1997

Exploration of subcutaneous bioavailability of neurotrophin

Dong Xie
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EXPLORATION OF SUBCUTANEOUS BIOAVAILABILITY OF A NEUROTROPHIN

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements of the Degree

Master of Science

by

Dong Xie

May 1997

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APPROVED FOR THE UNIVERSITY

ABSTRACT

EXPLORATION OF SUBCUTANEOUS BIOAVAILABILITY OF A NEUROTROPHIN

by Dong Xie

XNF, a neurotrophin required for the development and maintenance of the nervous system, has therapeutic potential for various diseases. However, its clinical application is undermined by poor subcutaneous (s.c.) bioavailability. To explore the fate of XNF after s.c. administration, four studies were conducted. The first was tissue distribution in rats; significant ^{125}I -XNF activity was found in the injection site. The second was a competition binding study at the injection site in order to examine the potential high-affinity binding to trkX (specific to XNF) and/or low-affinity binding to p75 (binds to all neurotrophins). Both types of binding did not significantly contribute to the disposition of XNF. In the third study, a protease inhibitor cocktail was coadministered with ^{125}I -XNF in mice; results suggested the importance of proteolysis and/or non-specific binding to extracellular matrix components. Finally, the cellular distribution study indicated that XNF was internalized and localized in secondary lysosomes of macrophages. Combined results suggest that internalization and subsequent degradation are important factors contributing to retention of XNF at the injection site.

To
my parents,
who have given me unconditional support

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INTRODUCTION

I. THE NEUROTROPHIN FAMILY

The development and maintenance of the vertebrate nervous system requires the continuous supply of a number of polypeptide hormones known as neurotrophic factors which are defined as the proteins capable of affecting survival, target innervation, and/or function of neuronal cell populations. They are a family of structurally and functionally related polypeptides, which include nerve growth factor (NGF), a prototypic neurotrophic factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), which is also known as neurotrophin-4/5. Among these, NGF was discovered first and has been the most intensively studied.

Nerve growth factor—first discovery of a neurotrophin

In 1934, Viktor Hamburger replaced the chick embryo for the amphibian larva in the study of the effects of limb bud extirpation on spinal motor neurons and sensory nerve cells innervating the limbs (Levi-Montalcini 1975). This work marked the beginning of a series of investigations in experimental neuroembryology. From his work, Hamburger concluded that the hypoplasia of motor nerve cells of the ventral horn and of other nerve cells of the same hemisection of the spinal cord resulted from lack of stimuli centripetally transmitted by nerve fibers of the first differentiated neurons. A reinvestigation of the effect produced by limb bud extirpation suggested an alternative control mechanism of the developing nerve centers by peripheral tissues. Multiple studies of silver-stained embryos were performed by Rita Levi-Montalcini, who joined the group in 1947. The conclusion was reached that the

severe hypoplasia of nerve centers deprived of their fields of innervation resulted from the death of differentiated neurons and not from the failure to recruit neurons from a pool of yet uncommitted nerve cell precursors.

An unexpected outcome occurred in 1948 when a former student of Victor Hamburger, Elmer Bueker, reported the results of an experiment consisting of the grafting of fragments of mouse sarcoma into the body wall of 3-day-old chick embryos (Levi-Montalcini 1975). The histological study of the embryos fixed 3 to 5 days later showed that sensory nerve fibers emerging from adjacent dorsal root ganglia had gained access into the neoplastic tissue, whereas no motor nerve fibers entered the tumor. Bueker concluded that histochemical properties of the fast-growing mouse sarcoma offered a favorable environment for the growth of sensory fibers. It was hypothesized that these anomalous effects could result from the release of a diffusible agent that altered the differentiating and growth properties of its target cells. This result was confirmed by Levi-Montalcini, who transplanted a mouse sarcoma onto the chorioallantoic membrane of 4- to 6-day chick embryos in such a position as to prevent direct contact between embryonic and neoplastic tissue. Embryonic and tumor tissues were in connection through the circulatory system. The finding that these extraembryonic transplants elicited the same effects as intraembryonic grafts confirmed the diffusible nature of the tumor nerve growth-promoting factor (Levi-Montalcini 1975).

The findings that mouse submandibular salivary gland extract added to culture medium elicits an even denser and more compact fibrillar halo marked the beginning of a systematic *in vivo* and *in vitro* analysis of NGF. The experimental evidence that nerve cell death is prevented by a supply of exogenous NGF (Levi-Montalcini 1975) demonstrates the vital role played by NGF in the life and differentiation of nerve cells. The subsequent

demonstration that labeled NGF is taken up by nerve endings of sympathetic or sensory fibers and is retrogradely transported to the cell provided strong support for the concept of NGF as a trophic messenger. Another important property of NGF is its ability to direct growing or regenerating axons of sensory and sympathetic fibers along its concentration gradient, which was established through various *in vivo* and *in vitro* experimental approaches.

Up to now, targets of NGF action that have been well characterized can be classified under three main categories:

- 1) neural-crest derivatives
- 2) central nervous system (CNS) neurons
- 3) cells of nonneuronal origin.

A general rule is that all cells are maximally responsive to the action of NGF during their early differentiation; the response undergoes progressive restriction in the adult. Long sympathetic neurons and sensory neurons provided a valuable system for demonstrating the three main activities of NGF:

- 1) its vital trophic role during early development stages
- 2) its property of enhancing differentiative processes such as neurite outgrowth
- 3) its property of guiding the growth or regeneration of neurites along its own concentration gradient.

In more recent years, two new populations came to the forefront of studies of NGF target cells: CNS neurons and cells originating from the hematopoietic system. Small and large

neuronal populations located in different brain areas have been shown to exhibit responses typical of sensory and sympathetic cells such as:

- 1) the presence of specific receptors
- 2) retrograde transport of NGF
- 3) increased neurotransmitter synthesis
- 4) trophic response manifested as protection against selective noxious treatments or surgical transections otherwise leading to death.

An analogous general consideration holds for the effect exerted by NGF on mast cells and possibly on other cells of the immune system. The number of mast cells increased *in vivo* and *in vitro* following NGF treatment (Aloe 1977), and NGF has an effect on histamine release (Bruni 1982, Mazurek 1986).

The sequencing of mouse submandibular gland NGF not only provided invaluable information on its primary structure but was instrumental in the preparation of synthetic oligonucleotides which resulted in the identification of NGF complementary DNA (Scott 1983). The cloning of mouse, human, bovine, and chick genes, which followed in rapid succession, demonstrated their high degree (about 50%) of homology. The NGF gene was located in the human species on the proximal short arm of chromosome 1 (Francke 1983). NGF is a dimer of two identical subunits held together by noncovalent bonds, although it has been demonstrated that a covalently cross-linked form of the dimer possesses full activity (Francke 1983).

The discovery of brain-derived nerve growth factor and cloning of neurotrophin-3 and neurotrophin-4/5

The dependency of many neurons on their target cells for normal development, and the restricted neuronal specificity of nerve growth factor suggested the existence of other such proteins. Therefore, an important progress in many laboratories, after NGF was discovered, was the search and characterization of NGF-like factors active on other neuronal populations. The factors may be divided into two major classes:

- 1) those coded by the NGF gene itself but processed through alternate post-transcriptional or post-translational pathways leading to polypeptide growth factors with somewhat different structure and function
- 2) other proteins or peptides having one or more of the trophic, chemotactic, and differentiative activities of NGF but encoded by other genes.

In 1978, Barde et al. first described the presence of a factor in a glioma-conditioned medium which supported the survival of embryonic sensory neurons and determined that the factor could be distinguished from NGF by immunological and functional criteria (Barde 1978). Furthermore, it was demonstrated that mammalian brain contained an activity similar to that of glioma-conditioned medium (Barde 1980). In 1982, Barde and colleagues reported the purification of this factor from pig brain and so named it “brain-derived nerve factor” (BDNF). It supported the survival and fiber outgrowth from cultured embryonic chick sensory neurons. Its activity migrated as one band on SDS-PAGE, where its molecular weight was determined to be 12.3 kDa (monomer) and its isoelectric point was greater than 10. It was found to promote the survival of neuronal populations that are located either in the central nervous system or are directly connected with it.

Unlike NGF, which is present in large quantities in some adult tissues that are of no relevance to the developing nervous system, the low abundance of BDNF has rendered its characterization difficult. Therefore, not until 1989 did Barde and colleagues clone the full primary structure, which is highly homologous to NGF.

Brain-derived neurotrophic factor is expressed predominately in the CNS (brain and spinal cord) and only detectable in heart, lung, and skeletal muscle in the periphery. During development BDNF is expressed in the brain at low levels initially but later increases to become the most widespread neurotrophin in the brain. BDNF has also been cloned from mouse, rat, and human (Leibrock 1989, Maisonpierre 1990).

