served. However, a more purified type I collagenase inhibitor and inhibitors against other types of collagenases, particularly type IV, which can degrade basement membrane collagen (*17*), should be tested. The high angiogenesis inhibitory activity present in shark cartilage should not only be helpful in exploring the enzyme inhibition profile of angiogenesis inhibitors in cartilage, but also in conducting antitumor studies.

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Functional α_1-Protease Inhibitor in the Lower Respiratory Tract of Cigarette Smokers Is Not Decreased

Abstract. *Cigarette smoking is the major risk factor for the development of pulmonary emphysema, a disorder that may result from an imbalance between the elastase and antielastase levels in the lungs. Decreased functional α_1-protease inhibitor, an inhibitor of neutrophil elastase, might render smokers susceptible to elastase-catalyzed destruction of pulmonary elastic fibers and the development of emphysema. Binding and inactivation of isotopically labeled porcine pancreatic elastase and human neutrophil elastase by α_1-protease inhibitor were measured in fluid obtained by bronchoalveolar lavage of volunteers. The inhibition of elastase-catalyzed solubilization of elastin and a tripeptide substrate were also determined. The mean level of functional α_1-protease inhibitor in the bronchoalveolar lavage fluid of smokers was found to be equal to or greater than that of nonsmokers, contradicting reports by other investigators. Increased elastase derived from pulmonary neutrophils, rather than decreased functional α_1-protease inhibitor, appears to be the main factor in the genesis of emphysema in smokers.*

An imbalance between elastase and antielastase in the lower respiratory tracts of humans is generally accepted as the basis for the enzymatic destruction of the elastic fibers in the walls of the air spaces in the lungs, a process believed to be central in the development of pulmonary emphysema (*1*). One proposed explanation for the association between cigarette smoking and the development of emphysema in humans is an increase in the elastase burden of the lungs of smokers as a result of the presence of larger numbers of neutrophils and alveolar macrophages (*1, 2*). α_1-Protease inhibitor (α_1-PI), the major antiprotease in the lungs, forms a covalent one-to-one complex with—and inactivates—a number of serine proteases, including neutrophil elastase. However, α_1-PI is made functionally inactive by oxidation of two methionine residues near its reactive site (*3*). Neutrophil myeloperoxidase–mediated oxidation of α_1-PI abolishes the formation of the α_1-PI–elastase complex (*4*) and promotes the retention of elastin-digesting activity by elastase in the presence of the modified α_1-PI (*4, 5*). It has been postulated that cigarette smoking decreases functional α_1-PI in the lower respiratory tract, either directly by chemical oxidation or indirectly by the release of oxidants from neutrophils (*6, 7*). Gadek *et al.* (*7*) reported a functional antielastase deficiency in the lower respiratory tract of cigarette smokers as compared with nonsmokers, although the two groups had similar levels of immunoreactive α_1-PI. Similarly, Carp *et al.* (*8*) reported a decreased elastase-inhibitory capacity in fluid obtained by bronchoalveolar lavage (BAL) of smokers and identified methionine sulfoxide residues in the α_1-PI from smokers' BAL. Using an elastase-binding assay for functional α_1-PI as well as an elastase-inhibitory assay, we have obtained data that contradict those findings.

Two groups of volunteers were studied: 16 nonsmokers [3 females and 13 males, aged 28 ± 1 years (mean ± standard error) and 21 smokers (9 females and 12 males, aged 25 ± 1 years). The mean number of pack years (years of smoking one pack per day) was 8 ± 1. Portions of the unconcentrated BAL fluid obtained from these subjects were assayed for functional α_1-PI (*9, 10*) by adding ^{125}I-labeled human neutrophil elastase (^{125}I-HNE) (*11*) or ^{3}H-labeled porcine pancreatic elastase (^{3}H-PPE) (*9*) and separating the α_1-PI–elastase complexes with molecular sieve chromatography. The PPE-specific α_1-PI measured in unconcentrated BAL fluid was 2.7 ± 0.1 percent of total protein for smokers versus 2.1 ± 0.2 percent for nonsmokers ($P < 0.05$); the ratio of smoker-to-nonsmoker values was 1.29. Comparable values for HNE-specific α_1-PI were 2.3 ± 0.1 versus 1.8 ± 0.2 percent, with a ratio of 1.28 ($P < 0.05$). Unconcentrated BAL fluid from smokers contained as much total PPE-specific α_1-PI as that from nonsmokers: 207 ± 21 versus 183 ± 23 μg, respectively. Comparable values for HNE-specific α_1-PI were 182 ± 20 versus 161 ± 22 μg, respectively. Functional α_1-PI measured by the binding of ^{3}H-PPE correlated well with that measured by the binding of ^{125}I-HNE ($r = 0.91$).

