Elastin Fragments Attract Macrophage Precursors to Diseased Sites in Pulmonary Emphysema

Abstract. *This study suggests one mechanism by which alveolar macrophages accumulate in the lung in pulmonary emphysema: elastin fragments generated at the diseased sites are potent chemoattractants for monocytes, the precursors of the macrophages. The most chemotactic elastin fragments have a molecular weight between 10,000 and 50,000 and are active at concentrations as low as 3 nanograms per milliliter. By comparison, elastin fragments with higher molecular weights and desmosines are active only at concentrations greater than 0.3 microgram per milliliter. In addition, preincubation of monocytes with the 10,000- to 50,000-dalton elastin impairs the ability of the cells to migrate toward elastin fragments but not toward activated serum. Fragments of tropoelastin are not chemotactic for monocytes. Because elastin, but not tropoelastin, contains lysyl-derived cross-links, these structures may be the active chemotactic site on the elastin fragments.*

Pulmonary emphysema is a chronic disorder characterized by destruction of alveolar walls, obstruction of the bronchioles, and trapping of air in spaces distal to the obstructions, with subsequent abnormal enlargement (*1–6*). There are increased numbers of alveolar macrophages in the emphysematous lung, especially at sites of disease activity (*5, 7–10*). This feature is particularly relevant to the pathogenesis of emphysema, since the macrophages are thought to be capable of injuring the lung parenchyma either directly or by attracting other inflammatory cells to the alveolar structures (*5, 8, 9, 11, 12*).

Since alveolar macrophages are derived from monocytes that originate in bone marrow and migrate to the lungs through the bloodstream (*13*), the presence of increased numbers of alveolar macrophages strongly suggests that chemotactic stimuli are generated at the sites of disease activity and attract circulating monocytes to the alveolar structures. Emphysema is probably associated with a disruption of the elastin network of the alveolar walls, since there is loss of the elastic recoil of the lungs (*1, 2, 5*). Consistent with this concept are animal studies demonstrating that placement of elastolytic enzymes into the lung results in a disease similar to emphysema and the release of elastin fragments (*2, 4–6, 14*). These observations suggest one possible mechanism by which monocytes are attracted to the lung in emphysema: elastin fragments, generated at sites of disease activity, are chemotactic for circulating monocytes.

To test this hypothesis, we evaluated activity of two forms of elastin (*15*): (i) fragments of tropoelastin, the non–cross-linked, 70,000-dalton, single-chain polypeptide that is the precursor of the elastin polymer and (ii) fragments of elastin, the insoluble polymer formed from tropoelastin by cross-linking of lysyl residues in the precursor molecule.

Fragments of tropoelastin or intact tropoelastin were not chemotactic for blood monocytes, but fragments of elastin were (Fig. 1). Although a spectrum of elastin fragments were capable of attracting monocytes, the peptides with the greatest activity were between 10,000 and 50,000 daltons in size. Elastin fragments of this size from human aorta and lung possessed similar chemotactic activity for monocytes (Table 1). Although it is likely that elastin fragments (10,000 to 50,000 daltons) generated by hydrolysis of elastin with oxalic acid or by digestion of elastin with purified leukocyte elastase are not identical, elastin fragments of this size, generated by either method, were equally chemotactic for monocytes. Such fragments attracted monocytes at concentrations as low as 3 ng/ml (range, 6×10^{-11} *M* to 30×10^{-11} *M*). By comparison, elastin fragments < 10,000 daltons or > 50,000 daltons were active only at concentrations from 0.3 to 3 μg/ml. "Checkerboard analysis" of the elastin fragments demonstrated that they preferentially stimulated direct rather than random migration of blood monocytes and thus fulfill the criteria for being a chemotactic factor (*16*).

The chemotactic activity of the elastin fragments was specific for monocytes. While the 10,000- to 50,000-dalton fragments attracted monocytes at concentrations as low as 3 ng/ml, these same peptides, at concentrations up to 30 μg/ml, did not attract neutrophils (Table 2). In addition, preincubation of neutrophils with the elastin fragments at concentrations up to 30 μg/ml did not alter the ability of the neutrophils to respond to a