Interestingly, the discovery of NT-3, a third member of the NGF family, was not achieved through classical protein purification procedures. Based on cloning strategies utilizing the partial sequence similarities between NGF and BDNF, the isolation of NT-3 was instead obtained by means of recombinant DNA technology by six independent groups (Rosenthal 1990, Maisonpierre 1990, Ernfors 1990, Kaisho 1990, Hohn 1990, Jones 1990). The DNA clones isolated from rat, mouse, and human encoded a protein having a strong sequence similarity to both NGF and BDNF. The distribution of NT-3 mRNA in the adult rat brain displays a high degree of regional specificity. It is predominately expressed in a subset of pyramidal and granular neurons in the hippocampus. The amino acid sequence of mature NT-3 is identical in rat, mouse, and human. That NT-3 was found to be 100% conserved between species suggests that it is a very ancient protein.

Further use of the conserved regions of NGF, BDNF, and NT-3 for polymerase chain reaction (PCR) searches of additional members of the NGF gene family has resulted in the

identification of neurotrophin-4 (NT-4) (Berkemeier 1991), described by full length sequence in the clawed toad *Xenopus* (Hallöök 1991). The biological activity of recombinant NT-4/5 from *Xenopus* is similar to that of BDNF with respect to stimulation of sensory neurons in culture. The mature human NT-4/5 has 66% homology to that of *Xenopus* and 50% homology to human NGF.

From studies of the recombinant neurotrophins it is evident that, although members of the NGF family share considerable sequence similarities, NGF, BDNF, NT-3, and NT-4/5 each have unique biological activities and they cooperate to support the development and maintenance of the vertebrate nervous system. The final number of neurotrophins involved in such a network of cell interactions functioning in brain development remains to be determined.

The biochemical characteristics and biological activities of neurotrophins

The male mouse submaxillary gland has been the most commonly used source for the isolation and purification of NGF (Bocchini 1969). The availability of milligram amounts of NGF from this organ has contributed greatly to our knowledge and understanding of neurotrophins in general. No comparable sources have yet been found for BDNF, NT-4/5, or NT-3. NGF occurs in gland homogenates, at neutral pH, as a complex of three subunits (alpha, beta, and gamma; stoichiometry: $\alpha_2\beta\gamma$) with a molecular weight of about 130 kDa and a sedimentation coefficient of 7S. The biological activity of NGF resides solely with the beta-subunit (beta-NGF). Beta-NGF is a dimer (26.5 kDa) of two identical 118-amino acid chains held together by non-covalent forces. Each chain has three disulfide bonds and their chemical reduction results in a loss of biological activity. The beta-NGF monomers are synthesized *in vivo* as large precursor molecules.

BDNF is present in extremely small amounts in pig brain where it was originally purified. The primary structure of mature BDNF dimer (27 kDa) shows a striking similarity to that of NGF. The commonality includes six cysteine residues, suggesting the presence of intramolecular disulphide bonds. As for NGF, BDNF is synthesized as a larger, precursor molecule.

The mature NT-3 dimer (27 kDa), which is probably derived from a larger precursor molecule, reveals more than 50 amino acids identical to mouse NGF and BDNF, including all six cysteine residues.

In 1993, Narhi and colleagues compared the biophysical characteristics of these three molecules by using sedimentation equilibrium and circular dichroism (CD), fluorescence, and Fourier transform infrared spectroscopy. Their studies indicated that the three proteins have similar, but not identical, secondary structures which contain primarily beta-sheets and irregular structures. NGF appears to contain the most beta-sheet while NT-3 contains a small fraction of alpha-helix. All three molecules contain disulfide bonds in similar environments, suggesting a resemblance in tertiary structure. Both NT-3 and BDNF have very hydrophilic surfaces at neutral pH, as indicated by low binding affinity to hydrophobic probes. The three molecules exist as strongly associated dimers in phosphate-buffered saline (pH 7.1).

Hundreds of studies on the biological activity of neurotrophins have been performed. Here is a summary of the specificity of neurotrophin action on peripheral and central neurons.

- 1) NGF: The responsive peripheral neurons are sympathetic, small-sized spinal sensory, and trigeminal ganglia. The responsive central neurons are basal forebrain cholinergic neurons.
- 2) BDNF: There are medium-sized spinal sensory, vestibule and nodose ganglia, and trigeminal ganglia peripherally and retinal ganglia cells, motor neurons, basal forebrain cholinergic, and substantia nigra dopaminergic neurons in the CNS.
- 3) NT-3: The peripheral neurons include large-sized spinal sensory, nodose ganglia and trigeminal ganglia neurons. The central neurons include motoneurons, locus coeruleus, noradrenergic, and cerebellar granule cells.

II. THE NEUROTROPHIN RECEPTORS

The trk proto-oncogene

The family of cell surface proteins with tyrosine protein kinase activity includes the receptors for known peptide growth factors such as insulin, insulin-like growth factor 1 (IGF-1), epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, and macrophage colony-stimulating factor (Ullrich and Schlessinger 1990). The function of other members of this family remains to be elucidated since their putative ligands have not yet been identified. These include the trk subfamily of tyrosine protein kinases such as the products of the mammalian trk (or trkA) (Martin-Zanca 1989), and trkB (Klein 1989) before their relationship with neurotrophins was found.

The *trk* gene was originally detected as an oncogene in focus formation assays conducted in 3T3 cells (Martin-Zanca 1986). The oncogene activity of *trk* resulted from a genomic rearrangement event in which nonmuscle tropomyosin sequences were aberrantly fused onto the transmembrane and cytoplasmic domains of *trk* (hence the name “tropomyosin receptor kinase”), yielding a 70 kDa cytoplasmic protein that created a mitogenic phenotype. Though the *trk* gene was discovered in a colon carcinoma, RNA analysis of a wide variety of tissues indicated that *trk* mRNA is present at low levels during development and absent in most tissues, including the colon. In situ hybridization has revealed that the highest levels of *trk* expression are confined to embryonic dorsal root ganglia and to other sensory ganglia such as the jugular and trigeminal ganglia (Martin-Zanca 1990). This important observation promoted the hypothesis that *trk* may be related to neurotrophin function. The findings of *trk* mRNA in sympathetic ganglia (Schechterson and Bothwell 1992) and basal forebrain cholinergic neurons (Holtzman 1992) have confirmed that *trk* expression is directly associated with being classic targets of NGF.

Furthermore, many studies had indicated that beta-NGF induces rapid phosphorylation of tyrosine residues when added to NGF-responsive PC12 rat pheochromocytoma cells (Maher 1988). Meakin and Shooter (1991) also reported that high-affinity NGF receptors could be immunoprecipitated with anti-phosphotyrosine antibodies. These phosphotyrosine containing NGF receptors exhibited a molecular weight of around 140 kDa–150 kDa (Massagué 1981, Hosang and Shooter 1985), a size similar to those of the *trk* family of tyrosine protein kinases (Barbacid 1991).

The trkA, trkB, and trkC receptors

In 1991, Klein and colleagues for the first time experimentally confirmed that the *trk* proto-oncogene encodes the high-affinity receptor of NGF (Klein 1991). They found that NGF binds to mouse fibroblast cells expressing *trkA* with high affinity ($K_d \approx 10^{-11}$ – 10^{-10} M). NGF could be chemically cross-linked to the endogenous *gp140^{trk}* present in rat PC12 pheochromocytoma cells as well as to *gp140^{trk}* expressed in mouse fibroblasts and in insect SF9 cells. The addition of NGF to PC12 cells elicited rapid phosphorylation of *gp140^{trk}* on tyrosine residues and stimulated its tyrosine kinase activity (Klein 1991).

The interaction of NGF with *trkA* receptors exhibits a high degree of specificity since it does not significantly bind to *trkB*, a highly related tyrosine protein kinase whose extracellular domain shares 57% homology with that of *trkA*. Similar observations have been made regarding the specificity of neurotrophic factor binding. Rodriguez-Tebar et al. (1990) have reported that competition of NGF binding at its high affinity receptors by the related neurotrophic factor BDNF requires a 1000-fold excess of protein. These levels of specificity reflect the highly specialized function of these neurotrophic factors.

The *trkB* gene encodes a transmembrane growth factor receptor-like protein closely related to *trkA* (Klein 1989, 1991; Middlemas 1991). As with *trkA*, the expression of *trkB* expression appears to be more widespread within both the peripheral and central nervous systems. Two months after the *trkA* receptor was identified, Klein et al. reported that the product of the *trkB* gene is a receptor for brain-derived neurotrophic factor and neurotrophin-3.

In the same year, the screening of a porcine brain cDNA library with a probe derived from the human *trk* proto-oncogene by Lamballe et al. (1991) revealed the existence of a third member of the *trk* family of tyrosine protein kinase genes, *trkC*. The product of the *trkC* gene was identified as a glycoprotein of 145 kDa. This *trkC* is a functional receptor for NT-3 and *trkC* does not bind the highly related neurotrophin factors NGF or BDNF. In proliferating cells, the interaction between *trkC* and NT-3 elicits a more efficient biological response than when NT-3 binds to its other receptors *trkB* or *trkA*. These results indicate that gp140^{*trkC*} (*trkC*) plays an important role in mediating the neurotrophic effects of NT-3.

