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SERIES "MATRIX METALLOPROTEINASES IN LUNG HEALTH AND DISEASE"
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Matrix metalloproteinases in COPD

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ABSTRACT: There is considerable evidence that matrix metalloproteinases (MMPs) are up- and/or downregulated in chronic obstructive pulmonary disease (COPD), particularly in emphysema, in which they probably participate in proteolytic attack on the alveolar wall matrix. Recent data suggest that MMPs also have major roles in driving inflammation or shutting it down, as well as modifying the release of fibrogenic growth factors, processes that are important in the genesis of the various lesions of COPD. In cigarette smoke-induced animal models of emphysema, MMP-12 appears to play a consistent and important role, whereas the data for other MMPs are difficult to interpret. In human lungs, evidence for a role for MMPs is more tenuous and there are numerous contradictions in the literature. Little is known about the effects of MMPs in small airway remodelling, smoke-induced pulmonary hypertension and chronic bronchitis, but MMP-12 participates in experimental small airway modelling. To date, the accumulated data suggest that selective inhibition of MMP-12 might be a viable therapy for emphysema and small airway remodelling, but subtle differences in the functions of MMP-12 in animals and humans mandate caution with this approach. Whether inhibition of other MMPs might be useful is unclear.

KEYWORDS: Cigarette smoke, chronic obstructive pulmonary disease, emphysema, matrix metalloproteinases, matrix metalloproteinase-12, small airway remodelling

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The lung matrix is a complex network of proteins and glycoproteins that includes multiple types of collagens, elastin, fibronectin, laminin, and several heparin and sulfate proteoglycans. The matrix is usually considered only as a support for cells and vessels, but it also has a major function as a storage reservoir for cytokines and growth factors, such as transforming growth factor (TGF)-β. Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade the matrix components both in normal physiological states and in abnormal pathological processes. MMPs have a complex relationship with cytokines and growth factors; they can both activate and deactivate these effector molecules and, conversely, some cytokines can cause secretion of MMPs or their activation [1]. Most MMPs are secreted as latent pro-enzymes and need to be activated by proteolytic conversion [1, 2], emphasising the fact that MMPs generally do not act alone but interact with other types of proteases.

The idea that inflammatory cells and their proteases play a role in chronic obstructive pulmonary disease (COPD) originated with the observations of P. Gross, who demonstrated that instilled elastases could produce emphysema in experimental animals. This idea was formally encoded as the protease–antiprotease hypothesis, which states first that smoke evokes an infiltrate of inflammatory cells in the lower respiratory tract, secondly that the inflammatory cells release proteases, and thirdly that these proteases degrade the alveolar wall matrix, leading to emphysema [3].

The effector cell in emphysema was originally believed to be the neutrophil and the major culprit, neutrophil elastase, but over the past 20 yrs it has been shown that MMPs, including MMP-1, -2, -7,

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-9 and -12 can degrade a variety of matrix components, including collagens and elastin, and the protease–antiprotease hypothesis has been, at least implicitly, reformulated to include MMPs. This idea was given explicit support by the report of HAUTAMAKI *et al.* [4], who demonstrated that MMP-12-/- mice did not develop emphysema after smoke exposure, and was further reinforced by the findings that a variety of broad spectrum MMP inhibitors prevent or significantly ameliorate emphysema in experimental animals (as has been recently reviewed [5, 6]; see also below).

While the importance of proteases in the genesis of emphysema is generally accepted, the possible role of proteases, and in particular MMPs, in the other anatomic and functional manifestations of COPD has received relatively little attention. Small airway remodelling is now firmly established as a cause of airflow obstruction in patients with COPD; indeed, it appears that in some patients with COPD, small airway remodelling rather than emphysema is the major cause of airflow limitation, although in most patients both small airway remodelling and emphysema play a role [7]. Cigarette smoke-induced pulmonary hypertension has also emerged as an important predictor of mortality in patients with COPD, and one that is probably independent of emphysema and small airway remodelling [8].

In this review, we will cover MMPs in emphysema, small airway remodelling and vascular remodelling associated with pulmonary hypertension, and make some short comments about chronic bronchitis. As will become evident, MMPs frequently function in concert with other types of proteases and these interactions will be described, to the extent they are known. At the same time it has become clear that MMPs perform a variety of signalling functions and can also interact with inflammatory chemokines to upregulate or downregulate inflammation.

A variety of experimental models of COPD exist but for the most part we will confine ourselves to cigarette smoke-induced models, since these appear to be the closest approximations to human disease, and many of the other models are of uncertain relevance. We have reviewed the general topic of proteases and emphysema fairly recently [5], and rather than repeat all of the contents of that review, here we will emphasise new developments.

MMPs IN EMPHYSEMA: MMP-12

Role of MMP-12 and other MMPs as mediators of inflammation in COPD

As noted in the introduction to this article, the central tenet of the protease–antiprotease hypothesis is that proteases released from smoke-induced inflammatory cells attack the alveolar wall matrix, leading to emphysema. While this is probably true in a global sense, the details are much more complicated than this simple formulation would indicate. In particular, there is increasing evidence that MMPs not only degrade matrix but are major players in initiating and maintaining, and perhaps also downregulating, inflammation after smoke exposure. Because MMP-12 has been most extensively investigated in animal models in this regard, we will consider this MMP first.

In experimental animals, exposure to cigarette smoke consistently upregulates MMP-12 production and release [9–24]; this phenomenon appears to be much more frequent than upregulation of any other MMP, although we make that statement with caution because MMP-12 has been examined much more frequently than other MMPs.

Interestingly, MMP-12 itself appears to be not only a direct cause of matrix degradation but also a pro-inflammatory substance: NÉNAN *et al.* [25] showed that instillation of the catalytic domain of recombinant human MMP-12 into mouse airways lead to an initial lavage neutrophilia, followed over time by an increase in macrophages, but no inflammatory response was seen if an inactive mutant form of the catalytic domain was used instead. The acute inflammatory response was accompanied by an increase in a variety of pro-inflammatory cytokines and MMP-9.

Tumour necrosis factor (TNF)-α is normally converted from its membrane-bound pro-form to the released active form by TNF-α converting enzyme (TACE), otherwise known as A disintegrin and metalloprotease (ADAM)-17, but several MMPs, including MMP -1, -2, -3, -7, -9 and -12 [26] have TACE-like activity *in vitro* against synthetic fusion proteins. We have previously shown that wild-type, but not MMP-12-/-, mice have increases in whole lung active TNF-α levels as well as neutrophils after cigarette smoke exposure, and that wild-type, but not MMP-12-/-, macrophages release TNF-α after *in vitro* cigarette smoke exposure, suggesting that MMP-12 actually functions as a form of TACE, and by releasing active TNF-α drives a whole host of pro-inflammatory reactions [26]. Along this line, LE QUÉMENT *et al.* [27] showed that MMP-12 can also cause production and release of interleukin (IL)-8 (CXCL8) from cultured epithelial cells *via* pathways involving epidermal growth factor receptor and extracellular-signal-regulated kinase (ERK)1/2 activation.

