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COMMENTARY

NERVE GROWTH FACTOR—RECENT DEVELOPMENTS AND PERSPECTIVES*

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Nerve growth factor (NGF) is a protein, discovered nearly 30 years ago, which has the remarkable property that it can stimulate rapid neurite outgrowth from embryonic sensory and sympathetic ganglia both *in vivo* and *in vitro* [1–3]. The factor was originally discovered as a soluble diffusible substance released by two mouse tumors (sarcomas 180 and 37), and subsequently it was found in much larger amounts in certain snake venoms and particularly in the male mouse submandibular gland.

In the review presented below, only the more recent studies on the chemical and biologic properties of NGF have been considered in detail. For information relating to the historical development of the field as well as earlier work, the reader is referred to several detailed reviews [4–7].

CHEMICAL PROPERTIES

Most information on the chemical structure of NGF stems from studies on the protein isolated from mouse submandibular gland. Nerve growth factor can exist in several molecular forms and this feature has led to the appearance of an unsystematic and rather confusing nomenclature. It apparently occurs in the glands as a high molecular weight form, which has been given the name 7 S NGF (referring to its sedimentation coefficient). This species is a complex of three different noncovalently linked proteins designated α, β, and γ [8], of which only the β-component displays the characteristic biological activity of NGF in producing neurite outgrowth *in vitro*. The function of the 7 S complex is not known, although it is possible that it is related to storage of NGF within the submandibular gland.

If the biologically active subunit of NGF is isolated from pure 7 S complex (following pH-induced dissociation), then the protein obtained has been designated βNGF. If, however, the same subunit is isolated from gland homogenates (without first purifying the 7 S complex), then the biologically active species obtained is called 2.5 S NGF [9]. This species closely resembles the β-subunit. It differs from βNGF only in that, during isolation, limited proteolysis occurs at both amino and carboxyl termini with the extent of proteolysis depending upon conditions of isolation. Since these chemical differences do not apparently affect biologic activity, we shall distinguish between 2.5 S- and βNGF only when referring to their structures.

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The complete primary structure of the biologically active protein from mouse submandibular glands is known [10]. It consists of two [11, 12] identical noncovalently linked polypeptide chains. Each chain contains 118 amino acids, 3 disulfide bonds, and a high amide content which accounts for its high isoelectric point [13]. As noted above, the proteolytic cleavages which distinguish the β-subunit from 2.5 S preparations occur at the amino and carboxyl termini [10, 14]. The amino terminal modification results in removal of the first eight residues of βNGF whereas the carboxyl terminal cleavage involves excision of the C-terminal arginine. Neither modification has been shown to have an effect upon biologic activity [14].

NGF is also found in relatively high levels in certain snake venoms [15], and the partial sequence of the factor from the venom of the cobra *Naja naja* has been determined [16]. Like mouse NGF, the snake protein is composed of two identical polypeptide chains but with only 116 residues/chain. The sequence positions of some 70 per cent of the residues have been established, and of these, the percentage of residues occupying identical sequence loci is about 65 per cent including the positions of the six half cystines—suggesting an identical disulfide structure. These studies indicate that the primary structure of NGF has been appreciably conserved over a wide range of the evolutionary scale.

MECHANISM OF ACTION

The molecular mechanism whereby NGF stimulates ganglionic neurite growth is not known. Several lines of evidence indicate that the initial event in the action of NGF is binding of the protein to the surface of sensitive cells in both sensory and sympathetic ganglia. For example, when NGF is covalently jointed to BrCN-activated Sepharose beads, the resulting Sepharose–NGF conjugate is biologically active in producing neurite outgrowth [17]. These results do not appear to stem from free NGF in the Sepharose–NGF preparation, although it is difficult to exclude the possibility that enzymic cleavage of the protein (or a biologically active segment of it) might occur at the cell surface. Preparations of NGF covalently linked (by glutaraldehyde) to bacteriophage T_4 are also biologically active in the sensory ganglion assay, although this sytem was not examined for leakage of free NGF during the biological assay [18].

