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12. L. C. Craig, *Methods Enzymol.* **11**, 870 (1967).
13. The manufacturer's stated exclusion limit applies rigorously to spherical, uncharged molecules. Although the sodium dodecyl sulfate present in the buffer should nullify charge effects, the data are interpreted in terms of relative, not absolute, size.
14. After 24 hours of dialysis, the nondiffusible material was recovered in only 0.1 percent yield; the extent of polymerization in 81 days is thus small at 65°C. However, 2.5 percent of the 85°C sample was nondiffusible, as was 5.0 percent of a "Fox-Windsor" preparation heated at 75°C for 197 days.
15. D. M. W. Anderson and J. F. Stoddart, *Anal. Chim. Acta* **34**, 401 (1966).
16. T-50 values were determined from plots of log percent remaining versus time. These plots were smooth but nonlinear, to be expected of heterodisperse systems.
17. Normalized values are presented because two dialysis apparatuses were used that did not give comparable results; replicate runs in each apparatus, however, were similar.
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19. D. L. Rohlfing, *Origins Life* **6**, 203 (1975).
20. J. P. Ferris, D. B. Donner, and A. P. Lobo [*J. Mol. Biol.* **74**, 499 (1973)] have correctly pointed out that the terms "polymer" and "polymerize" are used ambiguously (and optimistically). The exclusion limit of Bio-Gel P-2 would indicate (*13*) some molecular species in each preparation of at least about 1600 daltons, or in the neighborhood of 11 to 28 linked residues per molecule, depending on the predominant amino acid in each type of polymer [see (*1*, *10*)].
21. For the temperature range 65° to 85°C, an average activation energy of 24 ± 5 kcal/mole was calculated from the areas under the curves of Bio-Gel elution patterns and from absorbance values at 400 nm. For the range 160° to 190°C, a value of 5.5 ± 2.6 kcal/mole was obtained from published data on yield and molecular weight of thermal polyamino acids [K. Harada and S. W. Fox, *J. Am. Chem. Soc.* **80**, 2694 (1958); S. W. Fox and K. Harada, *ibid.* **82**, 3745 (1960)].
22. Kirk (*8*, p. 7). By comparison, only about 430 volcanoes have erupted in historical times [*Encyclopedia Americana* (Americana Corp., New York, 1973), vol. 28, p. 218]; hot springs and fumaroles would, of course, also afford thermal energy.
23. In almost all cases, glycine is the predominant amino acid found. A variety of sets rich in glycine, including the "Fox-Windsor" set of this study, polymerize at 175°C (*10*).
24. By using an activation energy of 24 kcal/mole (*21*), it is calculated that about 7 years at 35°C (95°F, that is, environmentally common) would yield the same extent of polymerization observed in 81 days at 65°C.
25. I thank Walter W. McAlhaney and Ricardo Moreno for capable technical assistance, John M. Herr for review of the manuscript, and Gerald T. Cowley for photographic services. Supported by grant NSG 7069 of the National Aeronautics and Space Administration.

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Isolation of a Cartilage Factor That Inhibits Tumor Neovascularization

Abstract. *A cartilage fraction isolated by guanidine extraction and purified by affinity chromatography inhibits tumor-induced vascular proliferation and consequently restricts tumor growth. This fraction contains several different proteins; the major one has a molecular weight of about 16,000. The fraction strongly inhibits protease activity.*

Tumor-induced neovascularization is inhibited by neonatal cartilage and tumor growth is thereby restricted (*1*). We now report isolation and partial purification of a cartilage fraction that inhibits the vascularization of tumors.

Segments of cartilage 1.5 by 8 cm were excised from the distal tips of scapulae of calves less than 2 weeks old and extracted in lactated Ringer solution or in 1*M* guanidine hydrochloride (0.02*M* MES, *p*H 6) for 24 hours at room temperature. Sorgente *et al.* showed that a 1*M* guanidine extract has protease inhibitory activity, and they suggest that this activity may be responsible for cartilage avascularity (*2*).

Extracts were dialyzed exhaustively against water at 4°C and centrifuged. The supernatant was lyophilized and fractionated. Sixteen fractions were obtained. The first was an aqueous extract from lactated Ringer solution. The remaining 15 were obtained from guanidine extracts. Five of these came from Sephadex G-100, three from G-200, six from ammonium sulfate precipitation, and one from the trypsin affinity column. All fractions were dialyzed exhaustively against water at 4°C, passed through 0.45-μm Millipore filters, and lyophilized. Sterile technique was followed thereafter.

