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# Neurite Outgrowth Induced by an Endothelial Cell Mitogen Isolated from Retina

**John A. Wagner* and Patricia A. D'Amore‡**

*Department of Biological Chemistry, and Division of Pediatric Oncology, Dana Farber Cancer Institute; and ‡Laboratory of Surgical Research, The Children's Hospital and Department of Pathology; Harvard Medical School, Boston, Massachusetts 02115

***Abstract.*** Retina-derived growth factor (RDGF) is a polypeptide growth factor purified from salt extracts of bovine retinas on the basis of its mitogenic activity for capillary endothelial cells (EC) and BALB/c 3T3 cells. RDGF is angiogenic in vivo. We show here that RDGF induces neurite extension by PC12 cells and that this neurite outgrowth is dramatically potentiated by heparin. Neurite formation elicited by RDGF in the presence of heparin cannot be distinguished from that elicited by nerve growth factor (NGF) either by the time course of neurite formation or by the morphology of the neurites at the level of the light microscope. Neurite outgrowth induced by either purified RDGF or by a crude retinal extract is not blocked by antibodies to NGF. Furthermore, neurite outgrowth induced by NGF is not potentiated by heparin and NGF is not mitogenic for capillary EC. Thus, RDGF has profound regulatory effects on cell types of very different embryonic origins. These results indicate that the physiological role for this growth factor may be far more complex than previously suspected and suggest that the formation of neural connections and the process of vascularization may unexpectedly share common regulatory elements.



RETINA-DERIVED growth factor (RDGF)[1] is an anionic endothelial cell (EC) mitogen (two forms: molecular masses of 16,500 and 18,000 D) that has been purified from bovine retina by taking advantage of its unusually high affinity for the negatively charged glycosaminoglycan heparin (1, 5). Based on its biological and biochemical similarities, RDGF appears to be closely related if not identical to anionic growth factors that have been purified from other tissues including the hypothalamus and the brain (1, 5, 22, 26). Interestingly, the mitogenic activity of RDGF, as well as an EC growth factor from hypothalamus, have been shown to be potentiated by the addition of heparin (28, 35), suggesting a role for the interaction of growth factors with heparin-like molecules.

We have assayed the ability of RDGF to stimulate neurite extension by PC12 cells. This cell line, cloned from a transplantable pheochromocytoma, has been a useful model system for the study of nerve growth factor (NGF) action and the molecular mechanism of neurite extension (14). PC12 cells have surface receptors for NGF (3); and, in the presence of NGF, extend long nervelike processes (neurites), become electrically excitable, increase the rate of neurotransmitter synthesis, make synapses with appropriate cell types in culture, and assume many of the characteristics of mature sympathetic nerves (3, 6, 30–32, 36).

Our results indicate that RDGF can induce neurite formation by PC12 cells and that this induction is strongly potentiated by heparin. These RDGF-induced neurites are similar to those induced by NGF both morphologically and in the time course of their formation yet the induction by RDGF is not blocked by antisera to NGF. These data indicate that RDGF may be a member of a new class of potent neuronal growth factors.

## Materials and Methods

### PC12 Cells

The PC12 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Flow Laboratories, Inc., McLean, VA) and 5% horse serum (KC Biological Inc., Lenexa, KS). For the neurite assay, PC12 cells were plated at $5\times10^4$ per well into 24-well plates and allowed to attach overnight. The test substances were then added, the cells were incubated for 62 h, and the number of cells with neurites were determined. The criteria for a neurite was a process with a distinct growth cone, attached to the cell body by a narrow, phase-dense process greater than one-half cell diameters in length. Processes >1½ cell diameters long were scored as long neurites. 250 cells were scored for each data point. NGF was purified as described elsewhere (27).

### Capillary Endothelial Cells

Capillary fragments were isolated from bovine adrenal medulla and the EC cultured as previously described (9). The assays measuring the effects of RDGF and NGF on the proliferation of subconfluent capillary EC and DNA synthesis by BALB/c 3T3 cells were conducted as described elsewhere (5).

1. *Abbreviations used in this paper*: EC, endothelial cell; FGF, fibroblast growth factor; NGF, nerve growth factor; RDGF, retina-derived growth factor.

