airways [55] and PAR-2 expression also increases on the respiratory epithelium of asthmatic subjects [57]. Increased PAR-2 expression in these conditions may potentiate inflammatory reactions in response to serine proteases. Furthermore PAR-2 activation, leads to sensitisation of the capsaicin sensitive vanilloid receptor, TRPV1 [59, 60], in primary sensory neurons and thus may play a role in the exaggerated cough observed in certain airways inflammatory conditions such as asthma and chronic obstructive pulmonary disease.

We have recently shown that PAR-2 activation exacerbates allergic airway inflammation due to sensitization to ovalbumin [54], indicating that PAR-2 activation participates in the development of allergic airway inflammation in sensitized individuals. This effect seems to be primarily mediated by TNF release. Our studies on the mechanism of PAR-2-mediated allergic sensitization in mice also indicate that PAR-2 activation in the airways leads to alterations in dendritic cell (DC) activation and in this way plays a prominent role in allergic sensitization. In particular, we have shown that murine lung DC express PAR-2 and administration of a PAR-2 activation stimulus in the airways increased the potential of lung DC to take up antigen and the rate of trafficking to the lymph nodes and spleen [61]. This alteration in DC activity leads to sensitization to simultaneously administered proteins. This result is similar to the effect of serine proteases sensitizing mice to concomitantly administered antigens [19, 20].

Therefore, PAR-2 may be one of the receptors mediating the effects of serine proteases on both allergic sensitization and the development of allergic airway inflammation and as such may be an excellent target to prevent or alleviate the consequences of allergy in the airways.

TARGETING PROTEASES AS TREATMENT FOR ASTHMA

A number of endogenous proteases have been shown to be important in the pathogenesis of asthma and allergic airway inflammation. Inhibitors of these proteases, released from inflammatory and other cells following contact with the inhaled allergen, have been tested as potential therapeutic options in asthma.

Targeting Endogenous Proteases

Tryptase inhibitors have been shown to be effective in animal models [62, 63] and an inhaled inhibitor of tryptase

has been shown to decrease the late asthmatic response to allergen challenge in humans [64]. Secretory Leukocyte Protease Inhibitor (SLPI) [65, 66] has also been studied as a therapeutic option. Although these options have not moved to therapeutics yet, the interest in this area continues. Furthermore, serine protease inhibitors nafamostat mesilate and gabexate mesilate prevented airway inflammation to HDM allergens [67] in a murine model of asthma. However, it is not clear if this was due to inhibition of HDM enzymatic activity or inhibition of endogenous serine proteases.

Interestingly, certain allergens can inactivate endogenous elastase inhibitors, such as elafin, SLPI and $\alpha 1$ proteinase inhibitor, and in this way they may promote allergic inflammation through the unopposed action of elastases [68]. Der p 1 can also inactivate another inhibitor, $\alpha 1$ -antitrypsin [69]. Therefore, although the inhibitors described in the previous paragraph technically do not inhibit the activity of allergen protease activity, they may still be useful in counteracting the effects of particular allergens.

Targeting Protease Activity of Allergens

Further inhibitors of endogenous proteases are being developed and tested [70] and while this field will certainly see a large expansion over the next few years, to date, little attention has been paid to the therapeutic potential of inhibiting the protease activity of allergenic extracts. While a number of approaches could be useful in preventing the detrimental effects of such allergens, including blocking of intrinsic protease activity or inhibition of the target (meaning primarily PAR) receptors, the major prerequisite for progress in this area is a full understanding of the protease characteristics and mechanisms involved. However, the literature on this subject is still very poor.

Inhibiting the protease activity of an allergen could function both as a long-term preventive therapy but more importantly it could be very effective for acute episodic allergic diseases, especially in cases of acute development of symptoms after exposure to an allergen with protease activity. In that case the inhibition of the protease activity may be enough to avoid the development of wheezing and/or shortness of breath in asthma and also prevent the development of allergic airway inflammation.

Most work that has been done has focused on the inhibition of HDM proteases and especially the main allergen of HDM, Der p 1, which has cysteine protease activity. Cystatin A is a potent skin-derived inhibitor of the cysteine protease activity of house dust mite allergens (Der p 1 and Der f 1) [71]. Human cystatin C also inhibited the function of Der p 1 [72] as did plant cystatins [73]. It is interesting that cystatin C was relatively inactive at inhibiting Der p 1 activity when it was used in whole HDM extract. This could be the result of inhibitors of the inhibitor being present in the extract or other protease degrading the inhibitor. It is also interesting that polymorphisms in Cystatin A that induce decreased RNA stability and therefore reduce the expression of the protein, are associated with atopic dermatitis, presumably through increased endogenous and exogenous protease activity that may affect skin barrier integrity [74]. Cystatin C is also synthesized by human bronchial epithelial cells [75], but is generally present in all body fluids. Therefore cystatin C

comes in contact with HDM allergens when they are inhaled. Increasing its activity in the airways may be beneficial in asthma.