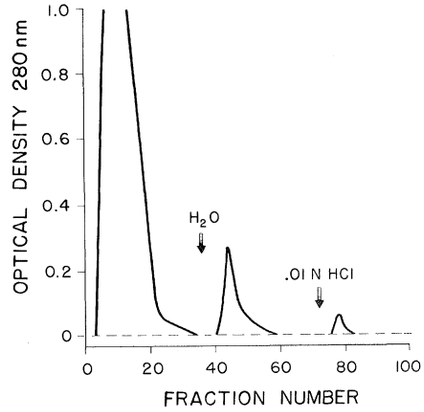

Fig. 1. Purification of protease inhibitor from cartilage by a trypsin-Sepharose affinity column. The column (1.5 by 15 cm) was equilibrated with buffer (0.05*M* tris, 0.5*M* NaCl, *p*H 8). The sample (1.45 g of guanidine extract of cartilage in 43 ml of buffer) was added at fraction 1, and buffer was added at fraction 7. The flow rate was 23 ml/hour at 4°C. Fractions of 7 ml were collected. High protease inhibitory activity was found in the HCl eluate.

Affinity chromatography with trypsin-Sepharose was used to purify the cartilage protease inhibitor. The affinity column was prepared as described by March *et al.* (*3*). We used 0.5 g of trypsin and 30 ml of packed Sepharose. Guanidine extract was passed over the affinity column, and the column was washed with buffer, distilled water, and 0.01*N* HCl (Fig. 1). The HCl eluate contained very high trypsin inhibitory activity. It migrated as one major and several minor bands on acrylamide (15 percent) slab gel electrophoresis. The major band fell between markers of myoglobin (molecular weight, 17,800) and egg white lysozyme (molecular weight, 14,400) on the gel. Approximately 500 g of cartilage produced 1 mg of this fraction.

Slow release polymers were made by mixing 5 mg of each dry fraction with 100 μl of 10 percent Elvax 40 (*4*) (an ethylene–vinyl acetate copolymer, 40 percent vinyl acetate by weight) in methylene chloride. The resulting dispersion was dried under vacuum, cut into 1-mm^3 pellets, and coated with a thin film of pure polymer. Polymer pellets containing lysozyme, alkaline phosphatase, and soybean trypsin inhibitor were prepared in identical fashion and tested in vitro to calibrate release rates and to ascertain that biological activity was not destroyed. Using agar gel diffusion assays (*5*), we found that polymers containing each of the three compounds released more than 100 ng/day of biochemically active material into aqueous media for periods longer than 100 days.

Implants of V2 carcinoma produce a neovascular response in the normally avascular rabbit cornea (*6*). Using this as a bioassay for tumor-induced vascularization, we assessed the inhibitory effect of 16 different fractions. Pellets of polymer and pieces of V2 carcinoma were placed into corneal pockets (Fig. 2A). The tumors grew as thin plaques, inducing vessels to sprout from the edge of the cornea 4 to 6 days after implantation. Vessel length and tumor diameter were measured four times each week by

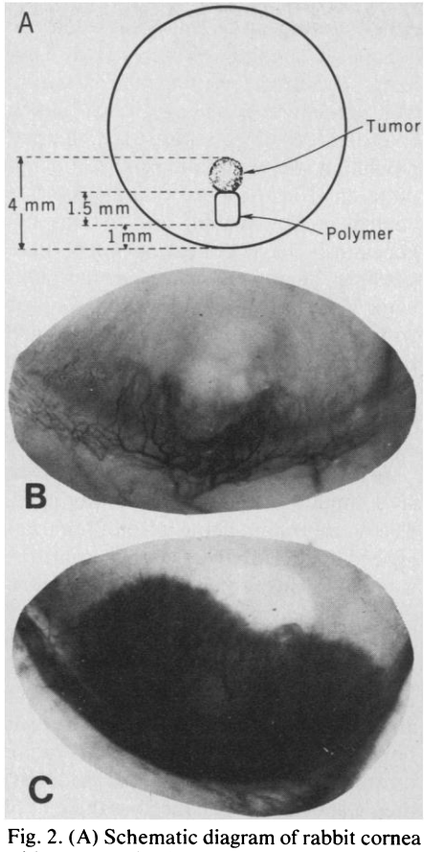

Fig. 2. (A) Schematic diagram of rabbit cornea with tumor and polymer. (B) Photograph of the lower third of a rabbit cornea containing tumor and polymer [12 days with inhibitor (the HCl eluate described in Fig. 1)]. Vessels are sparse and fail to grow in a zone surrounding the polymer. The tumor has not become vascularized. (C) Cornea containing tumor and polymer (12 days without inhibitor). Vessels appear as a dense carpet sweeping over the polymer. At this time, the tumor has already vascularized and is growing rapidly compared to that shown in (B) (~ × 7).

means of a calibrated slit-lamp stereomicroscope (accuracy, ± 0.1 mm). For each test fraction at least 12 corneas and an equal number of controls with identical tumor and empty polymer were used.