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## Purification of RDGF

RDGF used in most of the reported studies was purified ~200,000-fold by the methods of D'Amore and Klagsbrun (5) and was ~50% pure. Briefly, the retinas were extracted for 3 h into Hank's balanced salt solution followed by precipitation at pH 4. The acid-soluble material was then applied to a Bio-Rex cation exchange (Bio-Rad Laboratories, Richmond, CA) column, which bound all of the cationic components including basic fibroblast growth factor (FGF) if there was any in the preparation (33). The flow-through of the cation exchange column, containing all of the acidic 3T3 and EC stimulatory activity, was then applied to a heparin-Sepharose column and the activity was eluted with 0.9–1.1 M NaCl in a 0.1–2.0 M gradient. The peak of activity recovered from this column is the material used through these studies except where noted (i.e., Fig. 2). Final purification of the RDGF to >95% homogeneity was accomplished using a Mono S cation exchange (Pharmacia Fine Chemicals, Piscataway, NJ) or a DEAE anion exchange column (P. D'Amore, manuscript submitted). The Mono S column separates the major 16,500-D form from a minor 18,000-D form, whereas the DEAE column yields the purified doublet. Presumably RDGF binds to the Mono S column but not the Bio-Rex used earlier in the purification scheme because the former is a stronger cation exchange resin. The RDGF purified by either of these protocols has been shown to be at least 90% free of cationic FGF through studies using antisera directed against synthetic peptides specific for basic FGF (Sasse, J., and M. Klagsbrun, personal communication).

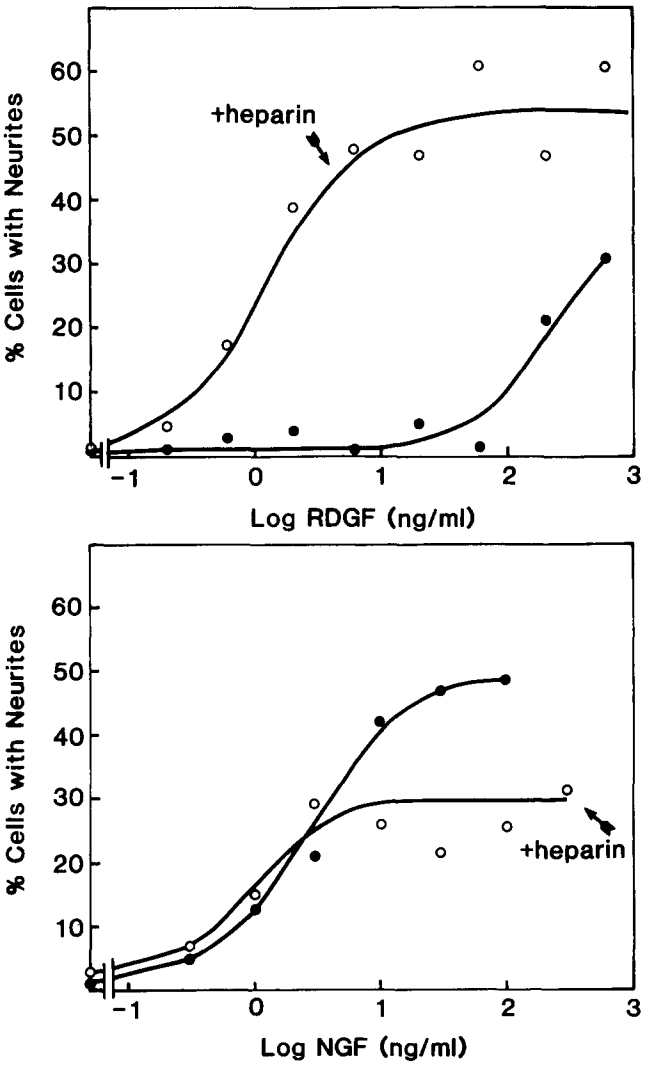


*Figure 1.* Heparin potentiates the action of RDGF but not NGF on neurite production by PC12 cells. PC12 cells were grown in the presence or absence of heparin (10 μg/ml) with increasing concentrations of RDGF (*A*) or NGF (*B*) as indicated, and assayed for the production of neurites 62 h after plating. The experiments described in this paper have all been repeated at least three times using at least two different preparations of RDGF with comparable results.