Knockout of MMP-12 protects mice against smoke-induced emphysema [4]; this process is accompanied by a marked decrease in lung macrophages. Treatment of guinea pigs with an MMP-9/MMP-12 inhibitor for 6 months of smoke exposure is similarly protective and is accompanied by major, but not complete, decreases in lavage neutrophils and macrophages [21]. In acute experiments, MMP-12-/- mice do not develop the usual lavage neutrophilia seen after cigarette smoke exposure, but the neutrophil influx can be restored by instillation of wild-type alveolar macrophages [26]. LE QUÉMENT *et al.* [28] showed that inhibition of the catalytic activity of MMP-12 with the selective inhibitor AS111793 in a 4-day acute model reduced lavage neutrophil numbers by about 50% and lavage macrophages by about 40%; this was associated with a reduction in soluble TNF receptors, macrophage inflammatory protein (MIP)-1γ, IL-6, KC, CXCL1, CXCL11, tissue inhibitor of metalloproteinase (TIMP)-1 and pro-MMP-9. In aggregate, these findings support the idea that MMP-12 functions as a direct mediator of inflammation (see further comments on this subject in the section entitled Differences between MMP-12 function in mice and humans and their implications for therapy).

In addition to direct release of pro-inflammatory mediators, proteolytic matrix attack by MMP-12 and other MMPs causes liberation of matrix fragments, and it has been known for many years that matrix fragments are in themselves pro-inflammatory [29, 30]. Elastin fragments specifically have been observed to attract monocytes but not mature alveolar macrophages or neutrophils [29, 30].

This idea has been examined in more detail recently. HAUTAMAKI *et al.* [4] found that macrophages did not accumulate in the lungs of MMP-12-/- mice after smoke exposure, although they could be induced to accumulate by administration of the chemoattractant chemokine monocyte chemotactic protein (MCP)-1 (CCL2), suggesting that the defect was not in the ability of MMP-12-/- macrophages to enter the lung, but rather a lack of a suitable chemoattractant. Monocytes express a surface elastin receptor, monocyte elastin binding protein. HOUGHTON *et al.* [31] showed that attack on mouse elastin by MMP-12 leads to liberation of elastin fragments containing the pentapeptides GXXPG or XGXPG, where X is a hydrophobic amino acid, and that an antibody that specifically recognises these sequences abolishes the chemotactic activity of the fragments toward monocytes *in vitro*, and ameliorates elastase-induced emphysema. A similar chemotactic sequence, VGVAPG, is found in human elastin. This finding suggests that a humanised antibody against such fragments might be useful as a treatment for COPD.

Other MMPs may, directly or indirectly, have similar pro-inflammatory roles. Recently, it has been reported that cigarette smoke exposure leads to release of the tripeptide proline-glycine-proline (PGP) from the lung matrix [32]. PGP functions as a neutrophil chemoattractant through binding to CXCR2, and repeated instillation of PGP produces emphysema in animal models. PGP is derived from collagen breakdown, and the release of PGP requires unmasking of the collagen motif by MMP-9, followed by actual excision of the peptide through the action of prolyl endopeptidase [32–35]. Interestingly, PGP is normally degraded by leukotriene A_4 hydrolase (LTR4H), limiting neutrophil influx, but cigarette smoke interferes with LTA4H-mediated degradation of PGP, while leaving intact the generation of leukotriene B_4, thus increasing neutrophilic inflammation in a positive feedback fashion [36].

Interactions of MMPs with their own cognate inhibitors as well as the inhibitors of other types of proteases can further modify the inflammatory response to cigarette smoke. Smoke-evoked neutrophils release the elastolytic serine proteases neutrophil elastase, proteinase 3 and cathepsin G. SHAPIRO *et al.* [37] showed that MMP-12 degrades α_1-antitrypsin, the major inhibitor of these enzymes, and especially of neutrophil elastase and, conversely, neutrophil elastase degrades TIMPs that normally inhibit MMP-12. This finding implies that neutrophil elastase and MMP-12 can interact to amplify the inflammatory response to smoke.

In aggregate, these observations indicate that, at least in mice, MMP-12 is central to a complex feedback loop (fig. 1), where smoke-mediated production of the enzyme can result in generation of neutrophil chemoattractants, matrix attack by proteases from chemoattracted neutrophils, matrix attack by MMP-9/prolyl endopeptidase liberating PGP and attracting more neutrophils, and matrix attack by MMP-12 itself, liberating macrophage-attracting matrix fragments (fig. 1). However, MMP-12 may also downregulate inflammation: we consider this idea in the section entitled Differences between MMP-12 function in mice and humans and their implications for therapy.

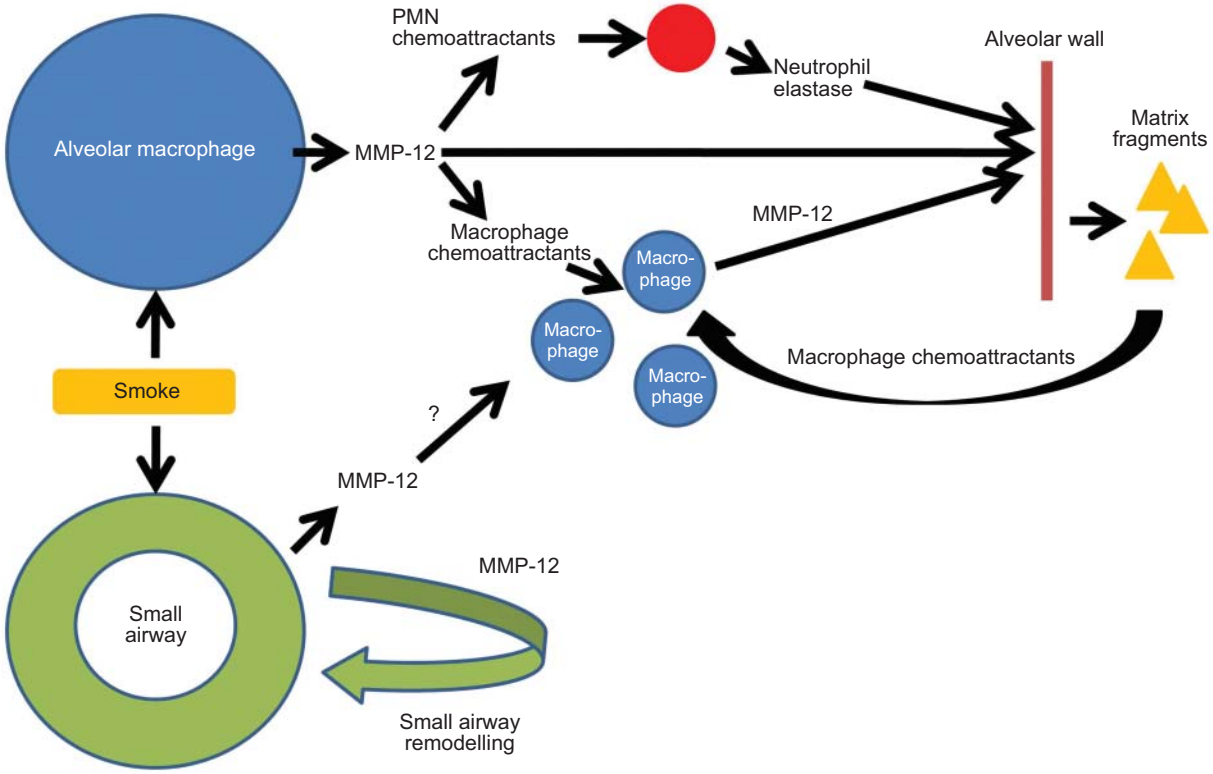

FIGURE 1. Effects of alveolar macrophage-derived and small airway-derived matrix metalloproteinase (MMP)-12. Macrophage-derived MMP-12 participates in attack on the matrix of the alveolar wall, leading to emphysema, and also amplifies the inflammatory response. The exact effects of small airway-derived MMP-12 in small airway remodelling are unclear, but it clearly has an important role, since knockout of MMP-12 or use of an MMP-9/-12 inhibitor prevents small airway remodelling in animal models. PMN: polymorphonuclear leukocyte.

