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Retinal Derived Growth Factor: A Regulator of Neural Regeneration and  
Revascularization in Wound Healing

Annual Report

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) Retinal Derived Growth Factor (a heparin binding growth factor that is identical to aFGF (acidic Fibroblast Growth Factor) stimulates neurite outgrowth <u>in vitro</u> . We have established a model system that will allow us to determine if a gradient of FGF can cause neurite outgrowth in the direction of the gradient. We have shown that aFGF can stimulate nerve regeneration <u>in vivo</u> and that this regeneration is predominantly due to increased neurite outgrowth by sensory nerves.					
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# Summary

Acidic Fibroblast Growth Factor (aFGF), which is identical to Retina Derived Growth Factor (RDGF), is a growth factor that stimulates neural differentiation and the formation of neurites. Work proformed under this contract is determining if a gradient of aFGF can stimulate neurite growth in the direction of increasing aFGF concentrations. Preliminary experiments establishing conditions for a slow release of aFGF over a period of several days to create such a gradient have been established. Subsequent work will determine if PC12 cells or rat sensory cells will extend neurites in response to this gradient.

We are also determining if aFGF can stimulate nerve regeneration in an in vivo animal model that measures nerve regeneration in a transected siatic nerve through a nerve guide tube. These experiments are providing evidence that purified aFGF can stimulate regeneration and that this regeneration is primarily due to an increase in neurite formation by sensory nerves.

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## FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Objective 1: To examine the effects of RDGF on the directionality of neurite extension by PC12 cells and sympathetic ganglia.

Rational and Experimental Design: We will determine if RDGF (which is clearly identical to aFGF (acidic Fibroblast Growth Factor)) can influence the direction of neurite outgrowth from when it is slowly and continuously released from a polymer of ELVAX (ethylene vinal acetate).

Results: We have successfully prepared RDGF and incorporated it into a slow release ELVAX polymer. When such a polymer is incubated with tissue culture media, RDGF is released in a graded fashion over a period of at least 3 days in a form that retains biological activity. The released RDGF can be assayed by measuring the ability to stimulate neurite outgrowth in PC12 cells as a function of concentration (Fig 1). Initial difficulties in getting the polymer to adhere to tissue culture dishes have been overcome with the discovery that either methylene chloride or crazy glue can be used to permanently fix the polymer to the dish.

Modifications of the original contract: Because of the ability of RDGF to stimulate the regeneration of sensory neurons in the rat (Objective 2), we intend to focus our efforts on the ability of the growth factor to influence the directionality of neurite outgrowth in PC12 cells and sensory (dorsal root ganglion) cells. This minor change in objectives discussed at the site visit (Nov 5, 1987) with Drs. Charles Wade and Philip Bowman who felt this was a wise change in objectives.

Objective 2: To determine if RDGF will enhance the efficiency of nerve regeneration in vivo.

Preliminary experiments: We carried out preliminary studies in mice to assess the effects of adding a laminin containing gel to the interior of a polyethylene tube. We quantified the number of myelinated axons and retrogradely labeled primary motor and sensory neurons at 2, 4, 6, 12, and 40 weeks (see table 1). These results suggested that the major effect of the protein additive was in the early stages of regeneration through the tubular prosthesis.

We attempted to obtain some electrophysiological data concerning transmission across the transection site and muscle reinnervation, but obtaining this data from mice proved to be too difficult. We decided to carry out further tests in rats since we were able to obtain electrophysiological measurements in these larger animals. The decision to change experimental animals and use rats instead of mice is a minor departure from the original contract that will allow us to carry out the scientific objectives of the contract more efficiently. We also chose to focus our initial efforts on the early time points.

Effects of acidic fibroblast growth factor (aFGF) added to the lumen of polyethylene tubes. The laminin-enriched gel mentioned above contains 80% laminin, heparan sulfate proteoglycans, Type IV collagen, and additional extracellular matrix components. We wanted to know if a more highly purified additive could stimulate PNS regeneration in a similar fashion.

Methods: Twenty-four adult male Sprague-Dawley rats were used in this experiment. Control animals were implanted with polyethylene tubes filled with Type I collagen alone (Vitrogen, N=6), or Type I collagen plus heparin (N=6) (Hepar Industries, Franklin, OH, 100 µg/ml). Experimental animals were implanted with polyethylene tubes filled with collagen containing heparin plus aFGF (N=12) (1000 units/ml). Six of the 12 experimental animals were processed for electrophysiological analyses, as reported in the quarterly report dated 6/1/87. The aFGF was purified from bovine retina or brain as described in detail in the original contract proposal. All solutions were made up in 0.1 M phosphate buffer (pH 7.4). The nerve guide tube was approximately 6 mm in length, and the nerve gap distance was approximately 4 mm. Animals were sacrificed four weeks following the initial surgery and processed as described above.