## Heparin

A previous publication by Folkman et al. (10) reported variability in various commercial preparations of heparin. The variability observed by these investigators concerned the ability of heparin to cause tumor regression when administered to animals along with cortisone. We have assayed heparins from a number of commercial sources for the ability to potentiate the mitogenic response of RDGF on EC and found no significant difference (P. A. D'Amore, unpublished data). We have therefore selected one brand of heparin (Hepar Industries, Inc., Franklin, OH), which is used in all of our reported studies.

## Results

Induction of neurite formation in PC12 cells by a preparation of RDGF that contained both high and low molecular weight forms of the factor was quantitated both in the presence and in the absence of heparin (10 μg/ml) (Fig. 1 *A*). In the presence of heparin, neurite outgrowth occurred at very low concentrations of RDGF; the concentration of RDGF required to produce half-maximal effect was ~1 ng/ml (60 pM). This was slightly less than the concentration of NGF required to elicit neurite outgrowth under identical conditions (half-maximal at 3 ng/ml; 240 pM), and was comparable to the concentration of RDGF required for half-maximal stimulation of cell division in capillary EC and 3T3 cells (1 ng/ml). PC12 cells responded to the addition of RDGF in the absence of heparin by extending neurites, but only at significantly higher concentrations (100 ng/ml). Neurite induction by RDGF in the absence of heparin was not as pronounced as the response in the presence of heparin and the effect was not saturable in this assay even at the highest concentrations tested (800 ng/ml) (Fig. 1 *A*).

Heparin potentiation of neurite outgrowth was specific for RDGF; heparin caused no significant increase in the potency of NGF (Fig. 1 *B*), and heparin alone had no effect on neurite formation. In fact, at saturating concentrations of NGF, heparin decreased the percentage of cells with neurites (Fig. 1 *B*). Furthermore, heparin did not increase the potency of two agents that are known to stimulate adenylate cyclase in PC12 (cholera toxin and the adenosine analogue, phenylisopropyladenosine) (7, 17) when neurite extension was assayed (data not shown). Thus, it appears that heparin is involved in the interaction between RDGF and the cell, rather than having an independent regulatory effect on PC12 cells.

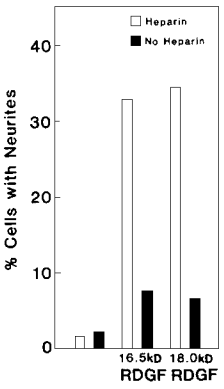


*Figure 2.* Neurite extension is elicited by both high and low molecular weight forms of RDGF. RDGF was further purified on a strong cation exchange column (Mono S, Pharmacia Fine Chemicals, Piscataway, NJ) as described in Materials and Methods. The ability of both forms of RDGF to cause neurite extension was measured in the presence and absence of heparin at 62 h after plating.

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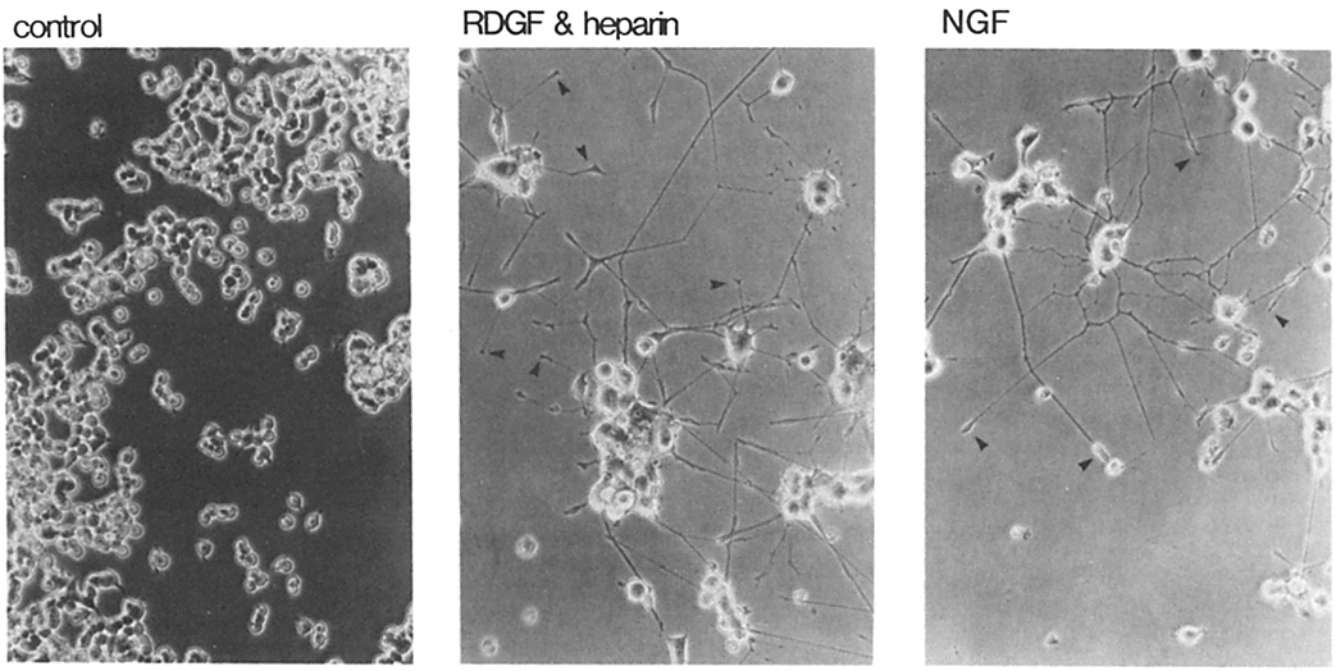