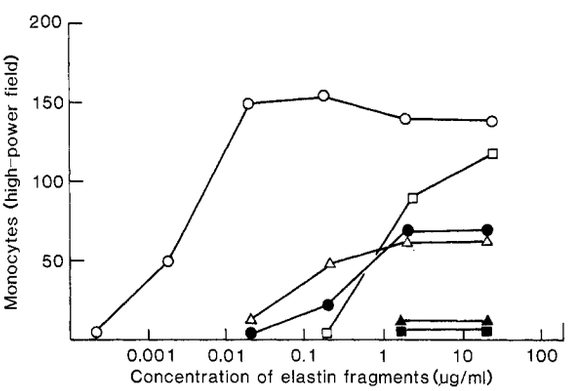

Fig. 1. Chemotactic activity of fragments of elastin and tropoelastin for peripheral blood monocytes. Human aortic elastin was prepared from postmortem specimens by extracting the tissues with 0.1*N* NaOH at 95°C for 45 minutes and defatting them with successive washes of ethanol, ethanol and ether, and ether (*22*). Partial hydrolysis of the elastin with 0.25*M* oxalic acid was then performed (*23*). The partially hydrolyzed elastin was dialyzed exhaustively against water and then subjected to ultrafiltration on an Amicon XM-50 membrane with a ≥ 50,000-dalton cutoff. Two size ranges of elastin fragments emerged: (i) 10,000 to 50,000 daltons, obtained from the material passing through the filter, and (ii) ≥ 50,000 daltons, obtained from the material retained by the filter. Both fragments were lyophilized and weighed. The purity of the elastin fragments was verified by amino acid analysis and enzyme-linked immunoassay. Tropoelastin was prepared by affinity purification of lathyritic sheep nuchal ligament extracted with 0.5*M* CH_3COOH in a CNBr-Sepharose column containing antibody to sheep elastin (*24*). Partial elastase digestion of insoluble elastin and tropoelastin was performed with purified leukocyte elastase (*25*) by incubating enzyme and substrate (100 μg/ml) in phosphate-buffered saline (PBS) (*p*H 7.4) in a dialysis bag. Simultaneous digestion and dialysis against PBS was continued for 18 hours at 24°C, after which the enzyme in the dialysis bag was inactivated by heating (100°C for 15 minutes). Concentrations of elastase-induced fragments of elastin or tropoelastin in the dialysis bags (> 10,000 daltons) and in the dialyzate (< 10,000 daltons) were determined by an enzyme-linked immunoassay. The chemotactic activity of various concentrations of elastin and tropoelastin fragments for peripheral blood monocytes was determined by a Nuclepore filter chemotaxis assay (pore size, 5 μm) (*26*). The chemotactic activity of elastin and tropoelastin was determined at concentrations ranging from 0.3 ng/ml to 30 μg/ml. Symbols: (○) elastin fragments of 10,000 to 50,000 daltons, (□) elastin fragments ≥ 50,000 daltons, (●) elastin fragments ≤ 10,000 daltons, (▲) tropoelastin fragments > 10,000 daltons, (■) tropoelastin fragments ≤ 10,000 daltons, and (△) desmosines (Elastin Products Co.). Below concentrations of 0.03 μg/ml, desmosines and ≤ 10,000-dalton elastin fragments were not chemotactic. Tropoelastin fragments and intact tropoelastin were not chemotactic at any concentration from 0.0003 to 30 μg/ml. The data represent the mean of five separate experiments.

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known chemotactic factor, activated serum. In contrast, preincubation of monocytes with the elastin fragments resulted in a decreased ability of the cells to migrate in response to elastin fragments (Table 2); the monocytes were deactivated by the fragments at concentrations as low as 3 ng/ml. However, preincubation of the cells with elastin did not decrease the ability of monocytes to migrate in response to activated serum. These observations suggest that the chemotactic receptors on monocytes for elastin fragments differ from the receptors for C5a, the primary chemoattractant in activated serum.

The inability of fragments of tropoelastin (compared to fragments of elastin) to attract monocytes suggests that a critical active site is present on elastin fragments but not on tropoelastin fragments. Unlike tropoelastin, elastin possesses desmosines, covalent lysine cross-links (*15*). This study and a study by Senior *et al.* (*17*) demonstrated that these lysyl residue–derived cross-links are chemotactic for monocytes, although they are not as active as the 10,000- to 50,000-dalton elastin fragments. The critical chemoattractant site on the elastin fragments probably contains lysyl-derived cross-links.