What causes MMP-12 production and secretion after cigarette smoke exposure?

Given the potential importance of MMP-12 in COPD, it is of interest to determine what drives its production and release (fig. 2). Smoke exposure causes protein leakage from the serum into the alveolar spaces. Among these proteins are plasminogen and prothrombin, which can be converted to plasmin and thrombin; the latter activate proteinase activated receptor-1 (PAR-1) and this leads to both secretion of preformed MMP-12 protein and activation of the secreted protein [38]. We found that this process could be demonstrated *in vitro* using smoke-conditioned medium and alveolar macrophages [22]: smoke caused the macrophages to secrete tissue factor, which in turn activated plasminogen and prothrombin, and plasmin and thrombin activated PAR-1. Plasmin and thrombin are serine proteases and α_1-antitrypsin, a serine protease inhibitor, blocked MMP-12 release, illustrating yet another potential interaction of MMPs and other proteases in the pathogenesis of emphysema.

BOTELHO *et al.* [10] showed that smoke upregulated production of granulocyte macrophage colony-stimulating factor (GM-CSF) in mice and that an antibody to GM-CSF or its receptor prevented smoke-mediated increases in MMP-12 release, while at the same time decreasing neutrophil and dendritic cell influx. Similar results using anti-GM-CSF antibodies were reported by VLAHOS *et al.* [18], who also noted that antibody treatment decreased production of TNF-α and the chemokine MIP-2, without decreasing MMP-9 production.

The tachykinins substance P and neurokinin A are present in sensory nerves, macrophages and dendritic cells. DE SWERT *et al.* [14] found that mice lacking the tachykinin NK1 receptor were protected against smoke-induced emphysema and showed decreased production of MMP-12 after smoke exposure. Alveolar macrophages express the neurokinin-1 receptor, and XU *et al.* [39] showed that treatment of alveolar macrophages with substance P greatly increased MMP-12 gene expression.

KASSIM *et al.* [40] observed that mice lacking gp91(phox), a component of NADPH oxidase, developed spontaneous airspace enlargement, but mice lacking both NADPH oxidase and MMP-12 did not. GP91(phox)-/- and wild-type macrophages produced equal amounts of MMP-12 protein, but the gp91(phox)-/- macrophages had greater MMP-12 activity, suggesting that oxidants derived from NADPH oxidase normally downregulate the activity of MMP-12; loss of this downregulation leads to matrix attack and emphysema.

Increased circulating levels of surfactant protein-D (SP-D) have been reported in patients with COPD [41]. TRASK *et al.* [42] noted that SP-D induces the secretion of MMP-1, MMP-3 and MMP-12 from isolated human alveolar macrophages. However, the role of SP-D *vis a vis* MMP-12 is uncertain, because YOSHIDA and

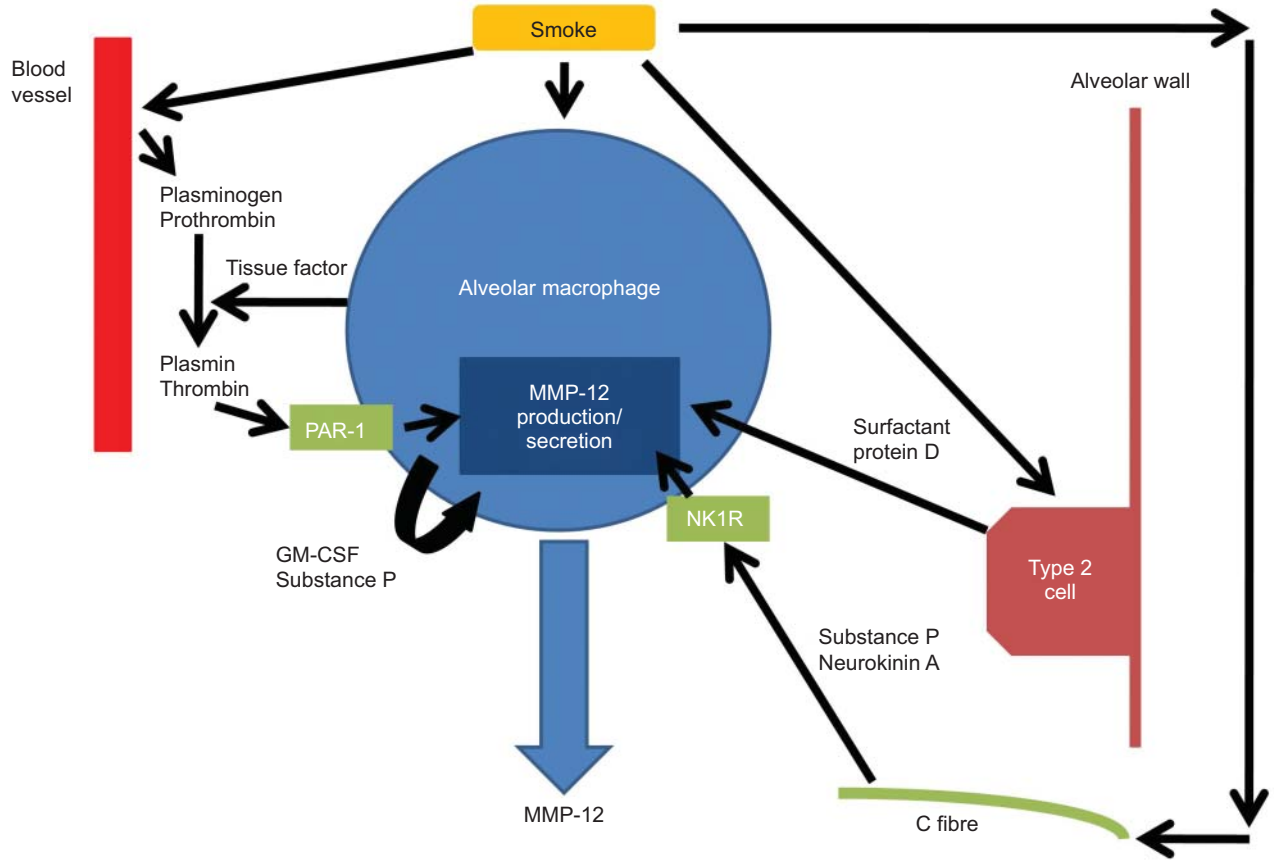

FIGURE 2. Factors that control matrix metalloproteinase (MMP)-12 production and secretion by alveolar macrophages after smoke exposure. Because of the numerous different mediators that drive MMP-12 production, inhibition of activity of the enzyme appears to be an easier approach than trying to block production. PAR-1: proteinase activated receptor-1; NK1R: neurokinin-1 receptor; GM-CSF: granulocyte macrophage colony-stimulating factor.