*Figure 3.* The morphological response of PC12 cells to RDGF is potentiated by heparin. PC12 cells were exposed for 10 d to NGF (50 ng/ml) or to RDGF (10 ng/ml) in the presence of heparin (10 μg/ml). The medium was changed every second day. *Arrowheads*, growth cones.

The two forms of RDGF can be separated by a strong cation ion exchange column as described in Materials and Methods. Neurites were induced in PC12 cells by both the high and low molecular weight forms of RDGF separated by this additional fractionation step, and the action of both forms was potentiated by heparin (Fig. 2). Both forms of RDGF also exhibited mitogenic activity on EC and 3T3 cells (data not shown), strongly supporting the conclusion that mitogenic activity and neurite inducing activity reside in the same peptide.

Fig. 3 shows micrographs of PC12 cells that have been treated with RDGF in the presence of heparin, cells that have been treated with NGF, as well as untreated PC12 cells. No consistent differences have been observed between neurites elicited by NGF and neurites elicited by RDGF. Each neurite is formed by a growth cone that is attached to the cell body by a narrow process. Although long neurites were induced by higher concentrations of RDGF (Fig. 4), the concentration of RDGF used in this experiment (10 ng/ml) caused formation of long neurites only in the presence of heparin.

Time course experiments revealed that there was a lag period before neurites (short or long) were produced in response to RDGF in either the presence or absence of heparin (Fig. 4). Such a lag period is also characteristic of the response of these cells to NGF (14, 36). In contrast, agents such as cholera toxin and adenosine that act by stimulating cAMP production are capable of stimulating the formation of only short neurites (i.e., processes that are <1½ cell diameters in length). These short cAMP-dependent neurites are formed more rapidly (within 8 h) than the processes formed in response to NGF (7, 17, 20) or RDGF (within 24 to 48 h); but, unlike the NGF-dependent processes, the cAMP-dependent processes do not elongate into the long neurites (>1½ cell diameters, Fig. 4). Interestingly, cAMP and cAMP agonists act synergistically with NGF to stimulate the formation of both long and short neurites. This suggests that the short, cAMP-dependent neurites may act as precursors to long neurites (17, 20). Furthermore, although RDGF alone stimulated the formation of long neurites at high concentrations (100 ng/ml), more cells produced long neurites