Results: The collagen alone control group was processed only for myelinated axon counts. The number of myelinated axons at mid-tube level is shown in Figure 2. Three unoperated rats were processed identically and revealed  $1071 \pm 300$  myelinated axons in the sciatic nerve. Significantly more myelinated axons were present in the experimental group with the addition of aFGF than in either of the control groups. A comparison of the number of retrogradely labeled primary sensory and motor neurons for animals which received collagen/heparin (N=6) vs. collagen/heparin/aFGF (N=6) is shown in Figure 2. Interestingly, more sensory neurons were labeled in the aFGF group, with no significant difference between the two groups in the number of motor neurons labeled.

The results of these experiments are in the final stages of being submitted to a scientific journal for publication. A copy of the final manuscript will be sent as soon as it is available.

Specific changes from the original contract that are to be implemented :

1) We will use rats rather than mice, and we will study 2, 4, 6, 12, and 40 weeks after surgery with a concentration of efforts on 12 weeks.

2) We will not test the effect of RDGF (aFGF) in the absence of heparin in tubes containing laminin because these gels already contain closely related glycosaminoglycan, so the experiment would not be meaningful (i.e., the original condition #2 will not be studied).

3) We will test the effect of aFGF in the presence and absence of heparin in unfilled nerve guide tubes at only two time points (4 weeks, when electrophysiology will be done, and 12 weeks, when axon counts and retrograde transport studies will be done). These times are chosen because these are the most interesting time points and because it would not be possible to do more experiments given the limited funds available.

#### Interpretation and Direction for 2nd Year

For the in vivo experiments during the second year of this contract we propose to focus on a 12 week survival time point. We will quantify the number of myelinated axons and retrogradely labeled primary sensory and motor neurons using the same methods as for our earlier studies. In addition, if additional funding becomes available (see below) we would be able to compare these results with animals which receive a standard peripheral nerve graft to repair the transected sciatic nerve. We will thus be able to determine if the entubulation results are comparable or superior to the standard method of repairing severed peripheral nerve. The results of these comparisons could have major implications for the repair of damaged human peripheral nerves.

The amount of manpower needed for the studies during the first year of this contract was much larger than originally requested. This point was thoroughly discussed during the site visit by Drs. Charles Wade and Philip Bowman. All parties agreed that the second year would move much more smoothly if a fulltime (as opposed to the originally budgeted 1/2 time) technician could be dedicated to Dr. Madison for this work. The cost over-runs for the first year were absorbed by other funds from Dr. Madison's laboratory. In addition, we would recommend Dr. Madison increasing his 10% effort to 25% effort for the second year.

The work is progressing nicely and is of major importance. These studies represent the second growth factor to be shown to have an in vivo effect on nerve regeneration; the first was nerve growth factor (NGF).