Although elastin fragments are probably generated at the sites of disease activity in pulmonary emphysema, the mechanisms underlying formation of the fragments are still unclear. However, such mechanisms seem to involve elastase, since mature cross-linked elastin is highly susceptible to degradation by this enzyme (*15*). Two candidates for the release of elastase in the alveolar structures are alveolar macrophages and neutrophils. Neutrophils are capable of storing and releasing large quantities of elastase (*18*) and are present in the alveolar structures of individuals susceptible to emphysema (*11, 12*). The role of the macrophage as a source of elastase in the alveolar structures of individuals with emphysema is not clear. Although alveolar macrophages from cigarette smokers spontaneously release elastase in vitro (*9, 10*), human macrophages have surface receptors for neutrophil elastase, suggesting that the elastase released by smokers' macrophages may represent ingested neutrophil elastase (*6, 19*).

Still, there is growing evidence that the increased number of macrophages may be important in the pathogenesis of emphysema. Alveolar macrophages from patients with emphysema appear to be in a state of activation compared to macrophages from normal nonsmokers (*8, 10–12, 20, 21*). The alterations that have been described for these macrophages include increased glucose utilization, increased secretion of various enzymes, changes in cell surface receptors, and spontaneous release of a factor chemotactic for neutrophils. Although the activation of these cells has been attributed to in vivo exposure to cigarette smoke, it is possible that the activation of the macrophages actually results from their exposure to elastin fragments. In this context, elastin fragments could serve as potent attractants for blood monocytes and may also play an important role in activating these cells. The presence of these fragments in the lung offers an explanation for the accumulation of alveolar macrophages in pulmonary emphysema and may help explain the chronicity of the lung destruction.

Table 1. Comparison of the capacity of elastin fragments (10,000 to 50,000 daltons) from human aorta and lung to function as a chemotactic factor for monocytes. Monocytes were isolated from the blood of normal individuals by Hypaque-Ficoll centrifugation (*27*). The chemotactic activity of various concentrations of elastin fragments for monocytes was then determined by a Nuclepore filter chemotaxis assay. The data are the number of monocytes migrating through the filter (pore size, 5 μm)

Source of elastin fragments*	Amount of elastin fragments (μg/ml)				
	30	3	0.3	0.03	0.003
Aorta	148	145	158	154	53
Lung	136	133	142	148	36

*The fragments were prepared by partial hydrolysis with oxalic acid (see legend to Fig. 1).

Table 2. Comparison of the capacity of elastin fragments (10,000 to 50,000 daltons) to function as a chemotactic factor for monocytes and neutrophils: deactivation effects. Before being used in the chemotaxis assay, the monocytes and neutrophils (each at 5×10^6 cells per milliliter) were incubated with 0, 0.003, or 30 μg of fragments per milliliter for 15 minutes at 37°C. The cells were then washed four times and exposed to 5 percent activated serum or elastin fragments (10,000 to 50,000 daltons; 30 μg/ml). Chemoattraction to serum or fragments was determined by a Nuclepore filter chemotaxis assay (*26*). The data are the number of cells migrating through the filters (pore size for monocytes, 5 μm; for neutrophils, 3 μm).

Type of cell*	Amount of elastin† incubated with cells (μg/ml)	Chemotactic factor	
		Activated serum (*28*)	Elastin
Monocytes	0	180	160
	0.003	175	48
	30	190	55
Neutrophils	0	220	3
	0.003	230	10
	30	210	6

*Both cell types were isolated from the blood of normal individuals after sedimentation in Plasmogel (H.T.I. Corp.) and Hypaque-Ficoll centrifugation (*27*). †Fragments (10,000 to 50,000 daltons) were prepared by partial hydrolysis of human aortic elastin with oxalic acid (see legend to Fig. 1).

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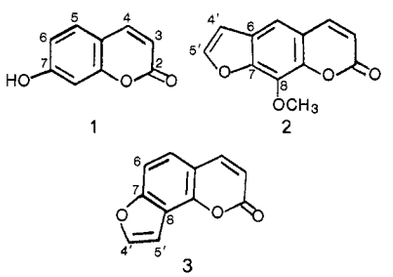
Toxicity of Angular Furanocoumarins to Swallowtail Butterflies: Escalation in a Coevolutionary Arms Race?