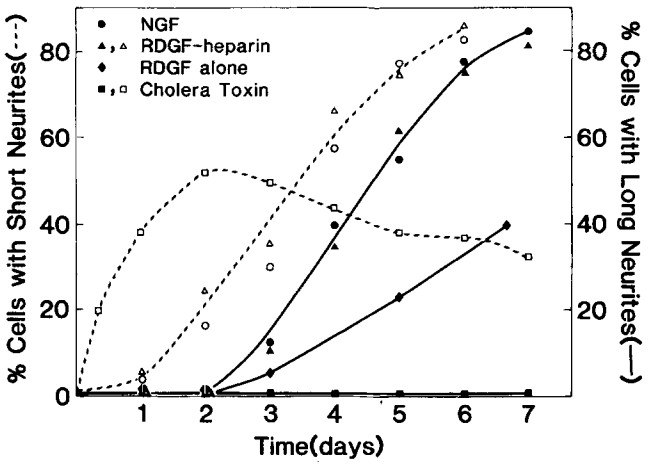


*Figure 4.* Time course of neurite extension by RDGF in the presence and absence of heparin. The time course of the formation of both short neurites (---, open symbols) and long neurites (———, closed symbols) of neurite extension is shown in response to NGF (20 ng/ml), (●-○); RDGF (10 ng/ml) plus heparin (10 μg/ml), (▲-△); and RDGF (100 ng/ml) in the absence of added heparin, (◆-◆) or cholera toxin ($3\times10^{-11}$ M), (■-□). A neurite longer than 1½ cell diameters was scored as a long neurite, while a neurite between ½ and 1½ cell diameters was scored as a short neurite. Media was changed at 48-h intervals. Using this criteria, neither adenosine nor cholera toxin effectively stimulates the formation of long neurites (16) (J. A. Wagner, unpublished data). Note that in the absence of heparin, 100 ng/ml is a subsaturating concentration of RDGF in this assay (Fig. 1 *A*).

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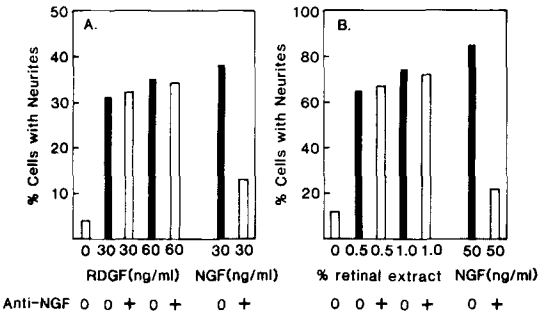


*Figure 5.* Antibodies to NGF do not inhibit the extension of neurites in response to either purified RDGF or salt extracts of retina. PC12 cells were incubated with either purified RDGF, a salt extract of retina from which RDGF is prepared (5), or purified beta NGF at concentrations indicated in the figure in the presence or absence of the monoclonal antibody (MCβ1) that blocks the biological activity of NGF (39), and assayed for neurite production at 48 (*A*) or 96 (*B*) h. The concentrations of NGF and RDGF were chosen so that a slightly subsaturating response was obtained. The concentration of MCβ1 was chosen to be three times that required to block saturating concentrations of NGF, and the antibody was preincubated with the factors for 5 h before addition to the cells.

in the presence of heparin even when 10-fold less RDGF (10 ng/ml) was used.

The possibility that RDGF was an alternatively processed form of NGF or that the RDGF preparation might have been contaminated with NGF was eliminated by three criteria. First, antibodies specific for NGF blocked neurite extension elicited by NGF, but did not prevent neurite outgrowth elicited by RDGF (Fig. 5 *A*). Thus, if RDGF is an alternatively processed form of NGF, it is immunologically distinct. Neurite-inducing activity was also detected in the salt extracts of retina from which RDGF was purified, and the neurite-inducing activity in this extract was also not inhibited by antibodies to NGF (Fig. 5 *B*). Second, SDS polyacrylamide gels revealed that, if NGF (12,500 D) is present in our preparations, it is present at <5% of the level of RDGF (data not shown); thus, contamination with NGF is probably not an explanation for the activity of RDGF in this assay unless an unusually active form of NGF is present. As a final criterion, NGF can be distinguished from RDGF because NGF did not have any mitogenic activity on either capillary EC or 3T3 cells (data not shown). Thus, NGF and RDGF appear to be distinct molecules that control an overlapping but nonidentical set of responses.