Of particular interest is the observation that the amino acid sequence of mouse NGF bears certain

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striking similarities to the primary structures of insulin and proinsulin [19, 20]. For example, three of the six half cystinyl residues which occur in both insulin and NGF are in identical positions, and two of these residues are paired in the same way in the two proteins. The sequence of mouse NGF can be aligned with human proinsulin with only five deletions required to yield the maximum similarity of 21 per cent identical residues. Moreover, the majority of identical residue positions are clustered in the segments of NGF which align with the A and B chain sections of proinsulin, separated by exactly the 35 residues required to accommodate the C-peptide of proinsulin. Taken together, these observations suggest that NGF and insulin are related in an evolutionary sense and may have arisen from a common ancestral gene. Moreover, the fact that insulin [21], as well as NGF, appears to act upon the cell surface and that both proteins elicit pleiotypic cellular metabolic events could mean that the two molecules possess a similar biological mechanism of action.

Recently, several studies have appeared in which the binding of NGF to NGF-sensitive nervous tissues has been measured. Frazier *et al.* [22] observed that binding of ^{125}I-NGF to sensory and sympathetic neurons is a non-saturable process which displays multiple apparent association constants ($K = 10^{10}$ to 10^{6} M^{-1}). Unlabeled NGF accelerates the dissociation of bound ^{125}I-NGF, and the authors suggested that NGF receptors might operate in a negatively cooperative fashion [22] similar to that proposed by De Meyts *et al.* [23, 24] for insulin receptors. Insulin can also reduce the binding of ^{125}I-NGF [22], and this effect could possibly be mirroring the structural similarities between the two proteins. In light of the observation that chick embryo sensory ganglia in culture respond maximally to NGF during days 7–9 of development [6], Frazier *et al.* [22] also measured ^{125}I-NGF binding to ganglia as a function of embryo age and binding per μg of tissue protein increased from 7 to 8 days and then declined (see also Ref. 25). Thus, the temporal variation of binding parallels that of biologic activity, and it may be that loss of biologic responsiveness stems from a decrease in functional receptors. It should be noted that two other studies of ^{125}I-NGF binding to ganglia or ganglionic membrane preparations have demonstrated saturable binding. In both of these [26, 27], the maximum concentrations of NGF employed were appreciably less than those used by Frazier *et al.* [22].

Although NGF is a dimer of identical chains at concentrations in the mg/ml range, a study of the gel filtration and sedimentation properties of the protein in dilute solution reveals that the dimer is in reversible equilibrium with monomer [28]. The association constant for the reaction is 9.4×10^{6} M^{-1} at pH 7.0, and this means that, at a total protein concentration of 1 ng/ml, the equilibrium mixture contains greater than 99 per cent monomer. Consequently, over the concentration range usually employed in bioassay (1–10 ng/ml), the monomer is the biologically active species. This finding raises the intriguing possibility that a monomer ⇆ dimer equilibrium might play some role in regulating the biological action of NGF and perhaps other noncovalently linked multisubunit hormones as well. Such a mechanism would require that the dimer be biologically inactive, yet capable of competing with monomer for cellular binding sites. In this regard, it should be noted that when the two NGF chains are covalently cross-linked, the resulting molecule is biologically active [29, 30]. However, the extent of cross-linking is not known and it may be that, at high dilution (1–10 ng/ml), the noncovalent forces between chains are still disrupted, with concomitant unfolding of the dimer and exposure of the active site(s) within individual monomer chains [28]. Finally, it is also possible that the biphasic tissue binding of ^{125}I-NGF discussed above may be reflecting the existence of a monomer ⇆ dimer reaction, wherein the monomer and dimer possess different association constants toward cell receptors.

In the course of studies on binding of ^{125}I-NGF to its known responsive target tissues, Frazier *et al.* [31, 32] also measured binding to a variety of non-neuronal tissues as well as brain, with the surprising result that all peripheral organs examined, including brain, display significant levels of ^{125}I-NGF binding. Moreover, this feature is not due simply to the presence of sympathetic nerve terminals in these tissues [32]. Consequently, the authors suggested that the existence of these binding sites in non-neuronal tissues might play a role in directing the growth of sympathetic fibers as well as maintaining functional innervation. Recent observations that NGF is absorbed by sympathetic [33] and sensory [34] nerve terminals and subsequently transported to the cell bodies by retrograde axoplasmic flow may bear upon this idea. However, there is another equally interesting possibility—namely, that these widespread tissue-binding sites might represent functional receptors for NGF on cells unrelated to the nervous system.