Portions of BAL fluid from 12 of the nonsmokers and 11 of the smokers were concentrated for the measurements of immunoreactive α_1-PI, albumin, functional α_1-PI, and total protein (Table 1). Concentrated BAL fluid from smokers contained as much PPE-specific and HNE-specific α_1-PI as did concentrated BAL fluid from nonsmokers (Table 1). Significant differences were not found, whether functional α_1-PI was expressed as a percentage of immunoreactive α_1-PI, of albumin, or of total protein. The PPE-inhibitory activity of the 23 concentrated BAL fluid samples was assessed as described earlier (*7*) by incubating increasing volumes of BAL fluid in 1 ml of buffer with PPE and measuring the

residual elastin-solubilizing activity in ^{3}H-labeled elastin substrate. Depending upon sample availability, nine different volumes of each lavage fluid were assessed in duplicate or triplicate, and inhibition curves were constructed. The mean of the 100 percent inhibition value and twice the 50 percent inhibition value were recorded for analysis. Using radial immunodiffusion plates to measure immunoreactive α_1-PI, we calculated that the amount of α_1-PI in the concentrated BAL fluid required to achieve complete inhibition of 1.0 μg of PPE was 4.4 ± 0.7 μg (mean ± standard error) for the smokers and 5.4 ± 0.8 μg for nonsmokers (not significantly different). Since the ratio of the molecular weight of α_1-PI to PPE is about 2.12, inhibition of 1 μg of PPE by 2.12 μg of α_1-PI would imply an inhibitory efficiency of 100 percent. Our values imply a 48 ± 8 percent and a 39 ± 6 percent inhibitory efficiency of α_1-PI in smoker and nonsmoker BAL fluids, respectively. When the peptide substrate succinyl-(L-alanyl)$_3$-*p*-nitroanilide (SAPNA) was used to measure residual elastolytic activity, a mean of 4.4 ± 0.3 μg and 4.6 ± 0.6 μg of α_1-PI was required to inhibit 1 μg of PPE by six concentrated smoker samples and seven nonsmoker samples, respectively. Inhibition of 1 μg of HNE by concentrated BAL fluid, as measured with ^{3}H-labeled elastin substrate required 3.8 ± 0.7 μg for concentrated smoker samples (N = 3) and 4.8 ± 0.4 μg for nonsmoker samples (N = 4). All of these measurements suggested that α_1-PI from smokers was at least as effective as α_1-PI from nonsmokers in inhibiting elastase.

In the following experiments, we were unable to detect oxidatively inactivated α_1-PI whose functional elastase-binding activity could be restored by chemical reduction of the BAL fluid. Portions of the BAL fluid from two smokers, both of whom smoked 1.5 packs per day and had smoked one cigarette 15 minutes before undergoing the lavage procedure, were dialyzed against the reducing agent sodium metabisulfite (0.025*M*), as described (*6*), or against dithiothreitol (0.001*M*). As measured with ^{3}H-PPE or ^{125}I-HNE, neither reducing agent raised the level of functional α_1-PI above that of the samples before dialysis or of the original sample dialyzed against buffer only. The level of ^{125}I-HNE–specific α_1-PI was not significantly increased by incubating a portion of the original sample at 37°C for 30 minutes with ^{125}I-HNE, as compared with incubating it at 22°C for 5 minutes. This result suggests the absence of oxidized α_1-PI (*8*).