The *trk* receptors are required for neurite outgrowth and cell survival. Binding of neurotrophins to members of the *trk* family produces biological responses through activation of the tyrosine-kinase domain, resulting in a rapid increase in the phosphorylation of selective cellular substrates, such as phospholipase C- γ and phosphatidylinositol 3'-kinase. Increased activity of the *ras* gene, a consequence of activation of all receptor tyrosine kinases, results from the activation of guanine nucleotide exchange factors coupled to adapter proteins which interact directly with *trk* after binding of ligand. Inactivation of *trk* tyrosine kinase by inhibitors of protein kinase, or by mutagenesis of key tyrosine residues in the cytoplasmic domain, or by disruption of the *trkA*, *trkB*, or *trkC* tyrosine kinase domains by homologous recombination results in inhibition of neurotrophin signal transduction and death of developing neuronal cells.

The non-specific receptor p75

The neurotrophin family possesses a unique receptor-signaling system that is composed of two transmembrane receptor proteins, between which there are no sequence similarities. All neurotrophins interact with these two different types of cell-surface proteins, the trk family of receptor tyrosine kinases with high affinity, and p75, the low-affinity receptor or nonspecific receptor, which was identified prior to the trk family.

The p75 receptor was originally defined by monoclonal antibodies that interfered with NGF binding and by affinity cross-linking experiments with ^{125}I -labeled NGF (Massagué 1981). p75 is a 75 kDa–80 kDa intrinsic membrane protein that binds NGF, BDNF, and NT-3 with comparable low affinity (K_d of 10^{-9} M). p75 has a relatively small cytoplasmic domain containing none of the structural motifs known to function in signal transduction for other receptors. It does not possess a tyrosine kinase domain and is expressed in all NGF-responsive cells, including sympathetic, sensory, and basal forebrain cholinergic neurons (Buck 1987, Patil 1990). This receptor belongs to a family of diverse cell surface proteins, including two tumor necrosis factor receptors and several T-cell antigens.

There are two models of p75 function. One model proposes that coexpression of both p75 and trk is required for high-affinity binding. Reconstitution studies using gene transfer and membrane fusion techniques in COS, 3T3, and melanoma cells, and in a mutant derivative of the PC12 cell have indicated that high-affinity binding requires coexpression of both receptors (Hempstead 1991). High-affinity binding appears to require a 10:1 ratio of p75 to trk, consistent with the *in vivo* levels of the two receptors in target cells. Kinetic measurements of ^{125}I -labeled NGF binding to cells expressing only trkA have indicated

that the on and off rates are both slow (half time > 10 min), accounting for the lower affinity interaction of NGF with p75 (Meakin 1991).

A second model proposes that high-affinity NGF-binding sites can be formed in the absence of p75. This model is supported by experiments performed with fibroblasts expressing only the trk receptor. These studies yielded low numbers of intermediate- or high-affinity binding sites for neurotrophins with a majority of low-affinity sites (Meakin 1992). Also, the use of blocking antibodies directed against p75 and the biological effects of mutant recombinant NGF have supported the conclusion that NGF binding to trkA, and not to p75, appears to be sufficient to elicit biological responses (Weskamp and Reichardt 1991).

Several functions have been suggested for p75. First, it may function as a presentation receptor to concentrate neurotrophins (Taniuchi 1988). Second, p75 may signal through a G protein-mediated mechanism. Third, p75 may be involved in retrograde transport of neurotrophins. Fourth, p75 may be associated with molecules capable of signaling or providing substrates for trk.

The parallel activities of neurotrophins

There are four members so far identified in the neurotrophin family: NGF, BDNF, NT-3 and NT-4/5. They all display similar biological effects on distinct but overlapping populations of neuronal cells (Table I). For example, all of the factors are capable of promoting survival and differentiation of sensory neurons, but they display markedly different activities within other cell populations. NGF, but not BDNF, enhances the

survival and outgrowth of neurites in sympathetic neurons, whereas BDNF, but not NGF, is capable of promoting the survival of nodose sensory neurons.

Table I. The Target Cell Populations of Neurotrophins

Neurotrophins	Peripheral Neurons	CNS
NGF	sympathetic	basal forebrain cholinergic neuron
	small-sized spinal sensory	
	trigeminal ganglion	
BDNF	medium-sized spinal sensory	basal forebrain cholinergic neuron
	vestibule & nodose ganglion	substantia nigra dopaminergic
	trigeminal ganglion	
NT-3	large-sized spinal sensory	motor neuron
	nodose ganglion	locus coeruleus
	trigeminal ganglion	noradrenergic
		cerebellar granule cells

Fibroblasts expressing the trk family members have been useful in demonstrating the potential relationships between the neurotrophins and their receptors (see Figure 1). By tests of cell transformation, increased autophosphorylation, and direct binding assays, many overlapping relationships have been discovered. These relationships are similar to those observed in the fibroblast growth factor and insulin growth factor families. Although NGF, BDNF, and NT-3 are the principal ligands for trkA, trkB, and trkC, respectively, trkA and trkB receptors are responsive to multiple neurotrophins (Klein 1991, Glass 1991, Soppet 1991). For example, autophosphorylation of trk in fibroblasts is induced not only

by NGF, but also by NT-3 and NT-4/5. NT-3 displays the greatest breadth, binding, and activation of each trk receptor family member when expressed in fibroblast (Figure 1).

p75, which binds all neurotrophic factors, may provide additional selectivity of binding, although its function needs to be clarified. Binding experiments with NGF, BDNF, and NT-3 have demonstrated that each neurotrophic factor binds to p75 with a low affinity, but the rates of association and dissociation vary markedly, with NGF having the fastest and BDNF the slowest off rate (Rodriguez-Tébar 1992).

Figure 1. Relationships between neurotrophins and their receptors.

Multiple interactions exist between neurotrophins and their receptor tyrosine kinases. The principal ligand-receptor interactions are denoted by heavy arrows, and weaker interactions are shown with thinner arrows. In addition, all neurotrophins bind p75 with low affinity.

III. THE THERAPEUTIC POTENTIAL OF NEUROTROPHINS

Neurons are among our most precious cells. Unlike the vast majority of other cells, however, most neurons can't be replaced if destroyed. The discovery and subsequent

characterization of a series of neurotrophins has brought great hope for people who are suffering from neurological disorders and injuries, for which current therapies are virtually nonexistent.

Peripheral nervous system

Several lines of evidence suggest that adult sympathetic and sensory neurons require target tissue-derived NGF for survival and/or function. Thus, all pathological processes which directly or indirectly affect retrograde axonal transport of tropic factors may produce alterations in these neurons. For example, sciatic nerve transection in adult rats results in modest cell losses and atrophy of the corresponding sensory ganglia. These lesion-induced deficits can be prevented by local application of NGF (Rich 1987).

Table II lists several diseases and injuries in which, researchers have proposed, neurotrophins might serve as therapeutic agents (Barinaga 1994).

In addition to physical/traumatic injuries to peripheral nerves, other circumstances may also induce altered trophic support from target tissue. These include toxic neuropathies caused by chemotherapeutic agents. Gao and colleagues reported in 1995 that NT-3 can reverse experimental cisplatin-induced peripheral sensory neuropathy. Cisplatin is a chemotherapeutic agent that is used extensively in the treatment of several types of cancers including bladder, testicular, and ovarian tumors (Williams 1979). The total dose of cisplatin that can be administered is limited by the toxic effects of the compound toward the peripheral nervous system (Mollman 1990). Although the mechanism of cisplatin neurotoxicity is not well understood, the pathological examination of cisplatin-treated patients reveals selective damage or loss of large myelinated sensory fibers, which results

in the loss of vibratory and proprioceptive sensations in corresponding distal parts of the body (Thompson 1984). In contrast, pain and thermal perceptions as well as motor nerve function are minimally affected by cisplatin treatment. Electrophysiologically, distal sensory latency is prolonged and sensory conduction velocity is slowed. As solid tumors have an apparent dose-related susceptibility to cisplatin, an agent able to reduce cisplatin neurotoxicity would be highly desirable.

Table II. The Therapeutic Potential of Neurotrophins on
Peripheral and Central Nervous Systems

DISEASE	NEUROTROPHIN
Peripheral Nervous System	
traumatic nerve injury	NGF
chemotherapy-induced peripheral neuropathy	NGF, NT-3
peripheral neuropathy associated with diabetes	NGF
amyotrophic lateral sclerosis	BDNF
Central Nervous System	
brain injury model	NGF
Alzheimer's disease	NGF, NT-3, BDNF
Parkinson's disease	NGF, NT-3, BDNF

The characterization of trk gene expression in dorsal root ganglion neurons has recently revealed that many small neuronal cells, which mediate pain and thermal sensations, express trkA, whereas the large dorsal root ganglion neurons with myelinated axons, which are involved in proprioception, express trkC. Therefore, NT-3 is a potentially

promising molecule to evaluate for protection of proprioceptive neurons from insults by toxins. Gao's study showed that NT-3 treatment *in vivo* restored to normal levels the reduced H-reflex-related sensory nerve conduction velocity induced by cisplatin (Gao 1994). Immunohistochemistry revealed morphological correlates to the electrophysiological results.