WHITSETT [43] found that SP-D-/- mice developed spontaneous emphysema and this was associated with increased production of MMP-2, -9 and -12, and was mediated by oxidant activation of nuclear factor-κB.

TNF-α and IL-1β are upregulated in human cigarette smokers and in animal models of smoke-induced COPD. LAPPALAINEN *et al.* [44] found that transgenic IL-1β over-expressing mice developed emphysema as well as small airway remodelling, along with increases in production of MMP-12, MMP-9 and a variety of neutrophil chemoattractants. Similarly, VUILLEMENOT *et al.* [45] showed that inducible transgenic TNF-α over-expresser mice developed emphysema along with increased gene expression of MMP-12 and a variety of pro-inflammatory chemokines.

Perhaps the most interesting finding in regard to regulation of MMP-12 is its connection to TGF-β1 activity. MORRIS *et al.* [46] showed that mice lacking the integrin $\alpha_v\beta_6$ fail to activate latent TGF-β1 and also express greatly increased levels of MMP-12 and develop emphysema. Emphysema does not develop in $\alpha_v\beta_6$-/-/MMP-12-/- mice, indicating that it is specifically MMP-12 that drives this process. TGF-β signals through phosphorylation of Smad3, and BONNIAUD *et al.* [47] noted that Smad3-/- mice express elevated levels of both MMP-9 and MMP-12 and develop spontaneous emphysema.

These observations suggest a number of potential interventions that could prevent MMP-12 release and/or activity. However, MMP-12 production is clearly complex and can be driven through a variety of pathways (fig. 2); given this variety, MMP-12 inhibition appears to be more likely to be useful than any attempt to selectively prevent its production and secretion. The $\alpha_v\beta_6$-/- and Smad3-/- mouse data also caution against attempts to interfere with TGF-β signalling as a way to prevent small airway remodelling (see below); such attempts might be successful, but could worsen emphysema, assuming, of course, that the same relationship of TGF-β signalling and MMP-12 release occurs in humans.

Does MMP-12 participate in human COPD?

Although the experimental animal showing a role for MMP-12 in emphysema are compelling, existing human data are hard to interpret. IMAI *et al.* [48] reported that MMP-12 was not upregulated in smokers and MMP-12 mRNA could not be found in most normal lungs. FINLAY *et al.* [49] cultured alveolar macrophages from emphysematous and control lungs, and found increases in production of MMP-9, but no differences in mRNA levels of MMP-2 and MMP-12, and no production of MMP-12 protein. LAPAN *et al* [50] pointed out that MMP-12 levels in induced sputum tend to be relatively low and developed highly specific assays to detect it; they were able to find MMP-12, but could not identify differences between control and COPD patients. GOSSELINK *et al.* [51] could not find any associations with parenchymal MMP-12 gene expression and Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage. LEE *et al.* [52] investigated the potential association of single nucleotide polymorphisms (SNPs) in MMP-1, MMP-9 and MMP-12 with COPD in a Korean population and found that only the MMP-9 -1562C→T showed a (protective) correlation; the MMP-12 N357S SNP was not associated with differences in COPD frequency. SCHIRMER *et al.* [53] examined MMP-3, -9 and -12 SNPs in a Brazilian population and failed to find any associations with COPD, although their sample size was quite small.

Conversely, ILUMETS *et al.* [54] looked at MMP-8, MMP-9 and MMP-12 levels in the sputum of COPD stage 0 and asymptomatic smokers and found that MMP-8 levels distinguished the two groups, but MMP-12 levels were also higher in stage 0 smokers compared to nonsmokers, and MMP-12 could be detected by immunohistochemistry in alveolar macrophages. DEMEDTS *et al.* [55] reported increased levels of sputum MMP-12 in COPD subjects compared to healthy smokers, ex-smokers, and never smokers. WOODRUFF *et al.* [56] used microarrays to examine alveolar macrophage gene expression in 15 smokers, 15 nonsmokers, and 15 subjects with asthma, and found a nine-fold increase in MMP-12 expression in the smokers. BABUSYTE *et al.* [57] found that the numbers of MMP-12 positive macrophages in lavage fluid was higher in current smokers with COPD than in ex-smokers with COPD or healthy smokers and never-smokers, and WALLACE *et al.* [58] also found higher levels of MMP-12 in the macrophages of current smokers compared to ex-smokers. VAN DIEMEN *et al.* [59] looked at SNPs in MMP-1,-2, -9 and -12, along with TIMP-1, in two cohorts; only TIMP-1 Phe158Phe was associated with loss of forced expiratory volume in 1 s (FEV_1).

HUNNINGHAKE *et al.* [60] investigated the association of SNPs in the MMP-12 gene in seven cohorts comprising 8,300 subjects and two COPD cohorts, and found that the minor variant rs2276109 (-82A→G) in the MMP-12 promoter was associated with a reduced risk of COPD. HAQ *et al.* [61] genotyped 26 SNPs in MMP-1, -9 and -12 in roughly 900 European COPD patients and 900 non-COPD smokers, and found that the A-A haplotypes of MMP-12 SNPs rs652438 and rs2276109 were associated with a risk of developing severe disease, while individuals with the minor G variants at either SNP had a lower risk. JOOS *et al.* [62] examined 590 continuing smokers and divided them into rapid and slow loss of function groups; they found that haplotypes consisting of alleles from the MMP-1 G-1607GG and MMP-12 Asn357Ser polymorphisms were associated with the rate of decline; they could not find an association with MMP-9 SNPs.

Differences between MMP-12 function in mice and humans and their implications for therapy

Given the clear role and apparently central location of MMP-12 in animal models of smoke-induced COPD, the demonstrable benefits of MMP-12 inhibition/knockout in mice and guinea pigs, and existence of some at least suggestive human data, inhibition of MMP-12 would appear to be attractive target for treating human disease. However, there are subtle differences in human and mouse MMP-12 function that need to be considered. Murine MMP-12 has a pro-inflammatory function through activation of the neutrophil chemoattractant CXCL5 (ENA-78) [63], so that in mice inhibition or knockout prevents lipopolysaccharide (LPS)-induced lung neutrophilia. However, human MMP-12 is not pro-inflammatory. Rather, following an initial neutrophil influx, human MMP-12 switches off neutrophil recruitment by inactivating a variety of CXC chemokines (CXCL1, 2, 3, 5, 6 and 8). This property is unique to MMP-12 and is not seen with macrophage-derived MMP-1 or MMP-9. In addition, fibroblast and epithelial cell derived MMP-1, 2, 3, 13 and 14, as well as MMP-12, inactivate macrophage chemoattractant chemokines (CCL2, 7, 8 and 13), downregulating

macrophage influx. On the basis of these findings, DEAN *et al.* [63] have proposed that the major role of human MMP-12 in innate immunity is actually to terminate neutrophil influx.