Our values for immunoreactive α_1-PI (3.9 ± 0.5 and 4.1 ± 0.4 percent of total protein in concentrated BAL fluid of smokers and nonsmokers, respectively) compare closely with reported values of 3.5 ± 0.3 percent (*12*). Cohen *et al.* (*13*) and Satoh *et al.* (*14*) found that 1.75 and 1.70 moles, respectively, of purified human α_1-PI was required to inactivate one mole of porcine pancreatic elastase; these values imply a 57 percent and a 59 percent functional efficiency for purified human α_1-PI. The excess of α_1-PI required was attributed to nonproductive proteolysis of α_1-PI by elastase rather than the presence of inactive α_1-PI. Our values for functional α_1-PI, measured by the elastase-binding method and expressed as a percentage of immunoreactive α_1-PI, are closely comparable to the results of those workers (Table 2). Our values from elastase-inhibitory assays are lower than their reported data, but we found comparable values for smokers and nonsmokers. Both Gadek *et al.* (*7*) and Carp *et al.* (*8*) found somewhat higher values for their smoking groups (76 ± 6 and 68 ± 6 percent, respectively). However, their calculated values for nonsmoking groups were 125 ± 4 and 118 ± 3 percent, respectively, values much higher than their values for smokers. The reasons for these discrepancies

Table 1. Functional α_1-PI in concentrated BAL fluid. BAL fluid was obtained through a flexible bronchoscope (*18*) from the right middle lobe of normal volunteers (16 nonsmokers and 21 smokers) under institutional board supervision. Four portions of 0.9 percent NaCl (60 ml) were instilled, with a return on aspiration of 66 ± 2 percent [mean ± standard error (S.E.)] of the instilled volume for the smoking group and 59 ± 3 percent for the nonsmokers (not significant, *t*-test). Portions of supernatant samples from the BAL fluid of 12 nonsmokers and 11 smokers were concentrated, and immunoreactive α_1-PI, albumin, and total protein were measured as described (*19*). α_1-PI plates were calibrated with a standard plasma sample and with purified human α_1-PI. Functional α_1-PI was measured by incubating portions of concentrated BAL fluid with 10 μg of ^{3}H-PPE or ^{125}I-HNE, which retained full catalytic activity after being labeled; molecular sieve chromatography was then used to isolate and quantify α_1-PI-elastase (*19*). In four separate determinations of functional α_1-PI on portions of the same sample, the S.E. was less than 10 percent. Lavage six times with 20 ml of saline rather than four times with 60 ml was performed in seven nonsmokers, and BAL fluid from five of the seven samples was used in studies of concentrated fluid. Functional α_1-PI, albumin, and total protein were not different in the two nonsmoking subgroups. Data are presented as means ± standard error.

Group	Functional α_1-PI			
	Total per sample (μg)	Measured as percentage of		
		Immunoreactive α_1-PI	Albumin	Total protein
		By ^{3}H-PPE binding		
Nonsmokers	134 ± 28*	56 ± 6	4.5 ± 0.6	2.2 ± 0.2
Smokers	141 ± 25	59 ± 6	4.9 ± 0.6	2.1 ± 0.2
		By ^{125}I-HNE binding		
Nonsmokers	128 ± 29*	52 ± 5	4.3 ± 0.7	2.1 ± 0.3
Smokers	135 ± 28	52 ± 4	4.6 ± 0.8	1.9 ± 0.2

*Values of functional α_1-PI are given for the BAL fluid samples from seven nonsmokers for whom the larger volume of saline was used (60 ml, four times).

Table 2. Functional α_1-PI as a percentage of immunoreactive α_1-PI in BAL fluid from smokers and nonsmokers and in purified human α_1-PI. For values shown in parentheses, human neutrophil elastase was used. All other values were obtained by using the elastase from porcine pancreas. Abbreviations: SAPNA, succinyl trialanine *p*-nitroanilide; Ac-(Ala)$_3$-OMe, *N*-acetyl trialanine methyl ester; NBA, *p*-nitrophenyl *N-tert*-butyloxycarbonyl-L-alaninate.

Method	Functional α_1-PI as percentage of		
	Immunoreactive α_1-PI in BAL fluid		Purified human α_1-PI
	Nonsmokers	Smokers	
Present study			
Elastase-binding	56 ± 6 (52 ± 5)	59 ± 6 (52 ± 4)	
Elastase-inhibitory, ^{3}H-elastin	39 ± 6 (38 ± 3)	48 ± 8 (48 ± 9)	
Elastase-inhibitory, SAPNA	46 ± 6	48 ± 3	
Gadek *et al.* (*7*)			
Elastase-inhibitory, ^{3}H-elastin	125 ± 4	76 ± 6	
Carp *et al.* (*8*)			
Elastase-inhibitory, SAPNA	118 ± 3	68 ± 6	
Cohen *et al.* (*13*)			
Elastase-inhibitory, Ac-(Ala)$_3$-OMe			57
Satoh *et al.* (*14*)			
Elastase-inhibitory, NBA			59

are not clear. Abboud *et al.* (*15*) reported that smoking two to four cigarettes produced a limited α_1-PI-inactivating effect.