Functional alterations of autonomic and/or sensory neurons occur with high incidence in diabetes mellitus (Westerman 1989). The most common form of diabetic neuropathy is distal symmetrical sensorimotor neuropathy. It develops insidiously, most commonly afflicts sensory, autonomic nerves, and affects neurons with the longest axons first. Thus loss of sensation begins in the toes and progresses in a proximal direction; motor dysfunction might compose part of the symptoms. The loss of sensation predisposes the diabetic patients to lesions, especially of the feet, which become infected readily and heal poorly. Thus, gangrene and amputation are integrally associated with progressive diabetic neuropathies. Experimentally, diabetic rats show reduced expression of target-derived NGF as well as reduced expression of neuronal genes that are responsive to NGF. The latter is corrected by administration of exogenous NGF (Apfel 1994). Thus, deficient neurotrophic support might contribute to the pathogenesis of diabetic neuropathy, and any successful treatment might include the administration of exogenous neurotrophins.

Amyotrophic lateral sclerosis (ALS) was the first disease to be targeted for the administration of neurotrophic factors. It is a fatal condition in which the motor neurons progressively degenerate, eventually depriving patients of all movement, even the ability to breathe. A number of growth factors including BDNF and non-neurotrophic factors such as IGF-I and ciliary neurotrophic factor (CNTF) have been shown to support the growth of motor neurons in laboratory cultures. Furthermore, BDNF has been shown to aid the

healing of injured motor neurons in rat and to improve the condition of mice with hereditary motor neuron diseases similar to ALS (Barinaga 1994).

Central nervous system

The fact that NGF supports the function of forebrain cholinergic neurons, and the pronounced loss of these neurons and their related biochemical markers, has focused attention on NGF both as a factor involved in the pathogenesis of the disease and as a possible tool for clinical treatment (Phelps 1989, Hefti 1986). Forebrain cholinergic deficits may be responsible for memory deficiencies observed in Alzheimer's disease. An increased availability of NGF could retard cholinergic degeneration and improve clinical manifestations related to dysfunctional NGF-sensitive neurons in the septal area and nucleus basalis of Meynert. In behaviorally impaired aged rats, continuous intraventricular infusion of NGF for 4 weeks partially reversed forebrain cholinergic neuron atrophy and, more importantly, improved the retention of a spatial memory task (Fischer 1987).

Another potential clinical indication for NGF is in Parkinson's disease. Although NGF does not affect mesencephalic dopaminergic neurons, it is known to affect the adrenal medulla, which has been used in clinical grafting to the striatum of Parkinson patients (Backlund 1985). It has been proposed that the concomitant infusion of NGF in the proximity of the adrenal medulla graft may greatly enhance the survival of transplanted cells and facilitate their transformation to a neuronal phenotype, as observed in experimental animal models (Stromberg 1985).

NGF also appears to affect the survival of rat retinal ganglion cells following optic nerve transection (Carmignoto 1989). The expression of trkA on retinal ganglion cells suggests a

direct effect of this trophic factor on these neurons. The clinical relevance of these data remains to be evaluated; however, the retina could be more accessible (by intraocular administration) to growth factors than other CNS components.

In addition to NGF, the *in vitro* responsiveness of dopaminergic and cholinergic cells to these neurotrophins has created an opportunity for studies with NT-3 and BDNF due to the involvement of dopaminergic and cholinergic systems in neurodegenerative disorders like Parkinson's and Alzheimer's disease, respectively. Dopaminergic neurons appear to be activated when exogenous BDNF and, to a lesser extent, NT-3 are provided to the substantia nigra of both intact and dopamine-denervated animals by supranigral infusion but not intra-aortical administration (Altar 1992). In intact rats BDNF and NT-3 infusion elevated dopamine metabolism in the ipsilateral neostriatum causing various behavioral responses (Lindsay 1993). Although the findings suggest a lower potency of BDNF and NT-3 relative to NGF towards brain cholinergic systems, further studies applying other administration routes and doses of novel neurotrophins may improve their effectiveness.

IV. THE METABOLISM OF MACROMOLECULES AFTER EXTRAVASCULAR ADMINISTRATION

Neurotrophic factors have therapeutic potential in numerous neurologic diseases, and some are presently being developed for clinical application. The first human studies with a new drug are generally performed to characterize the safety and pharmacokinetics (defined as the study of the time course of drug and metabolite concentrations in biological fluids, tissues, and excreta) of a new molecule. This information will be routinely employed in the design of dosage regimens, drug interaction studies, safety evaluation, and assessment of adverse

reactions. Some classic pharmacokinetic parameters are bioavailability, clearance, distribution volume, and elimination half-life. The route of administration of a molecule (i.e., intravenous injection, i.v., intramuscular injection, i.m., subcutaneous injection, s.c., intradermal injection, i.d., and oral administration, p.o.) are likely to affect the pharmacokinetics of a drug (Notari 1980).

Extravascular administration

For most macromolecular drugs which have a molecular weight greater than 1 kDa, extravascular administration has advantages over other routes. First, oral administration, most commonly used for small molecular drugs, is not a good choice for proteins since the drugs will be digested within the gastrointestinal tract and lose their physiological activity before reaching their target tissues. Even if the proteins escape this process, they still could be oxidized, hydrolyzed, or reduced by the liver's efficient system (first-pass effect). Extravascular administration bypasses the body's very efficient detoxification system within the gastrointestinal tract.

Second, extravascular administration could be of importance for the practical use of autocrine/paracrine molecules released by peripheral target tissues (Poste 1988). In this regard, endocrine and autocrine/paracrine molecules must be distinguished. Endocrine agents are released into the bloodstream at the site of production and are then distributed via the blood to their target tissues at distant sites. Therefore systemic administration such as i.v. application is a suitable way to administer endocrine drugs. In contrast, paracrine (acting on surrounding cells)/autocrine (acting on the secreting cell) mediators, such as growth factors, act only over limited regions and are often rapidly inactivated to minimize activities at distant sites. Therefore extravascular administration might be considered an

optimal delivery system that more closely mimics distribution of the autocrine drug in the body.

Third, i.v. administration of some therapeutic proteins such as cytokines can be accompanied by significant toxicity due to transiently high blood concentrations. Extravascular administration can improve the therapeutic index (ratio of dose at which adverse events occur to therapeutic dose) and safety of a protein drug by reducing peak concentrations and resulting in protracted absorption profiles.

Fourth, some proteins are rapidly inactivated following i.v. administration. NGF has a distribution half-life of about 5 minutes and an elimination half-life of 2.3 hours following i.v. injection. When administered by subcutaneous injection, the apparent half-life is prolonged to 4.5 hours. Hence, extravascular administration can reduce the dosing frequency.

Finally, extravascular administration such as s.c. injection would make it possible for those patients who require long-term treatment to self-administer the drugs at home.

Tissue distribution and metabolism of macromolecules after subcutaneous injection

After s.c. injection, macromolecules enter blood via two pathways. One is by direct transport in blood vessels, which is driven primarily by concentration gradients. The second is by absorption into the lymphatic system followed by transport into blood at the jugular vein where the thoracic lymph duct merges with the blood circulation (Figure 2).

At the injection site, the protein could be degraded by extracellular proteolysis, intracellular proteolysis, and phagocytosis following aggregation. The neurotrophins could bind to trk, p75, and/or non-specific extracellular matrix components (ECM). Some of these binding and aggregation processes are reversible.

Figure 2. Macromolecule fate following injection into subcutaneous space.

The lymphatic system

The lymphatic system consists of a whole-body network of vessels, lymph nodes, and lymph that originates in interstitial fluid which in turn is derived from plasma exudate. The major physiological function of the lymphatic system is to maintain the body's water

balance. Thus, lymph is a drainage system, which returns excess fluid, protein, lipophilic compounds, and waste products from the interstitial space to the vasculature.

Following extravascular administration, a drug can be transported into the general cardiovascular pool either by the blood capillaries or by the lymphatics (see Figure 2). As early as 1921, Lewis et al. injected horse serum s.c. into dogs and detected it in thoracic duct lymph after 40 minutes and in blood after 3.5 hours. Field and Drinker (1947) also demonstrated that horse or dog serum was absorbed mainly by the lymphatics in the dog when injected subcutaneously. Winstein and co-workers (1984) proved that after s.c. injection, monoclonal antibodies enter local lymphatic capillaries and pass to the draining lymph nodes, where they bind to target cells. Antibodies not bound in local nodes passed to more distant nodes. If still not removed from the lymph fluid, antibodies entered the blood through the thoracic duct (Ballard 1968).