The experiments of DEAN *et al.* [63] were performed using LPS as the pro-inflammatory agent, but their observations appear to explain our previous findings that MMP-12-/- mice do not develop a lavage neutrophilia after cigarette smoke and that the neutrophil response can be restored by intratracheal instillation of wild-type alveolar macrophages [64].

Additionally, HOUGHTON *et al.* [65] have shown that MMP-12 has an important role in killing of bacteria within murine macrophages; MMP-12-/- mice showed impaired bacterial clearance and increased mortality with Gram-positive and Gram-negative experimental infections. Interestingly, this effect is not mediated by the catalytic domain of the enzyme but rather by the carboxy terminal domain.

These observations imply that inhibiting MMP-12 might be contra-indicated in treating human COPD because such therapy could lead to increased neutrophil-mediated matrix destruction, and might or might not lead to increased lung infections, depending on whether an inhibitor selectively targeted the catalytic domain. However, HOUGHTON *et al.* [31] have suggested that MMP-12 attack on the alveolar wall matrix may be more important than attack by neutrophil-derived serine proteases simply as a matter of relative cell number, so that inhibition of MMP-12 should provide substantial protection against emphysema.

Thus, at this point it is unclear whether MMP-12 inhibition would be beneficial in treating COPD, quite apart from issues of potential drug-related toxicity. Some suggestive evidence in favour of MMP-12 inhibition therapy comes from our study of guinea pigs using an MMP-9/-12 inhibitor, where the inhibitor prevented smoke-induced inflammation, emphysema, and small airway remodelling [21], although it was obviously not possible to distinguish the role of MMP-9 *versus* that of -12, nor is it known whether guinea pig MMP-12 enhances or decreases neutrophilia. A recently published 6-week human trial using an MMP-9/-12 inhibitor, AZD1236 reported no decrease in inflammatory mediators, but no increase either [66].

EMPHYSEMA: ROLE OF OTHER MMPs

Collagen in emphysema

Although MMP-12 is an elastase, most MMPs have collagenolytic activity. The role of matrix collagen in emphysema is underappreciated. HOGG *et al.* [67] demonstrated physiologically that the walls of emphysematous spaces were actually stiffer than the adjacent lung tissue, and other workers have shown increased collagen content in emphysema [68–70]. Ultrastructural studies in humans [71] and animals [72] demonstrated changes in collagen in emphysema, and suggested that there was a dynamic alteration of the collagen matrix during the genesis of emphysema, a hypothesis that continues to be developed [73].

MMP-1

MMP-1 is also known as interstitial collagenase-1. It has an active molecular weight of 41 kDa, and is active against collagens I, II, III, VII, X, gelatin, pro-MMP-2 and pro-MMP-9. In the lung, it is secreted by bronchial epithelial cells, type II pneumocytes and alveolar macrophages.

In 1992, D'ARMIENTO *et al.* [74], using a haptoglobulin promoter, developed a transgenic mouse model that overexpressed human MMP-1 in the lung and developed emphysema shortly after birth. This provided evidence that the genesis of emphysema could be related to more than just elastin degradation by elastase, and brought attention to the importance of the lung matrix as a whole in COPD. Using a heterozygous line from this transgenic model, the authors also demonstrated late development of emphysema at 12 months, with a decrease in type III collagen by immunostaining and increases in lung compliance [75]; they suggested that loss of collagen III rather than collagen I was a major determinant of emphysema.

Increased whole lung MMP-1 mRNA and protein has been demonstrated in human and guinea pig emphysema [48, 76, 77], with increased immunoreactivity found in macrophages and in airway epithelium. GOSSELINK *et al.* [51] reported that MMP-1 gene expression increases in the parenchyma from GOLD stage 0 to 3/4, although the magnitude of the difference overall was fairly small. Similar changes have been reported in guinea pigs exposed to cigarette smoke [78], although there is dispute about whether guinea pigs actually have an MMP-1 that is comparable to the human enzyme. Mice lack MMP-1; the closest equivalent is MMP-13 and this is upregulated by cigarette smoke [79].

Cigarette smoke induced increases in MMP-1 appear to be driven primarily through mitogen activated protein kinase and specifically ERK pathways, although H_2O_2, a component of cigarette smoke, can increase MMP-1 expression through an ERK-independent pathway [80]. Mice exposed *in vivo* to cigarette smoke and human emphysematous lungs both have increased immunohistochemical expression of phospho-ERK in the airway epithelium and alveolar pneumocytes. Further work has demonstrated that induction of MMP-1 by cigarette smoke is regulated at the promoter site, and ERK1/2 signalling is required. In addition, the proximal binding sites for transcription factor activator protein 1 (AP-1) is required for both baseline and cigarette smoke induced activation of the promoter [81].

MMP-9

MMP-9 is also known as gelatinase B, and has an active molecular weight of 85 kDa. It has multiple potential substrates, including collagens IV, V, VII, X and XIV, gelatin, elastin, and pro-MMP-9 and -13 [1]. MMP-9 is secreted by bronchial epithelial cells, neutrophils, eosinophils, mast cells and alveolar macrophages. In the airway epithelium, serine protease activated protease-sensitive receptors induce the release of MMP-9, suggesting a possible additional relationship with neutrophils. MMP-9 expression is induced by IL-13. MMP-9 activates latent TGF-β, which then has a reciprocal role in activating production and secretion of MMP-9 [1, 2, 82]. A further feedback loop exists between IL-8, which induces the secretion of MMP-9, which then amplifies release of MMP-9 in addition to cleaving IL-8 into a more active form [83]. IL-1β and TNF-α are inhibitory for MMP-9 production, although the combination of PDGF, IL-1β and TNF-α induces MMP-9 activity.

There are a number of human studies which have searched for relationships between COPD and MMP-9. Plasma levels of MMP-9 appear to be increased in α_1-trypsin deficiency-associated emphysema and in COPD [84, 85], and were shown to correlate negatively with FEV_1, carbon monoxide transfer factor

and oxygen saturation, and were also able to predict both a decline in pulmonary function and numbers of exacerbations [84]. Induced sputum analysis has found increases in MMP-9 protein and MMP-9 activity [83, 85] in smokers with and without airflow obstruction. Interestingly, MMP-9 was increased in the sputum after smoking cessation compared to baseline, and this was not related to the numbers of neutrophils present [86]. Increased immunohistological staining of MMP-9 has been identified in the lungs of smokers with COPD [87] and in guinea pigs exposed to cigarette smoke [78]. Alveolar macrophages from cigarette smokers have been shown to release greater baseline and stimulated amounts of MMP-9 [88].

PCR analysis on bulk lung samples has shown upregulation of MMP-9 in smokers with severe COPD, with negative correlations with FEV_1, diffusing capacity of the lung for carbon monoxide and arterial oxygen tension, and positive correlations with arterial carbon dioxide tension [76]. GOSSELINK *et al.* [51], using laser capture microdissection, found upregulation of gene expression for MMP-9 in the parenchyma that correlated with the progression of GOLD categories of COPD. A separate microarray study showed increased expression of urokinase plasminogen activator and its receptor, genes involved in upregulation of MMPs [89], in patients with COPD.