The squamous cell carcinoma antigen 2 (SCCA2), an inducible protease inhibitor produced by bronchial epithelial cells, was also reported to inhibit the proteolytic activity of Der p 1 and Der f 1 [76]. In addition, $\alpha 1$ -antitrypsin can inhibit Der p 1-mediated CD23 cleavage [77]. The relevance of this is not clear especially since Der p 1 can in its turn cleave and inactivate $\alpha 1$ -antitrypsin [69].

Other synthetic inhibitors of the cysteine protease activity of HDM allergen have also been described [72]. One such inhibitor, PTL1028, a peptide based irreversible inhibitor, is selective for cysteine proteases and does not inhibit human serine proteases, such as elastase, thrombin or trypsin and has very limited activity against some cathepsins. This inhibitor decreases the ability of Der f 1 to cleave CD23 from human B cells and to increase the permeability of human bronchial epithelial monolayers. It also has similar effects on HDM extracts. However, there was no attempt to see whether other functions of HDM are inhibited. Also there was no attempt to study inhibition of HDM effects *in vivo*. Although Der p 1 has probably no serine protease activity, $\alpha 1$ -antitrypsin inhibits partially the effect of Der p 1 on bronchial cell monolayer permeability. This may be due to contamination of Der p 1 with Der p 3, as has been suggested [78]. Although there is some suggestion that Der p 1 can activate the serine protease receptor, PAR-2 [79].

Pollen grains contain proteases that are seen by the immune system at the same time with the pollen allergens. These enzymes can degrade tight junctions and increase airway epithelial permeability [80] but, significantly, are not inhibitable by the major endogenous inhibitors [81]. If these proteases play a role in the development of allergic inflammation then they would be quite difficult to target. The substrate specificity for a number of these proteases has been defined [11], but further studies are needed to identify specific inhibitors and ways to intervene.

While most studies and inhibitors have addressed the activity of the Der p 1 cysteine protease, there are many serine proteases and aspartic proteases within the allergen families. For house dust mite for example serine proteases, such as Der p 3 and Der p 9 have been shown to have effects through activation of PAR-2 [32, 78], a subject that will be discussed in detail in the next section.

Various serine protease inhibitors have been shown to be effective in animal models of allergic airway disease [62, 67]. In most cases the data indicate that these inhibitors block endogenous serine proteases released following exposure to allergens. However, some of these inhibitors may also attenuate the enzymatic activity of the allergens and, in this way, intervene at two separate points in the allergic inflammation cascade.

Unfortunately, in contrast to endogenous proteases, where much work has focused on designing specific inhibitors as well as isolation of endogenous inhibitors, little research has been published on the substrate specificity or potentially effective inhibitors of allergen associated serine proteases.

Targeting the Function of Serine Protease Receptors (PAR-2)

This is a relatively new field and the literature is limited. The current experimental evidence from murine models indicates that the system offers a lot of promise and the biotech industry has taken notice. A number of patents to use inhibitors of PAR-2 activation for treatment of diseases including airway inflammatory diseases have been approved.

The cardinal problem is that there is currently no sufficiently effective method for inhibiting PAR-2 *in vivo*. However, recently various novel methodologies have been tested, primarily *in vitro*, and the results have been relatively encouraging. Antisense and siRNA have been proven effective in various *in vitro* systems [82, 83] and there is limited evidence that they can be effective also *in vivo* in murine models of arthritis [84], and in models of immunity to tumors [85]. Other interesting developments include a cell permeable peptide that inhibit the function of the receptor [86] and a new small molecule PAR-2 antagonist that has recently been shown to be effective in arthritis [84, 87]. Given the recent increased interest in this field further developments should be forthcoming.

It will also be important to address the potential side-effects of PAR-2 inhibition. PAR-2 activation has acute bronchodilatory effects [88] and in some models PAR-2 activation has been associated with anti-inflammatory responses [89]. However, this appears to be the result of PAR-2 activation with very high doses of synthetic peptide agonists and its *in vivo* relevance is not clear.

There are only two *in vivo* studies we could identify in which PAR-2 was activated in humans [90, 91]. These did

not show significant side effects, but the activation was local and there was no study of potential systemic effects. Also there are no results on systemic inhibition of PAR-2 and the potential of that approach for serious side effects. However, as studies in animals show more encouraging results, human studies will follow and thus the presence or absence of toxicity of the method will require greater scrutiny.

OCCUPATIONAL ALLERGY

A field that is often overlooked when the immune mechanisms of allergic sensitization are discussed is occupational allergy. Occupational allergy is a very important and growing problem for a number of industries with many public health consequences [92]. Enzymes are some of the most common allergens associated with occupational allergy and asthma.