In 1988, Supersaxo et al. studied the effect of different parenteral administration routes on the cumulative recovery of human interferon alpha-2a in lymph and on its concentration in the blood and lymph of sheep. Blood samples were taken from the jugular vein, and lymph was collected via cannulation of the efferent vessel of the popliteal lymph node. The results showed that, following s.c. administration, approximately 59% of the dose of the interferon alpha-2a was recovered in the popliteal lymph of the cannulated leg. The authors suggested that, following extravascular administration, the molecule was transported mainly by the lymphatics draining the site of application.

A subsequent study by Supersaxo et al. (1990) was performed to assess the effect of molecular weight on the lymphatic transport of water-soluble compounds following s.c. administration. The results showed that in the molecular weight range used (200–19,000)

there is a linear relationship between the molecular weight and proportion of the dose transported lymphatically. Higher molecular weight was associated with enhanced lymphatic absorption.

The differential access of molecules with varying molecular weights from the tissue space to blood and lymphatic capillaries may also be related to the anatomy of the lymphatic system of skin and subcutaneous tissues as well as structural differences between blood and lymphatic endothelial cell lining. The skin contains a superficial lymphatic plexus in the dermis. The endothelial lining of the blood capillaries have a continuous and uninterrupted subendothelial basement membrane. In contrast, terminal lymphatics do not possess this membrane. The lack of a basal lamina within lymphatic capillaries may be of major functional importance in facilitating access of interstitial macromolecules to the lymphatic system. In addition, adjacent endothelial cells of terminal lymphatics may have gaps from 20 nm to 100 nm. These gaps make it possible for macromolecules and even larger particles of up to 1- μ m diameter to enter the lymphatics (Leak 1984).

In addition to absorption and distribution, the lymphatic system is important for the disposition of macromolecular drugs administered extravascularly. In general, the chemical composition of lymph is very similar to that of plasma and many components differ only in concentration. It contains all the protein fractions of plasma including albumin and macroglobulin, which are the most important binding proteins for drugs. Many of the enzymes present in plasma are also found in the lymph. In lymph draining tissue in which an enzyme is produced the concentration of the enzyme may be higher than in plasma. This is true for peptidases, hydrolases, and proteases, which are responsible for the biotransformation of therapeutic proteins via hydrolysis into peptide fragments (Yoffey 1970).

V. THE PHARMACOKINETIC PROFILE OF XNF

The intent of this thesis is to demonstrate the student's mastery of the scientific protocols, procedures, techniques, and analyses of neurotrophin distribution. To that end, any of the identified neurotrophins described in the literature could have been used. The actual neurotrophin used in demonstrating these abilities will be identified as XNF throughout the remainder of the paper. Where necessary, the identity of binding proteins, physicochemical characteristics, or other experimental variables which could reveal the identity of XNF are also masked.

Due to its broad therapeutic potential in peripheral neuropathy and degenerative diseases in the central nervous system, XNF has been pursued for clinical application by several biopharmaceutical companies.

In early development, a pilot study was performed to characterize the pharmacokinetics of XNF in a relevant species (rat). This information is critical for the preclinical development of a molecule. Two rats were used in the pilot study. Rat A was given 1 unit/kg XNF by i.v. injection through a femoral vein cannula. Rat B was given XNF by s.c. (5 units/kg) injection into the scapular region. Blood samples were collected from a jugular vein cannula for both rats at various timepoints (see Table III and Figure III). The XNF concentration was determined by an enzyme-linked immunoabsorbent assay (ELISA) with a sensitivity of 0.2 ng/ml.

The bioavailability of XNF following subcutaneous administration (F_{sc}), defined as $(AUC_{sc}/dose_{sc})/(AUC_{iv}/dose_{iv})$ where AUC is the area under the concentration-time

profile as measured by the trapezoidal rule (Rowland 1989), was 7%. On average, the Fsc of therapeutic proteins is around 70% (personal communication with Dr. V. Hale, Genentech). This means that 93% of s.c.-administered XNF did not reach blood. For that material, which did reach blood, the i.v. data show that the XNF concentration drops 100-fold within 30 minutes after administration, which indicates a very rapid clearance of XNF from blood. This extremely low bioavailability can limit a molecule's clinical utility if it is not very potent. The cause of the low bioavailability is unknown and because of the magnitude of the result, is worthy of further study.

Table III. The Plasma Concentration of XNF Following S.C. and I.V. Injection in Rats*

Time (hr)	IV concentration (ng/ml)	Time (hr)	SC concentration (ng/ml)
pre-dose	0	0	0
0.33	11729	1	99.1
0.83	6793	3	83.7
0.165	3913	5	61.2
0.25	1563	6	41.2
0.5	22.87	7	28.3
0.75	3.95	8	16.45
1	1.93	9	10
2	0.9	24	0.25
3	0.49	28	0.25
4	0.25	32	0.25
6	0.28	48	0.25
8	0.25	52	0.25

* Assay performed by G. Bennett, Genentech, Inc.
Abstracted from Genentech Report No. 95-297-1137.

Figure 3. Pilot XNF concentration data in rats (N=1). IV dose=1 unit/kg; SC dose=5 units/kg.

The objectives of this thesis project are:

- 1) to examine the distribution of XNF in rodents (rat/mouse) following s.c. administration.
- 2) to focus on the tissues that retain the largest quantities of XNF to explore the disposition of XNF related to:
 - a) binding: specific binding to trkX or non-specific binding to p75.
 - b) biotransformation: hydrolysis by proteases.
- 3) to visualize the cellular distribution of XNF in specific tissues/organs that retain XNF following s.c. dosing.
- 4) to identify the major causes of low bioavailability of XNF in rodents.

MATERIALS AND METHODS

I. RADIOLABELING OF XNF

Recombinant human XNF was obtained from Genentech. XNF was radiolabeled with ^{125}I using the lactoperoxidase method as described previously (Rohrer and Barde 1982; Rodriguez-Tebar 1992, Escandon 1993) with minor improvements. The purity of the protein preparations was confirmed by SDS-PAGE, which indicated >95% purity as judged by Coomassie blue staining of the gels (BioRad, Richmond, CA). Mass spectrometric analysis of the preparations was in agreement with >95% of the full-length, unprocessed form of protein. XNF was iodinated using Enzymobead reagent (BioRad, Richmond, CA).

Briefly, the beads were rehydrated in 2 ml of distilled water overnight at 4 °C, centrifuged, and resuspended in 180 μl of distilled water. Fifty microliters of the Enzymobead suspension was added to the iodination reaction as described by the manufacturer. About 20 μg of XNF in 20 μl excipient buffer (10 mM NaAcetate, 140 mM NaCl, pH 5.5, filtered) was mixed with 37.5 μl 0.4 nM NaAcetate (pH 5.6) and 15 μl lactoperoxidase (1 IU/ml), made by diluting 100x stock (Bovine Milk Lactoperoxidase (Calbiochem Lot No. 4267488) 100 IU reconstituted in 1 ml SQ (distilled) water, stored as 10- μl aliquots at -70 °C). After adding 20 μl ^{125}I -Na (2 mCi), the reaction was started by adding 15 μl hydrogen peroxide and subsequent vortexing and incubation for 5 minutes. This process was repeated once. The reaction was stopped by adding 15 μl of 20 mM N-acetyl-L-tyrosine (Sigma, St. Louis, MO), made up in SQ water, stored at 4 °C). Labeled XNF was

separated from free iodine using desalting columns (Pharmacia Biotech, Sweden). 5 µl of reaction mixture was diluted resulting in 1:20 serial dilution for the trichloroacetic acid (TCA) assay. At the end of the incubation period, about 90% of the total radioactivity could be precipitated by TCA. The mean specific activity of ^{125}I -XNF was found to be 1.5×10^5 cpm/ng, corresponding approximately to one radio-atom per monomer of XNF. The mean concentration of stock ^{125}I -XNF was about 13 ng/µl. ^{125}I -XNF was used within 14 days of preparation.

II. TISSUE DISTRIBUTION STUDY FOLLOWING SUBCUTANEOUS ADMINISTRATION OF XNF IN RATS

Animals

Four adult female Sprague-Dawley rats (Charles River, Hollister, CA) with an average body weight of 350 grams were divided into two groups of two animals with timepoints of 2 hours and 6 hours. All animals were acquired, cared for, and surgically handled in accordance with the guidelines of the National Institutes of Health (NIH) "Guide for the Care and Use of Laboratory Animals."

Subcutaneous administration of XNF

The rats were preblocked with a dose of 100 µl of Sodium Iodide (50 mg/ml, i.p.) 24 hours before the experiment. On the day of dosing, the rats were anaesthetized by Ketamine cocktail (composed of Ketamine, Xylazine, and 0.9% sodium chloride at 1:1:2)

(about 0.5 ml/rat) i.p. and subcutaneously administered with 200 μ l of 125 I-XNF at the right thigh region previously shaved to about 4 cm² . All animals were allowed to roam freely in their cages and were provided access to food and water throughout the experiment.