Tumor that was placed 2.5 mm from the edge of the cornea induced vessel growth rates nearly identical to those resulting from tumor and empty polymer. Empty polymer caused neither inflammation nor vessel growth. Of the 16 fractions tested, 8 caused severe inflammation, thereby preventing assessment of inhibitory activity. The remaining fractions were noninflammatory, and all except the HCl eluate from the affinity column had the same vessel growth rates as their controls.

Results obtained with the HCl eluate were highly significant. Vessel growth rates for 16 control and 21 experimental corneas with this fraction (Fig. 3) were nearly identical during the first week after the onset of neovascularization. During the second week, the rate of vessel growth in eyes with inhibitor was less than one-half that of the control group. By the third week of exposure to inhibitor, the tumor-induced vessels grew at one-third the rate of the controls and some vessels regressed (a t-test comparing controls to inhibitors in both the second and third weeks gave $P < .01$).

When polymers were empty, vessels appeared as a dense carpet sweeping over the polymer toward the tumor (Fig. 2C). When vessels penetrated the tumor, it grew rapidly into a large protruding mass occupying nearly the entire cornea, and necessitated killing the rabbits. In three rabbits, this stage was reached as early as the second week, and all rabbits with control polymers were dead by the third week. Very similar results were obtained when polymers containing fractions without inhibitory activity were tested.

By contrast, when inhibitor was present, vessels were sparse, grew slowly, and failed to grow in a zone surrounding the polymer (Fig. 2B). By the fourth week many vessels were regressing, and ten of the 21 tumors never became vascularized. Of those which were vascularized, vessels penetrated the tumor at a site remote from the inhibitor zone.

These studies show that a partially purified cartilage factor isolated by affinity chromatography will inhibit tumor-induced vascular proliferation and thereby restrict tumor growth. These data support the general concept (*7*, *8*) that (i) solid tumors pass through an avascular and a vascular phase and (ii) preventing tumors from achieving the vascular phase may be an effective approach by which to bring about tumor dormancy. This approach to inhibition of tumor growth is unique because it operates at the level of tumor vessels rather than tumor parenchyma, and because the inhibitor is isolated from a normal tissue.

The use of a noninflammatory polymer capable of releasing macromolecules over a prolonged period was critical to these experiments. This was true because fractions diffused away from the implant site rapidly and because minute quantities of inhibitor were available for testing.

Some tumors eventually became vascularized even in the presence of inhibitor. This occurred at a site remote from the polymer. One might hypothesize that the inhibitor diffused into a small zone around the polymer. A vessel growing through an area remote from this zone could reach the tumor successfully, although more time would be required because of the greater distance. Furthermore, over the time course of the experiment, the polymer is releasing a diminishing, or at best constant, amount of inhibitor, and is confronted by a gradually spreading tumor with an increasing capacity to stimulate vessels.

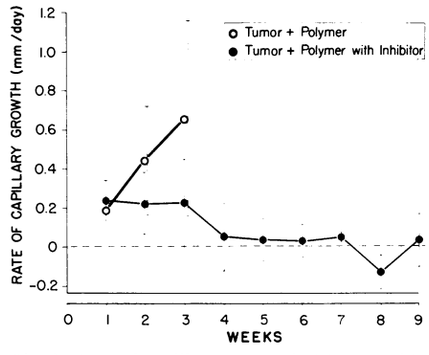

Fig. 3. Rate of capillary growth in 21 eyes with inhibitor (HCl eluate, Fig. 1) and 16 controls. Rates were determined by weekly differences in the length of the longest vessels. Calculation of rates began at the onset of neovascularization, which occurred 5 ± 1 days after implantation for each of the 37 corneas. ○, Vessel growth rates in the absence of inhibitor. All rabbits were dead by the end of the third week. ●, Vessel growth rates when inhibitor was present. By the fourth week some vessels were regressing.

Slit-lamp observations showed no adverse effect of the polymer-inhibitor combination on the avascular tumor itself. Histologic sections showed healthy tumor cells at the interface between tumor and polymer. Furthermore, the polymer-inhibitor did not retard growth of either V2 carcinoma or virus-transformed NIH 3T3 cells in culture.

The mechanism of inhibition of neovascularization by this cartilage material is unknown (*9*). These data do not provide any evidence that this inhibitor blocks tumor angiogenesis factor (*7*) directly. Furthermore, there is no evidence that this inhibitor is specific for tumor-induced vessels. Other forms of neovascularization (inflammation or wound healing) may also be inhibited. It is unclear whether antiprotease activity plays a role in this inhibition. It remains to be seen whether systemic administration of this inhibitor will suppress tumor neovascularization in a manner similar to that demonstrated here by local delivery.

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10. We thank Ivan Preis, Germaine Grant, Susan Clark, Paul Wesley, Steven Fleit, and Dr. David Tapper for valuable technical assistance.