A number of animal studies have shown increases in MMP-9 levels that accompany increases in MMP-12, as discussed above. In mice, VLAHOS *et al.* [17] found that MMP-9 increased after exposure to 9 cigarettes for 3 days, and there was a relative dose-dependent increase in MMP-9 in BALB/c mice exposed to 3–9 cigarettes for 4 days. FORONJY *et al.* [90] showed that transgenic overexpression of human MMP-9 in mouse alveolar macrophages leads to slowly developing emphysema, and that this process was associated with loss of parenchymal elastin but not collagen. As described in the section on MMP-12, we were able to prevent emphysema by treating guinea pigs with an MMP-9/-12 inhibitor [21], but we could not determine which of the two enzymes was playing a role.

Rather different results were seen with MMP-9 knockout mice. These animals have abnormal soft tissue collagen fibres with abnormal wound healing [91]. However, ATKINSON *et al.* [92] found that these mice had a similar inflammatory response profile and developed the same severity of cigarette smoke induced emphysema as did strain-matched wild-type mice. This study also evaluated MMP-9 in the lungs of humans with severe emphysema and demonstrated that macrophages were the primary source of MMP-9, but MMP-9 mRNA levels did not correlate with markers of continuing lung damage or with local degrees of emphysema. We consider these findings further, below.

Other MMPs

MMP-2 is also known as gelatinase A and has an active molecular weight of 66 kDa. Collagens I, II, III, IV, V, VII, X, XI and XIV serve as substrates, in addition to gelatin, elastin and fibronectin [1]. MMP-2 is secreted by bronchial epithelial cells and by airway smooth muscle cells where it is an important modulator of cell proliferation [1, 2]. SEGURA-VALDES *et al.* [87] described increased immunorreactivity for MMP-2 in the lungs of patients with COPD, mainly in alveolar macrophages and airway epithelial cells. This observation supports other studies in which increased expression and activity related to MMP-2 was identified in the lungs and sputum of patients with COPD [2]. We found increased MMP-2 protein [79] and MARCH *et al.* [93] observed increased MMP-2 activity in mice exposed long term to cigarette smoke. Interestingly, MMP-2 (and MMP-9) mRNA upregulation and increased protein in lavage and lung tissue has also been identified in emphysema induced by wood smoke [94].

In contrast, a study using laser capture microdissected samples of human parenchyma found that MMP-2 gene expression levels decreased with increasing GOLD stage, and MMP-2 protein levels correlated with gene expression levels [51]; of note, TIMP-1 expression decreased roughly in parallel.

MMP-8 and MMP-13 are collagenases with active molecular weights of 64 and 55 kDa, respectively, both sharing collagens I, II and III, and gelatin as substrates. There are few data on MMP-13 in COPD. We found that it was upregulated in mice by cigarette smoke with long-term exposure [21]. LEE *et al.* [95], using proteomic analysis of lung tissue, found upregulation of MMP-13 in the lungs of COPD patients. Expression was confirmed by immunohistochemistry, which showed staining of macrophages and type II alveolar epithelial cells. However, no differences in MMP-13 gene expression were found in human lung parenchyma across the different GOLD stages [51].

Data are also relatively sparse in relation to MMP-8. Increased MMP-8 has been identified in induced sputum [83], particularly during exacerbations [96] and was localised to neutrophils and macrophages. Levels of MMP-8 have been shown to correlate with airflow obstruction [83].

MMP-10 (stromelysin-2) has been little studied, but GOSSELINK *et al.* [51] found an increase in gene expression levels in the parenchyma with increasing GOLD stage.

ADAM-33 is a metalloprotease of unknown function. SNPs in the ADAM-33 gene have been strongly associated with asthma, and there is some suggestion that such SNPs are also associated with COPD [97–99]. GOSSELINK *et al.* [51] could not find any differences in ADAM-33 gene expression in the parenchyma in the various GOLD stages, although there were significant decreases in expression levels in the airways with increasing functional deficits.

EFFECTS OF THERAPY ON MMPs IN COPD

In humans, therapeutic endeavours such as steroids have shown no effect on sputum MMP-9 or MMP-1 levels [100] and in fact it has been claimed that, in rats, steroids can rapidly induce emphysema *via* an MMP-9 dependent pathway [101]; however, this finding has not been replicated in studies using steroids in mice or guinea pigs exposed to cigarette smoke.

A study of cigarette smoke-exposed guinea pigs [78] showed increases in MMP-9, accompanied by modest (30%) amelioration of emphysema by administration of CP-471,474, a broad-spectrum MMP inhibitor, but one with limited effectiveness against MMP-1. In mice, inhalation of GM6001 [102], another broad-spectrum MMP inhibitor, as well as systemic administration of RS113456 and RS132908l [103], also broad-spectrum MMP inhibitors, all provided quite significant protection against emphysema, as did the dual MMP-9/-12 inhibitor, AZ11557272, in guinea pigs [21].

The general topic of MMP inhibitors as therapeutic agents and the potential benefits and problems with such an approach is discussed in detail by VANDENBROUCKE *et al.* [6].

CONCLUSIONS REGARDING MMPs AND EMPHYSEMA

If one believes that proteolytic attack is the driving force behind emphysema (and not everyone does [104]), then MMPs are logical candidates to examine, since they can degrade most of the components of the alveolar wall matrix. However, the published data are confusing and often contradictory, and are complicated by differences in human lungs compared to animal models, the exact choice of tissue examined in molecular and biochemical assays, as well as the method of examination, and the choice of SNPs and populations for the genetic assays. Furthermore, genetic studies for the most part deal with COPD as an entity, rather than breaking it down into emphysema *versus* small airway remodelling. Since some patients predominantly have emphysema, some predominantly have small airways disease, and many have both, as noted above, it is entirely possible for an association to be present with only one of these lesions.

Therefore, determining which MMPs play a role in emphysema and what that role might be is extremely difficult; in particular, it is not clear what are epiphenomena and what events are directly relevant. It appears that many different MMPs are upregulated in the parenchyma, often in alveolar macrophages, both in humans with emphysema and in cigarette smoke-induced mouse and guinea pig models; and it is also clear that, in mouse models, overexpression of some MMPs, for example, MMP-1 and MMP-9, can produce emphysema, even without the presence of smoke [74, 90]. However, the lack of protection against emphysema seen in MMP9-/- mice [92] suggests that overexpression models are probably less informative than knockout models.

The human data are equally contradictory; for example, as previously noted, GOSSELINK *et al.* [51] suggested that the increased expression of MMP-9 in the parenchyma as GOLD stage increased supported a direct role for MMP-9 in human emphysema, but ATKINSON *et al.* [92] could not find increased MMP-9 gene expression that correlated with emphysema severity. The same situation may apply to MMP-1, and here the lack of a murine MMP-1 makes evaluation of the role of MMP-1 more difficult. We have discussed MMP-12 at length above, and this enzyme appears to be the most likely candidate for a direct role in emphysema, but the human data are far from unanimous.

An additional complication in interpreting the published data is that the exact role of MMPs in any given setting is not obvious. MMPs might directly degrade alveolar matrix, but it is clear that they can also serve primarily as signalling molecules, and there is considerable support for this idea in regard to MMP-12, as discussed in the section entitled Differences between MMP-12 function in mice and humans and their implications for therapy. A similar role has been proposed for MMP-2, which is able to cleave MCP-3 in such a fashion as to downregulate inflammation [105]. GOSSELINK *et al.* [51] suggest that their finding of downregulation of MMP-2 in the parenchyma with increasing GOLD stage could, thus, be a cause of persisting inflammation.