Tissue harvest and homogenization

Two hours and six hours after injection, the rats were deeply anaesthetized by Ketamine cocktail (Ketamine 90 mg/kg + Xylaine 10 mg/kg) i.p. (0.8 ml/rat). About 600 μ l of blood was withdrawn from the left ventricle of each rat and collected into EDTA-containing tubes and put on wet ice. Then the animals were immediately perfused transaortically with a mixture of 4% paraformaldehyde (room temperature) in 0.1 M phosphate buffer (pH 7.4) and the carcasses were stored at 4 °C. The cardiac blood was centrifuged at 3000 rpm for 5 minutes and the supernatant (plasma) was frozen for further processing.

On the following day, the target tissue and organs including adrenal gland, brain, heart, inguinal nodes, kidney, liver, lung, mesenteric nodes, pancreas, spinal cord, spleen, stomach, subcutaneous tissue (injection site), and thymus were excised. The subcutaneous tissue included stratum corneum, epidermis, dermis, and underlying connective tissue (fat, fascia). The fur on the skin was removed by scraping with a knife. Each piece of tissue sample was cut into two pieces. One-half was used for autoradiography and the other half was used for immunoprecipitation. These tissue portions were respectively weighed and counted in a gamma counter (Auto-Gamma counter 5000 series, Packard, IL) with an efficiency approaching 95%, washed with phosphate buffered saline (PBS), and subsequently stored at -70 °C.

Two days (48 hours) after perfusion (stored at -70°C), the tissue samples for immunoprecipitation were minced using scissors and soaked in a corresponding volume of ice-cold immunoprecipitation buffer (PBS containing 20 mM EDTA, 1% Triton X-100, pH 8.0, stored at 4°C until used). The volume of buffer in μl was 10 times the tissue weight in mg. The tissues were lacerated using a homogenizer (Tekmar, Cincinnati, OH) (bubble formation during homogenization was avoided) and subsequently centrifuged at 3000 rpm for 15 minutes at 4°C . 1100 μl of supernatant was centrifuged at 14000 rpm for 1 to 2 minutes and then 1000 μl of supernatant was placed over wet ice for further immunoprecipitation analysis.

Immunoprecipitation assay

The Preparation of Protein A-Antibody Complex

Protein A was purchased from Sigma Chemical Company (Catalog No. P-3391, Lot No. 64H0408) as 1.5 grams of lyophilized powder (1 g of powder swells to approximately 4 ml) with binding capacity of approximately 20 mg of human IgG per ml. One and one-half grams of protein A was washed with 40 ml of ice-cold PBS at least three times to remove stabilizers (dextran and lactose) by centrifuging at 3500 rpm for 5 minutes at 4°C . As the final step, the fresh, cold PBS with the equal volume of swelled dry protein A was added. This mixed volume was counted as the final volume of protein A solution.

The antibody was polyclonal anti-mouse anti-neurotrophin antibody (source masked to disguise identity) raised in rabbit. This antibody is known to be able to react with all human

recombinant neurotrophins. The antibody was reconstituted by adding 1 ml of distilled water to solubilize the protein. The 20- μ l aliquots were stored at -20°C until used.

The protein A solution was mixed with anti-mouse neurotrophin serum in a 10:3 ratio and incubated on a constant shaker at 4°C for 4 to 6 hours. The solution was centrifuged at 2500 rpm for 5 minutes at 4°C . The supernatant, which contained free antibody, was then removed. An equal volume of immunoprecipitation buffer was added. Care was given not to disturb the protein A-antibody complex on the bottom of tube. The complex was further examined by visual inspection and used within 1 week of preparation.

Immunoprecipitation Assay

The individual organ homogenates were counted (pre-immunoprecipitation count, preipp). 75 μ l of the protein A-antibody solution was added to each sample and gently agitated in a shaker at 4°C overnight. For plasma samples, 900 μ l of immunoprecipitation buffer was added to 100 μ l of plasma before adding protein A-antibody complex. On the next day, the samples were washed four times: briefly, after centrifuging the samples at 13,000 rpm for 2 minutes, the supernatant (containing the free ^{125}I) was aspirated and replaced with 1 ml of fresh, ice-cold immunoprecipitation except for the final step of washing where, instead of replacing fresh immunoprecipitation buffer, 40 μ l of SDS loading buffer was added (see details below) and the tissue or plasma were subsequently counted as immunoprecipitated XNF activity (post-immunoprecipitation count, postipp).

SDS-PAGE and Autoradiography

The SDS loading buffer (sample buffer, Novex, San Diego, CA) (Catalog No. LC 2676, Lot No. LSS60418) was diluted 1:2 from stock with SQ water and b-mercaptethanol (100%) in the hood in a ratio of 970:30. The loading buffer was stored at room temperature and used within 2 weeks after preparation. The SDS running buffer was diluted 1:10 with distilled water from 10x stock solution obtained from the Medium Preparation Department of Genentech. The molecular weight standard was purchased from BioRad (Catalog No. 161-0318, Lot No. 75372). The XNF control was prepared by diluting the stock ¹²⁵I-XNF, 2000-fold with XNF column buffer (1.0 mg/ml human albumin, 0.5 mg/ml protamine sulfate in NT-Y excipient buffer (10 mM Na-acetate, 140 mM NaCl, pH 5.5)). Both standard and XNF control were boiled at 100 °C for 5 minutes. The Tris-Glycine SDS mini gel (1.0 mm, 10 well size) was purchased from Novex and stored at 4 °C until used.

The immunoprecipitated samples (containing 40 µl of SDS loading buffer) were boiled in a 100 °C bath for 5 to 10 minutes and centrifuged at 13,000 rpm for 1 minute to separate the protein A-antibody complex from XNF.

20 µl of each tissue and plasma sample supernatant, 15 µl of molecular weight standard, and 15 µl of XNF control were placed onto 16% Tris-Glycine homogenous gels. No reducing agents were added. 140 volts was applied for about 1.5 hours. The gels were subsequently stained with 20 ml of Coomassie Blue and 20% glycerol and placed on the shaker at room temperature for about 1 hour. The destaining buffer (50% methanol, 10%

acetic acid) was replaced and agitation proceeded for an additional 1 hour at room temperature. The gels were then air-dried for 2 hours at 80 °C.

The dried gels were exposed to Kodak X-OMAT film (Catalog No. 165-1579) for a designated length of time (see gel picture in Results) in a light proof cassette containing an intensifying screen (Sigma). The films were developed in Kodak D19 Developer (Catalog No. 146-4593) and fixed in Kodak Fixer (Catalog No. 197-1746).

III. THE INJECTION SITE PROCESSING OF XNF: BINDING STUDY IN MICE

Based on the results from the Distribution Study, we further explored the disposition of XNF at the subcutaneous injection site. The pilot study of XNF bioavailability following subcutaneous administration in rats (see Introduction) showed that the XNF concentration in plasma reaches its peak about 1 hour after injection. Therefore, in order to explore the injection site processing as a function of time, 6 timepoints (10 min, 20 min, 30 min, 1.5 hr, 6 hr, and 24 hr) after injection were selected. There were three experimental groups:

Group 1 (control) : ^{125}I -XNF (2 $\mu\text{g/kg}$)

Group 2: ^{125}I -XNF (2 $\mu\text{g/kg}$) and excess cold XNF (200 $\mu\text{g/kg}$)

Group 3: ^{125}I -XNF (2 $\mu\text{g/kg}$) and excess cold NT-Y (200 $\mu\text{g/kg}$)

A total of 54 animals were studied: three groups at six timepoints and three animals per timepoint. Due to the large number of animals needed, the Genentech veterinary staff recommended that mice, rather than rats, be used

Animals

Female CD-1 mice were purchased from Charles River (Portage, CA). The age range was 7 to 9 weeks. All animals were acquired, cared for, and surgically handled in accordance with the guidelines of the NIH "Guide for the Care and Use of Laboratory Animals."

Organization of study groups

Within a particular experiment, there were three experimental subgroups, each receiving a different treatment. Within each experimental group, triplicate analyses were performed. After results were obtained, the triplicate individual samples were assigned a classification, of either high, medium or low (H, M, or L), based upon total count results. This assignment was performed for the purposes of accurate interpretation of gels (i.e., to avoid the comparison of an H sample value from one group to an L value from another when signals were low and could have been misinterpreted). The gel results shown here represent the M values, all of which were run on the same gel. The L and H gels showed similar results, but with different signal intensities.

All samples were placed over wet ice except during gamma counting which usually required no more than 30 minutes. Afterward, the samples were stored at -70°C . The samples were all analyzed within 2 weeks.