Many industrial fields use enzymes in their manufacturing practises. The detergent industry is the major user of alkaline enzymes, such as proteases, α -amylases, lipase and cellulases [93]. The high rate of allergic sensitization seen in the detergent industry is primarily sensitization to these enzymes [94]. The most potent enzymes used and associated with allergy are high alkaline proteases, which are serine proteases and belong to a subfamily of subtilisins. The first enzyme used was subtilisin Carlsberg, which was isolated from *Bacillus licheniformis*. Since then many other similar enzymes have been isolated and newer ones developed with genetic engineering. In addition to the enzymes used in the detergent industry, a number of allergens from fungi [95, 96], but also from foods [97], belong to the family of subtilisins. There is not a lot of information on the role of the protease activity of these allergens in human, but it should be

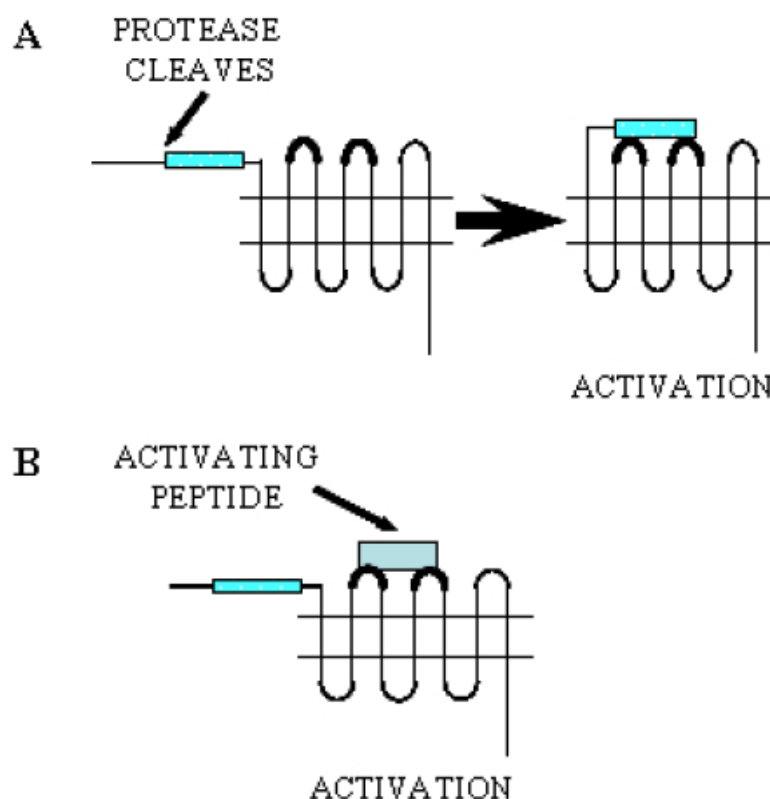


Fig. (1). Mechanism of PAR-2 activation by serine proteases (A) and PAR-2 activating peptides (B).

expected that the protease activity should be important for allergic sensitization. It is also possible that these enzymes mediate sensitization to other occupational allergens such as protein solubilized during manufacturing processes, as has been shown in murine systems for sensitization to protein antigens [19, 20], and avoidance of exposure to enzymes could have prevented sensitization to other allergens.

Sensitization to protease allergens accounts also for a significant part of occupational allergy in bakers [98]. In the baking industry, as in the detergent industry, enzymes are added in the raw material and become airborne during the manufacturing process. There are also reports of proteases used in specific occupations by small groups of people that cause high rates of allergic sensitization [99].

The recommendations for prevention of occupational asthma stress avoidance and generally environmental measures to reduce exposure to airborne enzymes and other allergens as the best way to prevent or treat the disease. There are no studies on the potential therapeutic role of inhibiting the function of the various enzymes after they become airborne or after they are inhaled. Although this approach is not easy it could be very useful for individuals with extreme sensitivity and could also be used to prevent allergic disease due to other aeroallergens with protease activity.

There are also no studies on the specific characteristics of enzymes required for their industrial use versus those characteristics that mediate sensitization. Identification of differences between these properties could be exploited to manufacture enzymes that would still be effective for their industrial use but not have the ability to sensitize the workers that inhale them. However our poor understanding of the enzymatic pathways that mediate allergic sensitisation hampers such an endeavour.

CONCLUSIONS

New approaches are needed for the treatment of allergic diseases. The protease activity of allergens is clearly very important both for allergic sensitization and the development of allergic airway inflammation in animal models. Although studies are limited, the same seems to be the case in human disease. Therefore, the approaches described here may prove useful in counteracting many of the effects of acute and chronic allergen exposure.

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