acoustic stimulus does not elicit a cochlear potential until day 12 (7, 19). However, we have recorded relatively infrequent action potentials from units in the cochlear nucleus of 10- or 11-day mouse pups, prior to ear canal opening, which could not be driven by our acoustic stimulus.

The results suggest that we have altered the normal development of synaptic connections in the auditory system despite the fact that neurons were active during ontogeny. Clopton and Winfield (20) have previously demonstrated that the response properties of neurons in the inferior colliculus may be biased by the rearing environment, but the rearing conditions were extremely complex, making it difficult to implicate a specific developmental mechanism. We used a very simple auditory stimulus. Repetitive click stimulation should largely mask persistent spontaneous activity, and, unlike deprivation, it avoids the potential degenerative affects of disuse (21). The locus of the connectivity changes we have detected remains to be determined, and we do not yet know the relative contribution of excitatory and inhibitory connections in producing the observed effects. Nevertheless, it seems likely that activity in the auditory system, as in the visual pathway, is critically involved in ontogenetic fine tuning.

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Shark Cartilage Contains Inhibitors of Tumor Angiogenesis

Abstract. Shark cartilage contains a substance that strongly inhibits the growth of new blood vessels toward solid tumors, thereby restricting tumor growth. The abundance of this factor in shark cartilage, in contrast to cartilage from mammalian sources, may make sharks an ideal source of the inhibitor and may help to explain the rarity of neoplasms in these animals.

Scapular cartilage in calves contains a substance that inhibits the vascularization of solid tumors (1). When this substance was infused into rabbits or mice, no toxic effects were observed in the animals, yet the growth of new blood vessels toward implanted tumors (V2 carcinoma and B16 melanoma) ceased and tumor growth stopped (2, 3). The single factor most limiting to the further study of this substance is its supply. Cartilage is present only in small quantities in mammalian species.

It occurred to us that sharks may be a potential source of this inhibitor because, unlike mammals, sharks have an endoskeleton composed entirely of cartilage. Cartilage composes about 6 percent of the shark's total body weight (4), compared to less than 0.6 percent in calves. In addition, some sharks are very large, about ten times heavier than calves.

Basking sharks (*Cetorhinus maximus*) 6.1 m long and weighing approximately 409 kg were obtained from Fresh Water Company, Boston. The fins and vertebrae were immediately excised, scraped with a scalpel blade to remove connective tissue, and stored at −20°C. To extract the inhibitor, a modification of the procedures used for calf cartilage (1–3) was employed. The shark fins were cut into 1-cm³ pieces and extracted in a solution containing $1M$ guanidine and $0.02M$ 2-(N-morpholino)ethanesulfonic acid (MES) for 41 days at room temperature. Extracts were dialyzed exhaustively against water by using membranes with a 3500-dalton cutoff and centrifuged. The supernatant was filtered through Whatman 1 filter paper and then lyophilized. Five hundred milligrams of cartilage yielded 1 mg of this extract.

The shark cartilage extract was incorporated into 1-mm³ pellets of ethylene–vinyl acetate copolymer (40 percent vinyl acetate by weight) (5) at a level of 300 μg of extract and 700 μg of polymer. The polymer pellets have been shown to release over 1 μg of biologically active molecules per day for over 100 days (3, 5). The pellets were implanted into corneal pockets in New Zealand White rabbits. Directly behind the pellets were placed 1.5-mm³ pieces of V2 carcinoma (1). These tumors induced vessels to sprout toward them from the edge of the cornea. The bioassay consisted of measuring the length of the single longest blood vessel with a slit-lamp stereomicroscope.

In experimental corneas, tumors and pellets containing shark extract were used. Control corneas were implanted with tumors and identical-sized pellets containing no extract. Earlier studies have shown that the rate of tumor neovascularization in such controls is statistically indistinguishable from the rate induced by (i) tumors and pellets containing extracts of calf cartilage with no biological activity, (ii) tumors and pellets containing proteins or polysaccharides, and (iii) tumors alone (1, 3, 6).