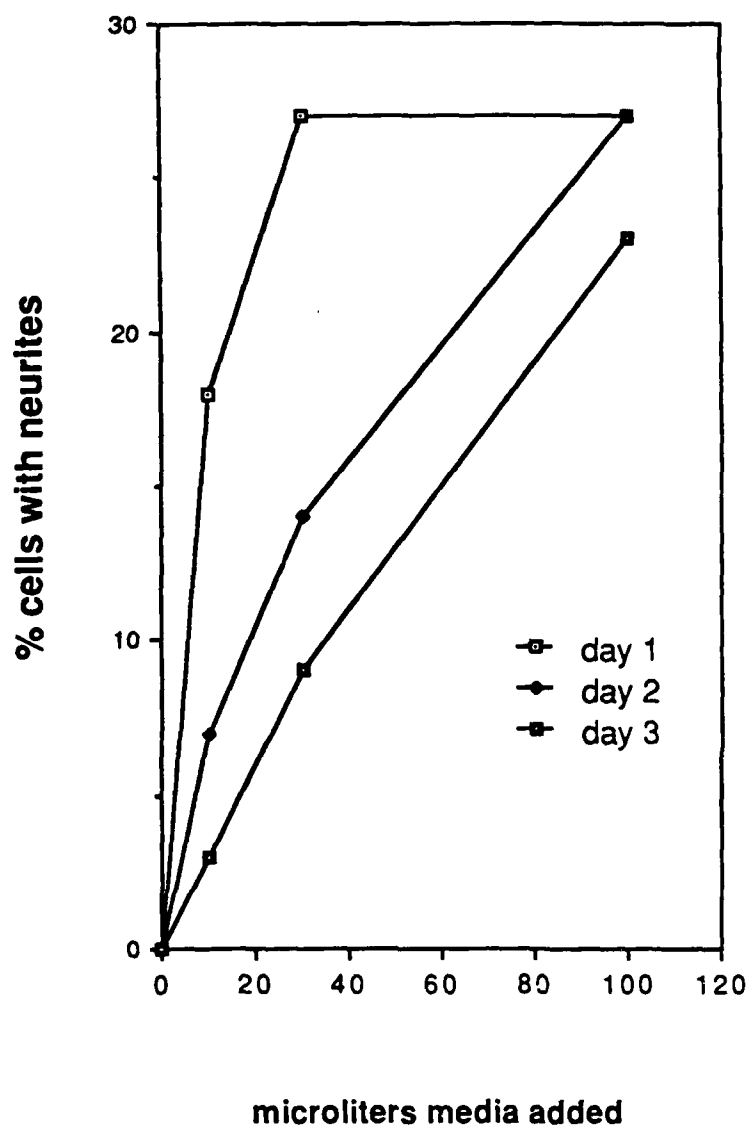


Fig 1: Release of aFGF from ELVAX. The amount of aFGF released into tissue culture media over three sequential 24 hour incubations was measured by titrating the released growth factor by measuring its ability to stimulate neurite outgrowth on PC12 cells. About 75% of the aFGF was released during the 3 days.

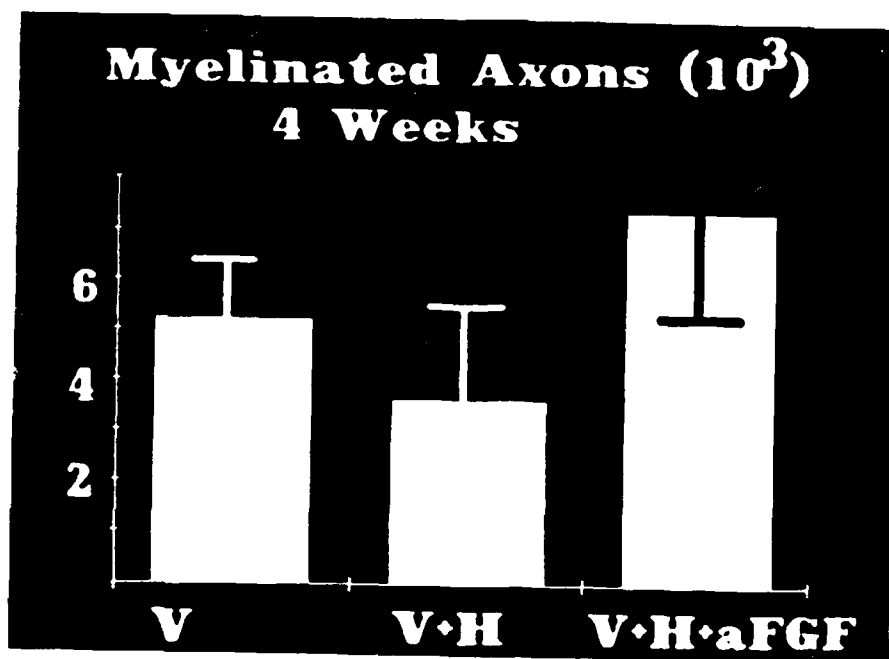


Fig 2: The number of myelinated axons crossing a nerve guide tube filled with collagen I (V), collagen I plus heparin (V+ H ), collagen I plus heparin and aFGF (V+ H+ aFGF). Note that 'V' represents vitrogen, a trivial name for a collagen I gel.