Dosing preparation

From a previously performed pilot study, 50 µl was determined to be an appropriate total volume for abdominal subcutaneous injection in mice. Group 1 received 50 µl of radiolabeled XNF solution. For groups 2 and 3, ¹²⁵I-XNF and competing molecule (cold XNF or cold NT-Y) were prepared separately as 25 µl. The solutions were then combined in a 1:1 ratio to make 50 µl of final dosing solution. No precipitation, viscosity, or color changes were visible after mixing.

The radiolabeled XNF (Lot No. 21599-23) was prepared 3 days before dosing. The concentration was 13 ng/µl with specific activity of 1.9×10^6 cpm/µl (0.146×10^6 cpm/ng). Cold human recombinant XNF (Lot No. 21592-50) was obtained as a clear liquid in a sterile glass vial containing approximately 5 ml of a 0.62 mg/ml solution (Genentech). The human recombinant NT-Y was also provided by Genentech as clear solution with a diluted concentration of 1 µg/ml (Lot No. M3/M3-RD 206). All drugs were stored at 4 °C until dosing.

Group 1: Dosed with ¹²⁵I-XNF alone:

Taking the average body weight (estimated) of mice as 30 grams and the dosing

as 2 µg/kg, the amount of administered ¹²⁵I-XNF was:

$$2 \mu\text{g/kg} \times 0.03 \text{ kg/mouse} = 60 \text{ ng/mouse}$$

The dosing concentration was:

$$60 \text{ ng/mouse} \div 50 \mu\text{l/mouse} = 1.2 \text{ ng/}\mu\text{l}$$

Group 2: Dosed with ^{125}I -XNF (2 $\mu\text{g/kg}$) and cold XNF (200 $\mu\text{g/kg}$, 100-fold excess)

For radiolabeled XNF: the concentration of pre-mixing solution was:

$$60 \text{ ng/mouse} \div 25 \text{ } \mu\text{l} = 2.4 \text{ ng/}\mu\text{l}$$

For cold XNF: the amount needed for each mouse was:

$$200 \text{ } \mu\text{g/kg} \times 0.03 \text{ kg/mouse} = 6 \text{ } \mu\text{g/mouse}$$

The concentration of pre-mixing solution was:

$$6 \text{ } \mu\text{g/mouse} \div 25 \text{ } \mu\text{l/mouse} = 240 \text{ ng/}\mu\text{l}$$

The final concentrations in mixed dosing solution of radiolabeled XNF and cold XNF were 1.2 ng/ μl and 120 $\mu\text{g/}\mu\text{l}$, respectively.

Group 3: Dosed with ^{125}I -XNF (2 $\mu\text{g/kg}$) and cold NT-Y (200 $\mu\text{g/kg}$, 100-fold excess)

The pre-mixing and post-mixing concentrations were the same as group 2. The post-mixing concentrations of XNF and NT-Y were 1.2 ng/ μl radiolabeled XNF and 120 ng/ μl cold NT-Y.

The drug administration and tissue harvest

The mice were injected s.c. with 2.5 mg of sodium iodide (50 mg/ml, i.p.) 24 hours and 30 minutes prior to dosing in order to saturate the thyroid to minimize non-specific absorption of ^{125}I . A 2.5 x 2.5 cm area on the belly was preshaved the day before the experiment. On the day of dosing, 50 μl of drug solution contained in a 0.5-ml Becton-Dickinson insulin syringe was subcutaneously injected in the shaved area of the belly. The circumference of the s.c. “bubble” was marked by a Sharpie lab marker. At 10 minutes, 20 minutes, 30 minutes, 1.5 hours, 6 hours, and 24 hours after dosing, the mice were deeply anaesthetized by an i.p. injection of Ketamine/Xylazine cocktail. Blood was harvested by

cardiac puncture and saved into an EDTA-containing tube on wet ice for later plasma separation. The injection site was dissected by cutting a circle approximately 0.5 cm outside of the lab-marker circumference and subsequently weighed and saved on dry ice. Based on the results from the pilot study, the injection site tissue which would be processed included epidermis, dermis, subcutaneous tissue (connective tissue below the skin), and underlying muscle. An attempt was made to exclude any fat tissue underlying the abdominal muscle. The animals were euthanized by cervical dislocation under CO₂. The dose injection syringes were counted before and after dosing.

Tissue homogenization

The injection site tissue samples were minced using micro-scissors and soaked in ice-cold immunoprecipitation buffer (for recipe, see Distribution Study). The volume of buffer calculated in microliters was 10-fold the tissue weight in milligrams. The resulting samples were homogenized and solubilized using the same methods described in the Distribution Study. After centrifugation at 3000 rpm for 15 minutes at 4 °C, the middle level (clearest) supernatant (indicative of lysis) was taken for further study, as shown below:

- 1) Trichloroacetic acid (TCA) precipitation: 100 µl
- 2) Immunoprecipitation: 600 µl for subgroups H and L, 200 µl for subgroup M due to the scarcity of lysis supernatant in this group
- 3) Straight method of SDS-PAGE without immunoprecipitation procedure: 50 µl

The remaining tissue and lysis supernatant were frozen at -70 °C.

TCA precipitation

The tissue lysis supernatant and plasma samples were mixed with TCA and Bovine Serum Albumin (BSA) as indicated below:

For injection site lysis: 100 µl lysis supernatant + 400 µl BSA solution (1%) + 500 µl TCA solution (10% tissue lysis supernatant solution)

For plasma: 50 µl plasma + 450 µl of BSA solution (1% in SQ water) + 500 µl of TCA solution (5% in plasma solution)

The total assay solution and final TCA concentration was 1 ml and 10% respectively. After mixing and vortexing, the samples were placed on wet ice for 15 minutes and subsequently centrifuged at 3000 rpm for 5 minutes at 4 °C. Pre-TCA counts of the samples were made by WIZARD 1470 counter (Wallace, MD). After aspirating the supernatant containing unprecipitated free ^{125}I , the pellet was counted as post-TCA counts (TCA-precipitated activity).

Immunoprecipitation and SDS-PAGE

For the preparation of protein A and anti-neurotrophin serum (antibody), see the “Immunoprecipitation Assay” section of the Distribution Study described above.

Each lysis and plasma sample was counted before the immunoprecipitation step as “preipp” activity. The procedure of immunoprecipitation of lysis supernatant and plasma samples were as follows:

For injection site lysis:

Subgroups H and L:

600 µl of lysis supernatant + 350 µl of immunoprecipitation buffer +
50 µl protein A-antibody = 1 ml

Subgroup M:

200 µl of lysis + 750 µl of immunoprecipitation buffer + 50 µl protein A-antibody = 1 ml

For plasma (subgroups H, M, and L):

100 µl of plasma + 850 µl of immunoprecipitation buffer + 50 µl protein A-antibody = 1ml

The samples were incubated on a constant shaker in a 4 °C room overnight. The next day, the samples were washed multiple times as described in the Distribution Study. At the final step of washing, instead of replacing with fresh immunoprecipitation buffer, 40 µl of SDS loading buffer was added as in the Distribution Study, and subsequently counted as immunoprecipitated XNF (postipp count).

Samples that went into SDS-PAGE directly without immunoprecipitation (preipp):
48 hours

For injection site lysis: 50 µl lysis + 50 µl SDS loading buffer

For plasma: 10 µl sample + 90 µl loading buffer

The loading buffer used in this experiment was 10.5 ml of 4x (four times the normal concentration) loading buffer containing 2.5 ml of 4x of upper gel buffer, 4 ml of 20% SDS, and 4 ml of 100% glycerol, which was diluted 1:1 with SQ water before use.

The ^{125}I -XNF control was prepared as described above. The molecular weight standard was purchased from Life Technologies, Inc. Gaithersburg, MD (Catalog No.26041-02, Lot No. DKN134) and prepared as instructed by the manufacturer.

All pre- and post-immunoprecipitation and samples as well as ^{125}I -XNF and molecular weight standard were boiled at 100 °C for about 5 minutes. No reducing agents were added. All samples were centrifuged at 13,000 rpm for 1 minute before being loaded onto two types of gels: Tris-Glycine 16% homogenous or 4–20% gradient. The gels contained 10 wells, 1 mm width. The gels were run under ~140–160 volts for 1.5 to 2 hours.

The loading amounts were as follows:

Post-immunoprecipitation: 20 μl supernatant (for both lysis and plasma)

Pre-immunoprecipitation: 20 μl of lysis, 10 μl of plasma

The resulting gels were incubated with about 5% glycerol in the constant shaker for 5 to 30 minutes and air dried at 80 °C for 2 hours. The dried gels were exposed to Kodak film (Catalog No. 165-1579) at –70 °C for various lengths of time (shown in the results) and developed for viewing.

IV. THE INJECTION SITE PROCESSING OF XNF: PROTEOLYSIS STUDY IN MICE

The objective was to assess the ability of protease inhibitors to enhance the recovery of intact XNF following s.c. administration.