Abstract. *Xanthotoxin, a linear furanocoumarin occurring in many plants of the family Umbelliferae, is not appreciably toxic to the umbellifer-feeding larvae of* Papilio polyxenes *(Lepidoptera; Papilionidae), whereas angelicin, an angular furanocoumarin found only in a few relatively advanced tribes of the Umbelliferae, reduces growth rate and fecundity. The biosynthetic pathway leading to angular attachment of the furan ring may thus have been a response within the Umbelliferae to selective pressures exerted by specialized herbivores that had adapted to feeding on linear furanocoumarins.*

Angular furanocoumarins, benz-2-pyrone compounds with a furan ring attached at the 7,8 positions, are formed by a biosynthetic pathway distinct from that which leads to the 6,7-substituted linear furanocoumarins, although both groups share the precursor umbelliferone (*1*). The linear furanocoumarins (*2*) occur in at least eight plant families, whereas the angular furanocoumarins (*3*) are reported to occur in only two families, the Leguminosae and the Umbelliferae (*2*). Even in the Umbelliferae, where they are most diverse structurally, angular furanocoumarins are found only in two relatively advanced tribes, the Apieae and the Peucedaneae (*2*). Although many plants synthesize linear furanocoumarins in the absence of angular furanocoumarins, few, if any, plants are known to produce angular furanocoumarins in the absence of linear furanocoumarins (*1*); this suggests that the biosynthesis of angular furanocoumarins evolved more recently.

The presence of angular furanocoumarins in advanced members of the Umbelliferae is enigmatic. The compounds seem more advanced biosynthetically than the more common linear furanocoumarins; yet they appear to be less effective as a defense against most organisms. Linear furanocoumarins are toxic to a variety of organisms because of their ability to cross-link strands of DNA in the presence of ultraviolet light (*3*). The double bond of the furan ring in the angular configuration, however, is largely ineffective at cross-linking DNA strands; as a result, angular furanocoumarins show little or no phototoxicity to viruses, to bacteria, or to plant or mammalian cell systems (*4*).



Insect herbivory is an important selective force in the ecology and evolution of plants (*5*). Prominent among the insects associated with the Umbelliferae are species in the butterfly genus *Papilio*. Most of these insects avoid feeding on umbellifers with angular furanocoumarins and at least one such plant (*Heracleum lanatum*) has been shown experimentally to support poor growth of *Papilio polyxenes* larvae (*6*). We demonstrate here that insects adapted to feeding on plants containing linear furanocoumarins are not necessarily adapted to feeding on plants containing angular furanocoumarins. This finding suggests that the biosynthetic pathway leading to angular furanocoumarins may have arisen in response to the selective pressures of insect herbivores, particularly those adapted to feeding on the more widespread linear furanocoumarins.

To compare the effects of angular and linear furanocoumarins on the growth of an insect adapted to feeding on linear furanocoumarins, we chose for bioassay the black swallowtail butterfly *P. polyxenes*. The larvae of this species feed on over 20 species of Umbelliferae, many of which contain linear furanocoumarins (*2*). Larvae were raised from eggs on the foliage of three different host plants: carrot (*Daucus carota*), which lacks furanocoumarins; parsnip (*Pastinaca sativa*), which contains only linear furanocoumarins in the foliage; and angelica (*Angelica atropurpurea*), which contains both linear and angular furanocoumarins in the leaves (*7*), yet is acceptable to *P. polyxenes* (*8*). By comparing the effects of angular and linear furanocoumarins on caterpillars reared on these three plant species, we could determine whether tolerance to the compounds is induced by exposure early during growth (*9*).

Freshly molted fifth-instar larvae from each of the three food plants were placed individually in plastic cups (7 by 3.5 cm) lined with moistened filter paper; the larvae were then starved for 24 hours. In each plant treatment group, three subgroups were formed. Controls received varying numbers of weighed leaf disks of the species on which the caterpillars were reared; 10 μl of acetone was applied to each disk with a microsyringe. The other subgroups received disks to which was applied either 5 μg of xanthotoxin (*2*), a linear furanocoumarin, or angelicin (*3*), an angular furanocou-

Table 1. Effects of prolonged ingestion of angelicin on growth rate and fecundity of *Papilio polyxenes*. Values are means ± standard error.

Treatment	Days to pupation*		Pupal weight*		Survival† (%)	Eggs (No.)	Caterpillars (No.)	
	Males	Females	Males	Females			Males	Females
Experimental (with angelicin)	22.67 ± 0.92	24.14 ± 0.63	0.896 ± 0.038	0.988 ± 0.020	78	125.28 ± 36.21	6	7
Control (without angelicin)	20.57 ± 0.81	22.22 ± 0.55	0.926 ± 0.032	1.113 ± 0.048	89	432.00 ± 66.90	7	9
P value	< .10	< .025	< .10	< .05	< .50	< .005		

*The Wilcoxon two-sample test was used to compare means. †A 2 × 2 test of independence with the *G* statistic was used to compare survival rates.

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