The extract of basking shark cartilage significantly inhibited tumor neovascularization. Three different tests were conducted, and inhibition was observed in every case (Fig. 1 and Fig. 2a). After 19 days all control corneas had large, three-dimensional tumors with an average maximum vessel length of 6 mm (half the diameter of the cornea) (Fig. 2b). In

contrast, none of the treated corneas had three-dimensional tumors. All treated corneas showed sparse vascularization, with zones of complete inhibition around the pellets (Fig. 2a). Average maximum vessel length was 1.5 mm, 75 percent shorter than in the controls (Fig. 1).

These results demonstrate that basking shark cartilage extract strongly inhibits tumor-induced neovascularization and that significant inhibition can be obtained with the extracts at a crude stage of purification. In contrast, calf cartilage extract must be highly purified by affinity chromatography before inhibition can be observed (1). [Improved purification procedures may exist; however, we have not as yet found one (1, 3).] It took 500 g of calf cartilage to produce 1 mg of a substance causing 70 percent inhibition of vascular growth (1), slightly less inhibition than observed here with one-thousandth as much starting material. Considering this observation along with the larger size and greater percentage of cartilage body weight in basking sharks as compared to calves, sharks may contain as much as 100,000 times more angiogenesis inhibitory activity on a per animal basis. Thus, with further study, shark cartilage may become a major source of angiogenesis inhibitor. Identification of this factor might provide insights into the tissue development of different species and into why elasmobranchs such as sharks, in contrast to mammals and even bony fish and amphibians, so rarely exhibit neoplasms (7).

The inhibitor does not appear to act directly on the tumor itself. Stereomicroscopic observations showed that in both control and treated corneas, the V2 carcinoma grew slowly in two dimensions in the collagen layers of the cornea, indicating that the tumor cells continue to proliferate even in the presence of the inhibitor. Histologic sections showed healthy tumor cells at the pellet-tumor interface, and the inhibitor did not affect the growth of V2 carcinoma cells in culture. We used the same methods and obtained the same results with respect to tumor cell growth as in previous studies with calf scapular cartilage inhibitor (1–3). Both inhibitors appear to act on capillary advancement rather than on tumor cell growth directly.

It is not known whether all sharks contain angiogenesis inhibitors or whether the same molecule in calf and shark cartilage is responsible for the inhibition of tumor angiogenesis. Shark cartilage contains many of the same biochemical activities as calf cartilage, including lysozyme activity (8), cell growth–promoting activity (9), inhibitory activity against type I collagenase (10), and inhibitory activity against proteases such as trypsin, chymotrypsin, and plasmin (11).

However, when the guanidine extracts of shark and calf cartilage are analyzed on sodium dodecyl sulfate gels (12), the patterns are different, with the calf cartilage extract showing more bands, particularly in the molecular weight range of 20,000 or greater. In addition, the protein content of the calf cartilage extract was 58 percent by weight, whereas the protein content of the shark extract was 20 percent (13). Direct comparison of the molecular characteristics of the two inhibitors must await their complete purification.

If shark cartilage is extracted for 1 day (the usual practice for calf cartilage) rather than 41 days, only a small amount of activity is detected. This may be because shark cartilage possesses a more tightly bound matrix than calf cartilage, as judged by (i) the resistance of shark, but not calf, cartilage to dissolution by $4M$ guanidine and (ii) histological examination by safranin O postextraction, which showed disruption of the collagen-proteoglycan structure in calf, but not shark, cartilage.

The concentration of guanidine is also important in extracting the inhibitor from shark cartilage. Guanidine concentrations of $2M$ and above extract more material, but some of the chemicals in this material cause severe inflammation in the rabbit cornea.

While the mechanism by which angiogenesis inhibitors function is unknown, a critical step in capillary advancement is the degradation of surrounding connective tissue (14). It has been speculated that angiogenesis inhibitors block proteolytic enzymes, such as trypsin or collagenase, responsible for this degradation (1, 15). We found that shark cartilage contains about one-fiftieth of the specific trypsin inhibitory activity of calf cartilage extracts. However, the shark extract had much more potent angiogenesis inhibitory activity than the calf extract. The result is significant because trypsin inhibition had been correlated with angiogenesis inhibition in calf cartilage (1, 15).