The finding that brain exhibits ^{125}I-NGF binding sites, taken together with the observation that NGF can stimulate regeneration of adrenergic neurons of the central nervous system [35], indicates that the action of the growth factor is not confined to the peripheral nervous system. Moreover, Merrell *et al.* [36] have studied the effect of NGF upon the surface specificity of chick embryonic tectal cells in culture. When dissociated 7-day-old embryonic tectal cells are treated with NGF in culture for 24 hr, they display a change in cell surface specificity characteristic of 8-day-old tectal cells. The concentration of NGF required (about 1.2 μg/ml) is nearly 100 times the concentration necessary to produce neurite extension from sensory ganglia, and the reasons for this are not clear.

NGF does not appear to operate upon the adenosine 3′,5′-monophosphate (cyclic AMP) system of sensory ganglia. It has been recognized for some time that cyclic AMP (at rather high concentrations) can also stimulate neurite extension from embryonic sensory ganglia in culture, although the morphologic effects are considerably less striking than those produced by NGF [37–39]. However, subsequent studies revealed that NGF displays no effect upon intracellular levels of cyclic AMP or upon the activity of adenylate cyclase of sensory ganglia [40, 41]. Consequently, it appears unlikely that cyclic AMP is a second messenger for the action of NGF.

BIOSYNTHESIS OF NGF

The problem of the relationship of NGF and the mouse submandibular gland (and its high concentration in the gland) is as perplexing now as when this tissue was first discovered to contain large amounts of the growth factor. Several lines of evidence suggest that NGF may be synthesized by the gland, although its removal appears to have no obvious adverse effects upon the mouse, and other mammalian submandibular glands do not seem to contain the factor [6]. Furthermore, the radioimmunoassay studies of Hendry and Iversen [42] demonstrate that the submandibular glands cannot be the sole source of NGF in the mouse, since serum levels of the factor decrease for a short period and then return to normal 2 months after removal of both glands. Although NGF or NGF-like biologic activity has been detected in a variety of animal tissues and sera, only recently have several cellular sites of synthesis been identified.

In 1974, the highly malignant mouse L cell line (which produces fibrosarcomas *in vivo*) was shown to secrete rather large amounts of a protein which is biologically active in producing intense neurite outgrowth in the sensory ganglion assay and which by two independent immunoassays is immunochemically indistinguishable from mouse submandibular gland NGF [18]. This finding is reminiscent of the early observation on the presence of NGF in mouse sarcomas 180 and 37 [1–3]. However, although the early NGF literature consistently refers to these tumors as sarcomas, further historical search indicates that they did not originate as such. "Sarcoma" 180 was originally described in 1914 as an axillary carcinoma of a male mouse and "sarcoma" 37 arose in 1908 as an adenocarcinoma of the mammary gland [43, 44]. Thus, the precise nature of these tumors is unclear.

Further studies were undertaken to examine some chemical properties of the L-cell NGF [45]. L cells were grown in the presence of radioactive amino acids, after which the cells were removed and the culture fluid was treated with nonradioactive submandibular gland NGF as carrier. Ion-exchange chromatographic plus electrophoretic analyses of these solutions demonstrated the presence of a component indistinguishable from NGF itself.

The observation that L cells secrete NGF (or a molecule closely similar to it) is pertinent to the well-established finding that these cells are a source of so-called conditioned medium. That is, medium harvested from L-cell cultures contains factors which stimulate proliferation of other unrelated cells (see, for example, Ref. 46). In this connection, it is possible that L-cell NGF could be responsible, or partly so in concert with other secreted L-cell growth factors, for some of the conditioning effects observed.

L cells are a transformed and malignant cell line and secretion of NGF could be simply a function of this property. Consequently, other cells in culture have been examined. Table 1 presents a list of those cells which so far have been shown to produce a biologically active nerve growth factor which reacts with monospecific antibody to mouse NGF. Several features emerge from Table 1. First, both biological activity (chick sensory ganglion system) and immunoreactivity are preserved over a wide species range. Second, primary as well as transformed cells synthesize and secrete an NGF-like molecule. Third, this property is not confined to a particular cell type. Taken together, the information of Table 1, while not extensive, suggests the possibility that many (and conceivably all) cells have the capacity to secrete NGF.