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Conditioning of *Pleurobranchaea*

The development of the mollusk *Pleurobranchaea californica* as a preparation for the study of brain-behavior relationships was a notable achievement of Davis and Mpitsos (*1*). *Pleurobranchaea* has large identifiable nerve cells, has complex behaviors (*2*), and can be studied as a "whole-body preparation" in which electrophysiological manipulations can be performed on the nervous system of an animal with almost complete freedom of action (*3*). Reports of conditioning with this species are, therefore, of considerable interest because they suggest the possibility of a neurophysiological analysis of learning at the cellular level.

The recent report of Mpitsos and Collins (*4*) and an earlier report of Mpitsos and Davis (*5*) are concerned with this problem, but should not yet be accepted as incontrovertible proof that *Pleurobranchaea* is capable of higher forms of learning. In the earlier work, on classical conditioning (*5*), the unconditioned stimulus (US) and conditioned stimulus (CS) were combined. A glass rod (providing the CS) was dipped into a liquid food substance (squid homogenate, the US) and then stroked on the chemosensitive oral veil. Although the response of the experimental group was appropriate for classical conditioning, a likely alternative explanation for the results is sensitization. Findings suggesting sensitization are that the CS occasionally (4.5 percent of the time) produced the same response as the US and that control subjects, with separate presentation of CS and US, showed a marked increase in response. The control groups used by the authors—CS alone, and CS alone followed in 3 to 4 hours by US alone—were inadequate to eliminate sensitization as an explanation. The required control group to demonstrate true classical conditioning is one in which the US and CS are presented alternately; and, in such controversial experiments as these, additional control procedures would be valuable.

Two other weaknesses of the experimental design should be noted. A particularly serious problem was the short intertrial interval, which was 30 to 60 seconds. We have observed, in attempting to replicate this experiment and in other work (*6*), that squid-elicited feeding responses last on the order of 30 to 60 seconds, or even longer. Therefore, the unconditioned response of the previous trial might often have occurred during the CS of the following trial. Another procedural difficulty was that the US used for the controls was not the same as that used for the experimentals because it was delivered by "decanting in the vicinity of the oral veil" rather than being carried directly to the surface of the oral veil by the rod. The CS also was not the same, since it would have a different texture due to the squid homogenate.

In the Mpitsos and Davis report (*5*) an avoidance conditioning procedure was described. Animals previously "conditioned" were subsequently divided into two groups: an avoidance group, in which animals failing to withdraw within 5 seconds after rod stimulation (previous CS) were shocked, and a control group which received a series of shocks followed in 2 to 3 hours by the CS test stimuli. Here again, the control group used was not appropriate. For a control, the shocks should have been alternated with the CS so that one could see if the avoidance contingency and not just the general effect of the shocks was the factor in decreasing the response.

One possible alternative explanation for the results that the authors cited was that the decreased feeding rate was caused by short-term shock-induced inhibition. This is a likely alternative explanation and the only argument that the authors can make on this point is that the depressed feeding rate—for two subjects—persisted for 4 days during a control procedure involving touch alone. Even if more subjects were used, this manipulation still would not provide the rigorous kind of evidence that appropriate control groups would, and it does not necessarily have a bearing upon other possible explanations. Furthermore, even if the experiment were improved with proper control groups, what would be demonstrated is only the facilitation of the extinction of a classically conditioned—or perhaps, sensitized—response.

In the more recent report (*4*), an "aversive conditioning" paradigm was used. The entire training consisted of ten trials spaced 1 hour apart. On each trial, the subject was presented with squid homogenate and was immediately shocked if it responded with the usual response of a bite or strike. This is very clearly a punishment procedure. However, there was an additional contingency: if there was no bite or strike, or "sustained" withdrawal response within 180 seconds, then a shock was delivered at the end of this 180-second period. This is clearly an avoidance contingency in which the response is a withdrawal and the warning signal is the squid stimulus.

Claims by the authors that these procedures particularly "resemble" classical conditioning are misleading. *All* signaled avoidance paradigms, not just this one, have some elements in common with classical conditioning. The procedure is instrumental conditioning (actually a combination of two paradigms), because the subject's response determines the occurrence of the reinforcer.

Let us now consider whether the authors have rigorously demonstrated a behavioral change classifiable as "conditioning." First of all, there seems to be some inconsistent information regarding the control procedure. Controls "received as much stimulation" as the experimentals, "but they were given food and shock alternately (unpaired) every half hour." Note that experimentals were not always shocked and there was some variability in the shocking procedure: "contact was often unavoidable." We have found that the exact location of electrodes can have a profound effect on the efficacy of the shock. Were these factors taken into consideration when the controls were run? Another point is that although the "observations were conducted blind," the person presenting the shocks had to be aware of the contingencies, and, because of the variable na-