We also found, using a film plate assay (16), that shark cartilage contains inhibitory activity against type I collagenase from rabbit cornea. For example, 3.75 mg of extract per milliliter caused 44 percent inhibition of 0.05 U of collagenase. However, when the specific activity of the shark extract was increased 20 times by fractionation on a Biogel A 1.5-m column (maximum collagenase inhibition was observed at 35,000 daltons) and this material was tested for angiogenesis inhibitory activity in the rabbit corneal assay, no significant inhibition was ob-
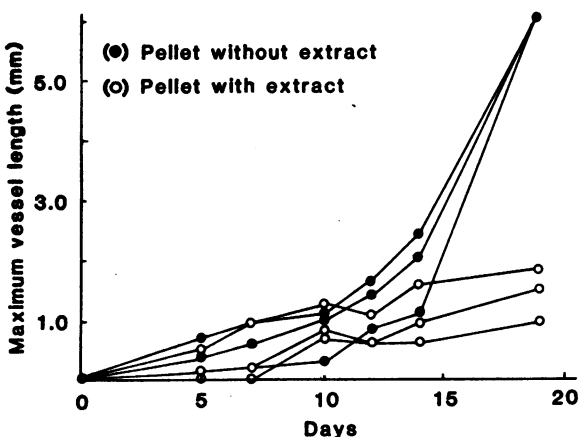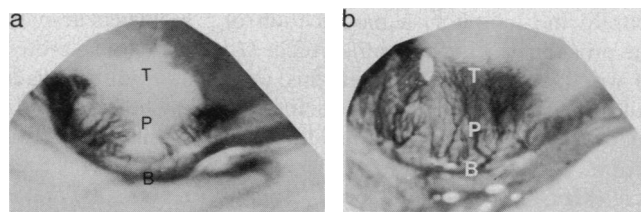

Fig. 1. Inhibition of capillary growth induced by V2 carcinoma by polymer pellets containing shark fin extract. The left corneas of three rabbits were implanted with tumors and empty polymer pellets and served as controls. The right corneas were implanted with tumors and polymer pellets containing the shark cartilage extract. The rabbits were killed on day 19 because the tumors in all the left eyes became three-dimensional and necrotic.

Fig. 2. Lower halves of rabbit corneas 19 days after the implantation of V2 tumor (T) and a polymer pellet (P) containing the inhibitor (a) or V2 tumor and a pellet without the inhibitor (b). The tip of the tumor was initially placed 2.0 mm from the edge of the cornea, and the pellet (surface area, 1 mm²) was placed directly below it with its tip 1.0 mm from the corneal edge. The blood vessels appear as a black sheet sweeping over the polymer pellet and the tumor in the control (b). However, they do not grow nearly as rapidly in the experimental cornea (a) and form a zone of inhibition around the pellet. B, bottom of cornea.

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 α1-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 α1-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 α1-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 α1-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 α1-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). α1-Protease inhibitor (α1-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, α1-PI is made functionally inactive by oxidation of two methionine residues near its reactive site (3). Neutrophil myeloperoxidase–mediated oxidation of α1-PI abolishes the formation of the α1-PI–elastase complex (4) and promotes the retention of elastin-digesting activity by elastase in the presence of the modified α1-PI (4, 5). It has been postulated that cigarette smoking decreases functional α1-PI in the lower respiratory tract, either directly by chemical oxidation or indirectly by 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 α1-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 α1-PI from smokers' BAL. Using an elastase-binding assay for functional α1-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 α1-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 α1-PI–elastase complexes with molecular sieve chromatography. The PPE-specific α1-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 α1-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 α1-PI as that from nonsmokers: 207 ± 21 versus 183 ± 23 μg, respectively. Comparable values for HNE-specific α1-PI were 182 ± 20 versus 161 ± 22 μg, respectively. Functional α1-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 α1-PI, albumin, functional α1-PI, and total protein (Table 1). Concentrated BAL fluid from smokers contained as much PPE-specific and HNE-specific α1-PI as did concentrated BAL fluid from nonsmokers (Table 1). Significant differences were not found, whether functional α1-PI was expressed as a percentage of immunoreactive α1-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