Table I. Cells which screte NGF in culture

Cell type	Source
L cells [18]	Mouse
3T3 cells [18]	Mouse
SV40 3T3 cells [18]	Mouse
Primary fibroblasts [47]	Chick
Neuroblastoma [48]	Mouse
Melanoma*	Mouse
Myoblasts†	Rat
Glioma [49]	Rat
Glioblastoma [49]	Human
Primary skin fibroblasts [50]	Human
Primary synovial fibroblasts [50]	Human

* R. A. Murphy, S. M. Krane, B. G. W. Arnason and M. Young, unpublished observations

† R. A. Murphy, J. Singer, J. D. Saide, B. G. W. Arnason and M. Young, unpublished observations.

As shown in Table 1, rat C-6 glioma cells in culture [49] as well as rat glioma tumors [51] produce an NGF-like factor, and these findings are pertinent to recent studies on the effects of glial cells upon neuronal cells in culture. For example, glial cells can promote nerve fiber production by dissociated chick sensory ganglion cells [52] and they can also induce morphologic changes in mouse neuroblastoma cells [53]. Thus, it could be that one function of glial cells *in vivo* is to supply neurons with NGF. If so, then one might predict that neuronal cells would not secrete NGF. However, as shown in Table 1, mouse C1300 neuroblastoma cells (albeit, a malignant line) do synthesize and secrete considerable amounts of the factor into their culture medium [48]. This raises an interesting and unanswered question: do certain normal untransformed neurons secrete NGF (either *in vivo* or *in vitro*) to which they are also functionally responsive? In the case of neuroblastomas (both mouse and human), several pieces of information indicate that these cells in culture do respond to NGF [54, 55]. Moreover, mouse neuroblastoma cells display surface receptors for NGF—and this property is a function of the interphase cell cycle, with receptors appearing largely in late G_1 [56–58]. This raises several interesting possibilities. One is that certain cells within the clone secrete NGF and other cells respond to it. Alternatively, a given cell may secrete the factor at one stage of the cell cycle and respond to it at another stage—perhaps as part of an autoregulatory growth mechanism [48]. Whether the capacity to secrete and to utilize NGF is a property not only of neuroblastoma but also of other cells which produce it is not known.

Table 1 reveals that certain human cells in culture secrete an NGF-like molecule. By immunologic and biologic criteria, the factor is also present in human serum [50]. Based upon radioimmunoassays, sera of normal males and females contain 40–80 ng/ml of a

substance which is immunologically similar to mouse NGF [50]. Moreover, the serum levels of this factor are consistently and greatly elevated in at least one disease process, Paget's disease of bone. This disease is characterized by intense osteoblastic and osteoclastic activity in the affected areas of bone. By radioimmunoassay, of 32 patients with Paget's disease, all displayed serum levels of an NGF-like immunoreactive substance which were up to five times as high as the levels found in normal subjects [50]. After prolonged treatment with diphosphonates, 28 of 32 patients' serum levels decreased markedly and in most cases to below 100 ng/ml [50]. The meaning of these findings is not clear, but it could be that enhanced secretion of an NGF-like molecule by cells occurs when they are undergoing extensive proliferation—perhaps in the malignant state as well. If so, then a central question is why do activated cells secrete increased amounts of the factor and what does this have to do with the nervous system?

FUTURE PROJECTIONS

Despite the considerable progress that has been made in defining the chemical properties of NGF and its physiologic role, many questions remain unanswered. The most important of these, perhaps, is the physiologic scope of its action. The early studies on the biologic effects of NGF revealed that only the sympathetic nervous system was under its direct influence *in vivo* [6]. But, then, why is it that so many different kinds of cells (at least *in vitro*) secrete the factor? Is it because these cells in some way require it themselves? Do they secrete it in order to communicate chemically with other cell types, or only to maintain a functional sympathetic innervation *in vivo*? What is the biologic meaning of NGF in serum? In this regard, it will be very important to understand the biologic and structural properties of cell-secreted and serum NGF in light of our knowledge of the mouse factor. It may be that these properties are closely similar to those of submandibular gland NGF, yet slightly but significantly different. NGF is clearly part of a larger group of trophic substances that have been termed growth factors, and it is possible that many of these substances will be found to be structurally as well as functionally related.

A central question that arises with any hormone or hormone-like substance, namely its mechanism of action, remains unsolved with NGF since no "second messenger" has been defined for any NGF effect. This problem is complicated by the possibility that NGF may have more than one mechanism of action.

At present, the role of NGF in disease and its possible therapeutic uses are unknown. Nonetheless, we believe that this protein is one of the more interesting substances with which to explore the growth and metabolic regulation of cells.

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