## Discussion

These data raise questions about the potential physiological role(s) of RDGF. RDGF (and the related acidic FGFs) have been shown to stimulate a wide variety of EC functions in vitro including proliferation (5), migration (19), and protease production (29), as well as angiogenesis in vivo (4, 12, 25). Thus, it was previously suspected that the primary target cells for RDGF were of mesenchymal origin. Yet, we show in this report that it also has profound effects on PC12 cells, which are derived from the neuroectoderm. This suggests that it may regulate the differentiation of diverse cell types. There is clearly a potential role for a number of different growth factors in regulating neural development (2). Investigators have noted the parallel nature of blood vessel and neural growth during development (34). Our finding that RDGF, an angiogenic factor, is also capable of inducing neurite outgrowth, provides one potential explanation for the apparent parallel between the development of the vascular and neural networks.

Although there is no consensus about the mode of action of NGF, it appears that NGF regulates both transcriptional and nontranscriptional pathways including patterns of protein phosphorylation (15, 18). Neurite production in PC12 cells is regulated by at least two distinct pathways. NGF stimulates the production of both long and short neurites after a lag of 2 d (36). On the other hand, cAMP can rapidly stimulate the formation of short neurites in the absence of NGF or act synergistically with NGF to stimulate the formation of long neurites (16, 20). The natural regulator of cAMP production in PC12 cells is thought to be adenosine (7, 17), whereas NGF action is apparently mediated by a distinct second messenger (38). Because neurite formation in response to RDGF occurs only after a lag period (Fig. 4) and because RDGF stimulates the formation of both long and short neurites with a time course similar to that of NGF, we suggest that RDGF regulates neurite production in a manner similar to NGF and distinct from adenosine.

Previous studies have suggested that brain or pituitary FGF, a cationic heparin-binding growth factor that is also an EC mitogen, stimulates neurite outgrowth by PC12 cells. The neurite outgrowth stimulated by this basic factor was reported to be transient and resemble that elicited by cAMP rather than NGF. Thus the effects of RDGF, an acidic heparin-binding peptide, appear to be qualitatively different than the reported effects of basic FGF (37).

Based on their biological and biochemical similarities, RDGF appeared to be closely related to acidic EC mitogens isolated from hypothalamus (22) and brain (8). Antisera prepared against synthetic peptides of acidic FGF cross react with purified RDGF (Sasse and Klagsbrun, personal communication). Amino acid composition (26) and sequence information (1, 8) suggest identity between these molecules though the possibility of tissue-specific posttranslational modifications have not been eliminated. Cationic FGF and RDGF (and acidic FGFs) are similar in that they both bind strongly to heparin (5, 13, 26, 33). However, the anionic and cationic EC mitogens appear to be distinct molecules by four criteria: (*a*) the salt concentration required to elute the factors from heparin (5, 13, 22, 26); (*b*) their isoelectric points (5, 13, 22); (*c*) their amino acid compositions (8, 26); and (*d*) their amino acid sequences (1, 8). We have carefully avoided the possibility that RDGF is contaminated with basic polypeptides by including, early in our purification scheme, a cation exchange column known to bind the basic factors (13, 33).

There is a precedent for the association of growth promoting activities with basement membrane. A basement membrane produced by corneal EC has been shown to promote neurite extension by PC12 cells (11, 23). A neurite-inducing factor was recently found in conditioned media associated with laminin, a major component of the basement membrane (24). These effects of extracellular matrix on cell function could be partially explained by the association of an RDGF-like molecule with one of the structural components of the matrix. Heparan sulfate and heparin-like molecules are pre-

dominant components of basement membrane glycosaminoglycans and are accessible to cells of both the vascular and neural system (21). The observation that both the mitogenic effects and the neurite inducing effect of RDGF are potentiated by heparin, taken together with the fact that RDGF binds with high affinity to heparin, suggests that the association of RDGF with heparin-like components on cell surfaces and/or in basement membranes may regulate the presentation of RDGF to vascular and neural cells in vivo.

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*Note Added in Proof*: Two recent publications have clearly demonstrated that acidic and basic heparin-binding growth factors are distinct gene products (Jaye, M., R. Howk, W. Burgess, G. A. Ricca, I.-M. Chiu, M. W. Ravera, S. J. O'Brien, W. S. Modi, T. Maciag, and W. N. Drohan, 1986, *Science (Wash. DC)*, 233:541-545; Abraham, J. A., A. Mergia, J. L. Whang, A. Tumolo, J. Friedman, K. A. Hjerrild, D. Gospodarowicz, and J. C. Fiddes, 1986, *Science (Wash. DC)*, 233:545-548).

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