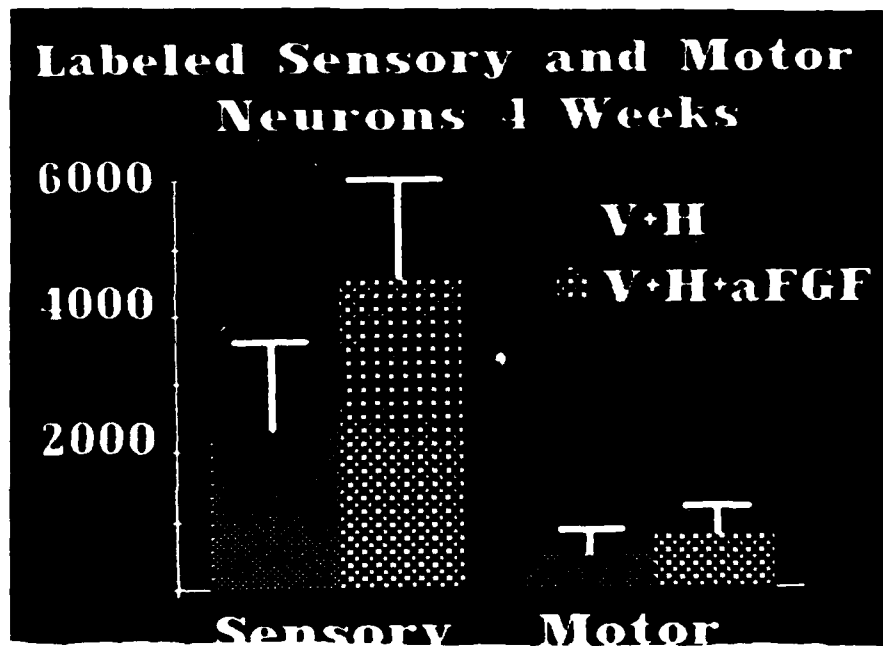


Fig 3: Labeled sensory and Motor Neurons was quantitated at 4 weeks by retrograde labeling with Horse Radish Peroxidase. Symbols are as in figure 2.

Table 1. Number of HRP-labeled cells in the spinal cord of mice with polyethylene tube implantation. Totals represents mean  $\pm$  S.E.M.

TYPE OF IMPLANT	SURVIVAL TIME (WEEKS)				
	2	4	6	12	40
EMPTY TUBES	632	666	526	690	731
	373	452	757	609	795
	607	777	748	582	700
	<u>363</u>	<u>758</u>	<u>621</u>	<u>878</u>	<u>856</u>
	494 $\pm$ 73	663 $\pm$ 74	663 $\pm$ 55	689 $\pm$ 67	770 $\pm$ 35
TUBES WITH LAMININ GEL	685	706	759	868	750
	782	758	651	823	836
	662	732	808	865	877
	<u>502</u>	<u>778</u>	<u>803</u>	<u>875</u>	<u>864</u>
	658 $\pm$ 58	743 $\pm$ 16	755 $\pm$ 36	857 $\pm$ 12	831 $\pm$ 29

Table 2. Number of HRP-labeled cells in the L3-L5 DRG of mice with polyethylene tube implants.

TYPE OF IMPLANT	SURVIVAL TIME (WEEKS)				
	2	4	6	12	40
EMPTY TUBES	1269	1516	742	1475	2330
	279	768	1772	1788	2796
	1121	1017	1060	1499	2483
	<u>546</u>	<u>1350</u>	<u>908</u>	<u>2067</u>	<u>2689</u>
	804±234	1163±167	1120±227	1707±139	2574±104
TUBES WITH LAMININ GEL	2332	1403	1373	1508	2739
	2088	1609	1331	1778	2487
	1348	1033	1789	2225	2364
	<u>990</u>	<u>1476</u>	<u>2043</u>	<u>1522</u>	<u>2508</u>
	1687±311	1380±123	1634±171	1758±167	2524±78

Table 3. Number of myelinated axons in the modportion of regenerated nerve cable of mice with polyethylene tube implants.

TYPE OF IMPLANT	SURVIVAL TIME (WEEKS)				
	2	4	6	12	40
EMPTY TUBES	581	1532	781	2180	1539
	67	206	1729	1825	1454
	209	1498	1371	1852	1697
	<u>69</u>	<u>1538</u>	<u>922</u>	<u>2067</u>	<u>1236</u>
	231±121	1193±329	1200±216	1981±86	1481±96
TUBES WITH LAMININ GEL	2122	1752	1979	2526	2237
	1724	1592	1313	2207	1505
	1322	1248	2066	2447	1802
	<u>957</u>	<u>1970</u>	<u>2769</u>	<u>2149</u>	<u>1764</u>
	1531±252	1640±152	2032±298	2332±91	1827±152

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