Since mouse, rat, and guinea pig models of chronic smoke exposure produce mild forms of emphysema, as well as small airway remodelling and vascular remodelling associated with pulmonary hypertension, *i.e.* since they reproduce the changes of human COPD, it is likely that the underlying mechanisms are largely the same in animals and humans, at least for early disease, and the animal models should be accorded considerable weight. The fact that knockout of selective MMPs and MMP inhibitors can completely or largely ameliorate emphysema in animal models is strong evidence that MMPs play an important role in human disease. However, as described, there are some documented subtle but important differences in the exact functions of murine MMPs (little has been studied about the details of rat and guinea pig MMPs) and human MMPs, and these differences may be extremely important when considering the role of a given MMP and, particularly, in selecting an MMP to inhibit with a synthetic inhibitor.

MMPs IN SMALL AIRWAY REMODELLING

Small airway remodelling is characterised the development of fibrotic, thick-walled and distorted small airways (bronchioles), accompanied by mucus metaplasia of the epithelium, mucus plugs in the lumens, and a chronic inflammatory infiltrate in the airway walls. Although small airway remodelling is an important cause of airflow obstruction, and the predominant cause in some patients, very little is known about its pathogenesis, and the scanty published data on MMPs are difficult to interpret.

MMP-12, MMP-9, and MMP-2 are normally produced in the airways: MMP-12 in both airway epithelial cells and airway smooth muscle cells [106, 107] and MMP-9 in airway epithelial cells [108]. MMP-2 is found in bronchial epithelial cells and airway smooth muscle cells and may be induced by TGF-β or release active TGF-β, depending on the model. MMP-9 may play a role in bronchiolar epithelial cell migration and may also release TGF-β from the matrix [108]. The usual function of MMP-12 is obscure, although the observations of DEAN *et al.* [63] suggest that it might serve to modulate inflammation. Another possibility is that it controls release of pro-inflammatory cytokines, such as TNF-α (see section entitled Differences between MMP-12 function in mice and humans and their implications for therapy). Gene expression data indicate that MMP-10, ADAM-33 and TIMP-1 are also present in human small airways [51], and in rats MMP 7, 16, 19 and 28 are normally present in the tracheal wall (used as a model of a large airway) [109].

Tracheal explants are free of exogenous inflammatory cells and thus can be used to model intrinsic events in the airways. Using this model, we found that acute *in vitro* smoke exposure significantly upregulated gene expression of MMP-10 (stromelysin-2), 19 and 28, and downregulated MMP-7 (matrilysin) and -16, but MMP-12 was not significantly changed [109]; these findings suggest that smoke can act directly on the cells of the airway wall to modulate expression of some MMPs, but changes in production of other MMPs probably require signalling from either evoked inflammatory cells or other lung compartments. However, LAVIGNE *et al.* [107] were able to induce gene expression of MMP-12 using cigarette smoke in primary normal human bronchial epithelial cells; they showed that the pathways involved included NADPH oxidase, AP-1 and TNF-α, and appeared to be dependent on smoke-induced production of hydrogen peroxide.

Somewhat different results are present in long-term (6 months) *in vivo* models. We have observed, using laser captured microdissected samples, that, compared with air-exposed mice, MMP-12 is markedly upregulated in the small airways after chronic cigarette smoke exposure and MMP-9 is downregulated (fig. 3). MMP-12 knockout mice are completely protected against small airway remodelling (fig. 4), and a combined MMP-9/12 inhibitor provided 100% protection against small airway remodelling in guinea pigs after 6 months of smoke exposure [21]. There do not appear to be any published data on small airway remodelling in MMP-9-/- mice.

However, human studies using laser capture microdissection have produced different findings. GOSSELINK *et al.* [51] failed to find an association of MMP-12 or MMP-9 gene expression in laser capture microdissected human small airways and GOLD stage but they did find that MMP-2 expression levels decreased with increasing GOLD stage, as did ADAM-33. Comparisons of gene expression to FEV1/forced vital capacity ratios are considerably different from comparisons of air- and smoke-exposed animals or comparisons of actual airway remodelling, and of course GOLD stage may reflect small airway remodelling and/or emphysema, so that it is difficult to correlate these results specifically with small airway remodelling.

Experiments using rat tracheal explants suggest that the fibrosis of cigarette smoke-induced small airway remodelling is driven, at least in part, by smoke-mediated release of TGF-β [110]. In this regard, the observation that MMP-12-/- mice are protected against small airway remodelling is in sharp contradistinction to the lack of protection of MMP-12-/- [111] (or MMP9-/- [108]) mice against bleomycin-induced fibrosis, even though bleomycin also upregulates MMP-12 (and MMP-9) production and TGF-β release [108, 111]. MMP-9 is required for alveolar bronchiolisation after bleomycin, possibly because it facilitates migration of bronchiolar cells into regions of injury [108], and it is conceivable that a lack of MMP-9 might also interfere with repair of cigarette smoke-induced airway epithelial injury.

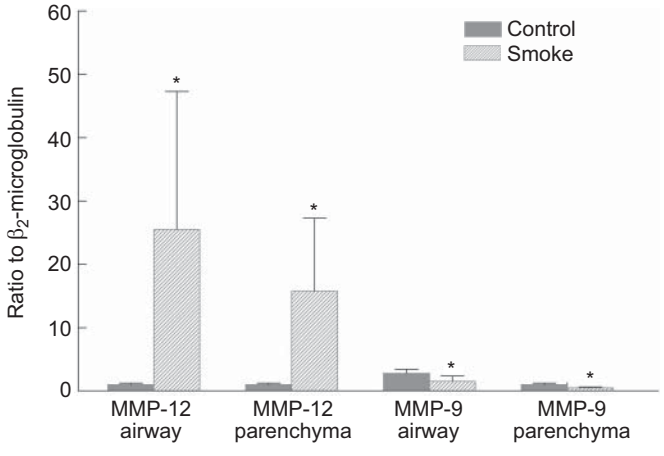

FIGURE 3. Comparative gene expression data from C57Bl/6 mice exposed for 6 months to cigarette smoke. Small airways and parenchyma were isolated by laser capture microdissection. Matrix metalloproteinase (MMP)-12 is significantly upregulated by smoke in both the small airways and the parenchyma, whereas MMP-9 is significantly downregulated. *: significantly different from control.

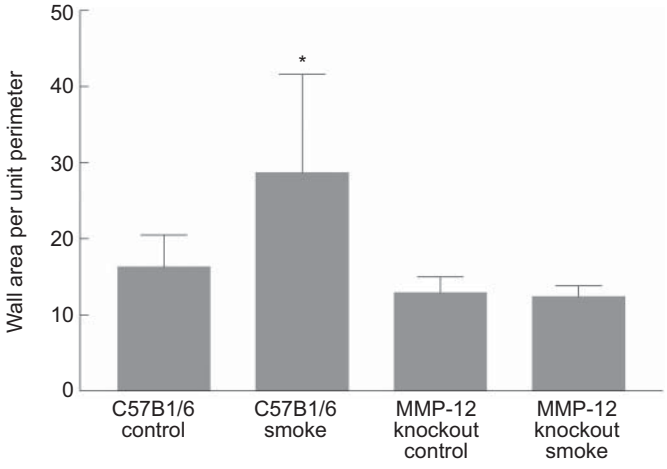

FIGURE 4. Effects of cigarette smoke on small airway remodelling (determined by morphometry on histologic sections) in C57Bl/6 and matrix metalloproteinase (MMP)-12-/- mice. In C57Bl/6 mice smoke exposure causes an increase in wall area per unit perimeter (*i.e.* small airway remodelling), whereas MMP-12-/- mice are completely protected against small airway remodelling.