We have investigated two differences in experimental design between our study and the previous studies but could not account for the differences in findings. First, the smoking group in the study by Gadek *et al.* (*7*) and by Carp *et al.* (*8*) had a more extensive smoking history (27 ± 7 and 18 ± 1 pack years, respectively, compared with 8 ± 1 for our smoking group). However, we found no significant difference in the mean functional α_1-PI as a percentage of protein calculated for the six heaviest smokers in our study (1.5 to 2 packs per day for 9 ± 2 years) as compared with our entire smoking group (2.9 ± 0.1 versus 2.7 ± 0.1 percent, respectively, for ^{3}H-PPE–specific α_1-PI). The addition of sodium azide, a bactericidal agent, to the BAL fluid samples did not account for the difference in findings. Before the addition of azide, we found peroxidase-like activity, with *o*-dianisidine used as substrate, in concentrated BAL fluid samples from smokers and less activity in samples from nonsmokers. This activity was suggestive of neutrophil-derived myeloperoxidase, which can readily inactivate α_1-PI in the presence of H_2O_2 and a halide but is inhibited by sodium azide (*4*). However, Nauseef, using a sensitive procedure developed in his laboratory (*16*), did not detect immunoreactive myeloperoxidase in portions of BAL fluid representing 6 percent of the samples from two smokers. Furthermore, incubation of portions of BAL samples for several weeks at 4°C in the absence of sodium azide failed to depress the level of functional α_1-PI in comparison with portions of the same samples to which sodium azide had been added.

Although the peroxides in smoke from different cigarettes are thought to variably reduce the elastase inhibitory capacity of α_1-PI (*17*), we did not investigate the effect of cigarette brand or the presence of filters on levels of functional α_1-PI in the BAL fluid of smokers. Also, our study does not exclude the possibility of a very rapid recovery of functional α_1-PI in the lungs after cigarette smoke–induced oxidation or of a significant smoke-induced depression of functional α_1-PI restricted to small localized portions of the lower respiratory tract.

Even though the lower respiratory tracts of smokers and nonsmokers may contain comparable levels of antielastase activity, smokers presumably are still at greater risk of developing emphysema because of an imbalance in the elastase-antielastase system. The sedimented BAL cells from our smoking group contained, on average, more than five times as many neutrophils and alveolar macrophages as we found in nonsmokers. If cells obtained by lavage are representative of the numbers of cells within the lungs, the elastase burden of smokers is clearly increased. Our results suggest that additional studies of the effects of cigarette smoke on α_1-PI in the lower respiratory tract are needed and that antioxidants might not offer protection against the development of emphysema in smokers.

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Allometry and Reorganization in Horse Skull Proportions

Abstract. *Allometric analysis of skull proportions in 25 species of fossil equids indicates that both scaling effects (allometry) and reorganization were factors in the evolutionary transformation of horse skulls. A relatively longer preorbital portion of the skull resulted from the ventral and forward displacement of the tooth row relative to the jaw joint and the orbit when high-crowned teeth evolved. Correlated with the increased distance between jaw joint and tooth row is an increase in the relative size of the attachment areas of masseter and internal pterygoid muscles.*

Horses have one of the longest and richest fossil records of any group of mammals. Their record extends back 55 million years and documents major morphological changes in feeding and locomotor systems. For these reasons, the fossil record of horses has been an important source of textbook samples of evolutionary phenomena (*1*). Most work on fossil horses has dealt with details of dental anatomy (*2*). There have been a few major studies of the evolution of the limbs (*3*) and of differences in skull morphology among closely related species or genera (*4*).

Our knowledge of the evolutionary transformation of horse skulls has for the past 40 years rested primarily on two studies, which came to contradictory conclusions. Robb (*5*), who analyzed changes in skull proportions in horse evolution by comparing preorbital length to total skull length in 13 species of fossil horses, concluded that the relatively long muzzles of modern horses resulted entirely from allometry—size-related changes in proportions that occurred during evolution from small ancestral horses to the large modern ones. Seven years later, Reeve and Murray (*6*) reanalyzed Robb's data. They compared preorbital length to braincase length (that is, skull length minus preorbital length) and concluded that there had been a reorga-