In summary, MMPs are produced in the airways in the normal state and their production is modified by cigarette smoke exposure, but there is no consensus in the literature concerning exactly which MMPs are important and exactly how they operate in airway remodelling. However, the fact that knockout of MMP-12 or inhibition of MMP-12/MMP-9 prevents small airway remodelling in animal models suggests that further investigation of this area might be useful in developing therapies for COPD. Some care must be taken in selecting an inhibitor of any kind to prevent small airway remodelling, since the same mechanism that inhibits airway fibrosis might decrease repair in the alveolar walls and exacerbate emphysema. Inhibition of MMP-12 is thus a particularly attractive target, since it prevents both lesions.

MMPs IN VASCULAR REMODELLING ASSOCIATED WITH PULMONARY HYPERTENSION

In humans, MMPs, particularly MMP-1, -2 and -9, are important during the vascular remodelling that is a normal part of lung development [112]. Although MMPs have been shown to have a role in vascular remodelling in a variety of animal models of pulmonary hypertension, including hypoxia and monocrotaline induced pulmonary hypertension, we will confine ourselves here to the vascular remodelling seen in COPD, or to relevant data gleaned from isolated cell experiments.

Endothelial dysfunction and, ultimately, pulmonary hypertension arises through a number of complex pathways, with interactions between cellular components such as inflammatory cells, endothelial cells, smooth muscle cells, vascular matrix and a number of cytokines [113, 114]. In vessels, MMPs can be produced by a variety of cell types, including smooth muscle cells, fibroblasts and endothelial cells, and their secretion appears to be related to complex signalling pathways.

In some forms of pulmonary hypertension, the vessels are remodelled with a combination of matrix production and destruction which appears to be related to protease, and

particularly MMP-2, activity. In a vascular smooth muscle cell culture model [115], angiotensin II and endothelin-1 (both proteins thought to be involved in pulmonary hypertension remodelling) were shown to increase MMP-2 secretion; this appeared to be associated with activation of the RhoA/ROCK pathway, as it could be blocked by statins, knockdown of RhoA, and inhibitors of ROCK. The RhoA/ROCK pathway has been shown to be important in endothelial dysfunction and vascular remodelling [113, 116, 117].

All forms of pulmonary hypertension appear to be associated with changes in the vascular apoptosis/cell proliferation index [118]. Serine elastase inhibitors have been shown to be effective in reversing pulmonary hypertension in non-cigarette smoke models [119], and both serine and MMP inhibitors were able to induce apoptosis and reduce cell proliferation in a pulmonary artery explant model [120]. These actions appeared to be related to their effects on tenascin C, with osteopontin acting as an alternate cell survival stimulus.

MMPs may also be important in endothelial to mesenchymal transition, a process that has been proposed as an important facet of vascular remodelling related to pulmonary hypertension [121]. This hypothesis suggests that, as a part of the response to injury, loss of cell-to-cell or cell-to-matrix contacts allows endothelial cell migration and differentiation, so that they acquire mesenchymal characteristics. The RhoA pathway is involved in this process, and there is some evidence which also implicates MMP-2 in allowing cell migration [121].

The extent to which these processes occur in cigarette smoke-induced vascular remodelling is not known. Our data, using a mouse model of chronic cigarette smoke exposure, has shown that there was a transient increase in small pulmonary artery gene expression for MMP-2, -9, -13 and -12, as determined by laser capture microdissection and vascular immunohistochemical staining for these MMPs; however, long-term smoke exposure was associated only with small increases in MMP-12 [122]. Upregulation of MMP production appeared to be TNF-α dependent, since it did not occur in TNF-α double receptor knockout mice [122]. However, in a guinea pig model, a combined MMP-9/-12 inhibitor prevented both emphysema and small airway remodelling, but did not prevent the development of pulmonary hypertension and vascular remodelling [20].

Overall, while MMPs may play a role in the vascular remodelling associated with cigarette smoke-induced pulmonary hypertension, there is little evidence at present that MMP inhibition prevents the development of pulmonary hypertension.

MMPs IN CHRONIC BRONCHITIS

The definition of chronic bronchitis is clinical, related to cough and sputum production, and is not linked to airflow obstruction. While mucus plugs may be important in poor survival in patients with severe airflow obstruction [7], most of the airflow obstruction in chronic bronchitis is secondary to small airway remodelling, which includes increased mucus production. Thus, the finding of inflammatory cells and various proteases in sputum is not necessarily indicative of the site of origin; for example, the study of VIGNOLA *et al.* [123] demonstrated increased levels of MMP-9 and TIMP-1 in the sputum of chronic bronchitics, but it is not clear whether these substances are related to large or small airway pathology.

There is little information reported about possible relationships between mucus and MMPs in cigarette smoke-associated disease. Rodent animal models are dependent primarily upon assessment of tracheal and small airway goblet cell metaplasia because bronchial glands are too sparse in rodents to produce chronic bronchitic-like effects. STEVENSON *et al.* [124] exposed rats to cigarette smoke and found an increase in goblet cell density and bronchoalveolar lavage (BAL) mucin after 2 days of cigarette smoke exposure, and in a dose-dependent manner during a 3-day exposure, and these changes appeared to mirror acute inflammatory mediators and neutrophilia; macrophages were not increased in this model. Interestingly, although goblet cell metaplasia was identified with as few as two cigarettes, BAL mucin did not significantly increase until a dose of five cigarettes. Use of a CXCR2 inhibitor had an inverse dose effect upon goblet cell metaplasia and this was opposite the effect on neutrophils.

Although the above study did not address the role of MMPs, a later report showed whole lung MMP12 gene upregulation which was progressive over the 34 weeks of the study in addition to acute inflammatory genes and stress genes; goblet cell metaplasia and BAL mucin had similar patterns [125]. These studies show at best indirect relationships between MMPs and airway mucin, but there are some data to suggest that MMPs could be involved directly in mucus production induced by cigarette smoke. Epidermal growth factor is involved in mucin secretion [126], and MMP-9 has been shown to activate this receptor and induce mucin 5AC expression [127], probably by cleaving pro-TGF-β to its active form.

In an IL-13 overexpressor model, ZHU *et al.* [128] demonstrated emphysema and mucus metaplasia, and increases in MMP-2, -9, -12, -13 and -14, but MMP inhibition did not affect mucus metaplasia. Other workers have shown that mucin production induced by acrolein-fog or LPS inflammatory stimuli appears to be related to MMP-9 activity [129–131], but the relationship of such models to smoke-induced lesions is uncertain.

STATEMENT OF INTEREST

Statements of interest for A. Churg and J.L. Wright can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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