Group p1 (control): Dosed with ^{125}I -XNF (2 $\mu\text{g/kg}$)

Group p2: Dosed with ^{125}I -XNF (2 $\mu\text{g/kg}$) and protease inhibitor cocktail (see details in “Drug Preparation” below).

Animals

Female CD-1 mice were used (Charles Rivers, Portage, CA). The age range was 7 to 9 weeks. The timepoints 10 minutes, 20 minutes, 30 minutes, 1.5 hours, 6 hours, and 24 hours were selected. With three mice at six timepoints for two groups, a total of 36 mice were used for this study.

All animals were acquired, cared for, and experimentally handled in accordance with the guidelines in the NIH “Guide for the Care and Use of Laboratory Animals.”

Drug preparation

50 μl of injection volume was employed in this study, and the radiolabeled XNF dose was the same as in the Binding Study for ease of comparison.

Group p1: Dosed with ^{125}I -XNF alone. Taking the average body weight (estimated) of mice as 30 grams and the dosing as 2 $\mu\text{g/kg}$, the amount of administered ^{125}I -XNF was:

$$2 \mu\text{g/kg} \times 0.03 \text{ kg/mouse} = 60 \text{ ng/mouse}$$

The dosing concentration was:

$$60 \text{ ng/mouse} \div 50 \mu\text{l/mouse} = 1.2 \text{ ng}/\mu\text{l}$$

Group p2: ^{125}I -XNF (2 $\mu\text{g/kg}$) and protease inhibitor cocktail:

^{125}I -XNF (2 $\mu\text{g/kg}$): 2.4 $\text{ng}/\mu\text{l}$, as for group 1.

The protease inhibitor cocktail consisted of:

- 1) Aprotinin: Bovine lung, solution as Kallikrein inactivator purchased from Calbiochem Biochemicals (San Diego, CA) (Catalog No. 616399, Lot No. B13876).
- 2) Leupeptin: purchased from Sigma Chemical Company (Catalog No. L-00649, Lot No. 45H6832)
- 3) AEBSF [4-(2-Aminoethyl)-benzenesulfonylfluoride, HCl]: purchased from Calbiochem Biochemicals (San Diego, CA) (Catalog No. 101500, Lot No. 1585916).

All protease inhibitors were prepared and diluted using XNF column buffer. 25 μl of XNF solution was combined with 25 μl of the protease inhibitor cocktail. No precipitation, color change, or viscosity change was detected after mixing with ^{125}I -XNF solution. The final concentrations of the protease inhibitors were 5 mg/ml for Aprotinin, 3 mg/ml for Leupeptin, and 5 mg/ml for AEBSF.

Drug administration and tissue harvest

The iodine preblocking, shaving, and subcutaneous injection procedure were generally the same as described for the Binding Study.

Organization of study groups

We reassigned the animal subgroups H, M, and L as described in the Binding Study and employed the same methods for immunoprecipitation.

Tissue homogenization

Homogenization was performed as described in the Binding Study with the exception of a change in the volume of samples used in the immunoprecipitation segments:

- 1) TCA solution: 100 μ l
- 2) Immunoprecipitation: 500 μ l tissue supernatant for each subgroup, H, M, and L.
- 3) Pre-ipp for SDS-PAGE: 50 μ l.

TCA precipitation

Same methodology was employed as described in the Binding Study

Immunoprecipitation and SDS-PAGE

For the preparation of protein A and anti-neurotrophin serum (antibody), see the section “Immunoprecipitation Assay” of Distribution Study described earlier. The lot numbers of protein A and antibody purchased were the same as those used in the Binding Study.

For injection site lysis:

Subgroups H, M, and L:

500 µl of lysis supernatant + 450 µl of immunoprecipitation buffer + 50 µl protein A-antibody solution = 1 ml

For plasma:

Subgroups H and L:

200 µl of plasma + 750 µl of immunoprecipitation buffer + 50 µl protein A-antibody = 1 ml

Subgroup M:

100 µl of plasma + 850 µl of immunoprecipitation buffer + 50 µl protein A-antibody = 1 ml
(because less than 200 µl of plasma was collected from one mouse in this group)

The samples were incubated on a constant shaker in a 4°C room overnight. On the next day, the samples were washed as described in the Distribution Study. At the final step of washing, instead of replacing with fresh immunoprecipitation buffer, 40 µl of SDS loading buffer (same as in Distribution Study) was added and subsequently counted as immunoprecipitated XNF (postipp counts).

For those samples that went into SDS-PAGE directly without immunoprecipitation:

For injection site lysis: 50- μ l lysis solution + 50 μ l SDS loading buffer (1: 2 dilution)

For plasma: 1- μ l sample + 50- μ l loading buffer (1:6 dilution)

The loading buffer used in this straight method was composed of 10.5 ml of 4x loading buffer containing 2.5 ml of 4x of upper gel buffer, 4 ml of 20%, and 4 ml of glycerol were diluted into 1x with SQ water before being used.

The ^{125}I -XNF control was prepared as described above. The molecular weight standard was purchased from Life Technologies, Inc., Gaithersburg, MD (Catalog No. 26041-02, Lot No. DKN134) and prepared as instructed by the manufacturer.

All pre- and post-immunoprecipitation and samples as well as ^{125}I -XNF and molecular weight standard were boiled at 100 °C for about 5 minutes. No reducing agents were added. All samples were centrifuged at 13,000 rpm for 1 minute before being loaded onto Tris-Glycine 16% homogenous gels. The gels were 1 mm in width and were run under ~140–160 volts for 1.5 to 2 hours. The loading amount were as follows:

Immunoprecipitation: 15 μ l supernatant was loaded (for both lysis and plasma).

Pre-ipp samples: lysis 15 μ l, plasma 10 μ l

The resulting gels were incubated with about 5% glycerin in the constant shaker for 5 to 30 minutes and air dried at 80 °C for 2 hours. The dried gels were exposed to Kodak film

(same as that used in Distribution Study) at -70°C for various lengths of time (explained in Results) and developed for viewing.

V. THE MICROAUTORADIOGRAPHY OF XNF DISTRIBUTION IN THE SUBCUTANEOUS SPACE AND LYMPH NODES OF RATS

This study was designed to visualize the time-dependent distribution of XNF in the skin and draining lymph nodes of animals subcutaneously injected with tracer doses of XNF. Rats were used in this study because of the technical difficulty of harvesting lymph nodes from mice, and because rats are the species in which the low bioavailability of XNF has been extensively studied.

Animals

Four male Sprague-Dawley rats were purchased from Charles River (Hollister, CA). The average body weight was 300 g. The animals were allowed to access food and water freely before experiments. All animals were acquired, cared for, and surgically handled in accordance with the NIH "Guide for the Care and Use of Laboratory Animals."

Drug preparation and administration

The XNF was radiolabeled as described earlier and used within 7 days after preparation. The stock solution had a specific activity of $1.9 \text{ cpm} \times 10^6/\mu\text{l}$ and an original concentration

of 13 ng/ μ l, which was subsequently diluted with XNF column buffer to a final dose solution of 0.1 mCi/ μ l.

The animals were anaesthetized by an i.p. injection of 0.7 ml Ketamine cocktail and subsequently shaved for at least 5 x 5 cm area at the injection site chosen on the right lower leg beneath the knee, an area thought to be drained partially by inguinal nodes (Tilney 1971). The injection bubble was marked with a Sharpie lab marker after subcutaneous administration of 200 μ l of dosing solution. The average dosing activity was about 20 mCi per rat. After injection, the animal was recovered and allowed to access food and water freely. At 30 minutes, 1.5 hours, 6 hours, and 24 hours after injection, the rats were deeply anaesthetized by Ketamine cocktail i.p. and perfused with heparinized saline followed by 10% neutral buffered formalin.

The tissues, including the injection site (2-cm diameter around the center of the marked injection point, contained skin, subcutaneous space (connective tissue), underlying muscle (2-cm diameter of one layer), right popliteal nodes, and right inguinal nodes. The corresponding tissues of left contralateral side were also harvested, with liver, spleen, and right kidney. The fixed tissues were processed into paraffin blocks by the Department of Pathology at Genentech using routine methods. Multiple sections were cut from each tissue block to allow a range of exposure times (3 days and 1, 2, 3, 4, and 5 weeks). Immunohistochemical and special staining procedures and routine hematoxylin and eosin staining were performed. Slides for autoradiography were dipped in Kodak NTB3 (Catalog No. 1654441) and exposed for the designated length of time in a light-proof container at 4 °C. Slides were developed in Kodak D19 and fixed in Kodak Fixer. Following counterstaining, the slides were evaluated.

RESULTS AND DISCUSSION

I. DISTRIBUTION STUDY

The objective of this study was to identify the body tissues which are quantitatively most important for XNF disposition following s.c. administration. At 2 or 6 hours after subcutaneous injection of ^{125}I -XNF, blood was harvested from the rats. After perfusion, a total of 14 tissues were dissected from each of four rats. They were subsequently counted and normalized by weight as total tissue radioactivity remaining in tissues ("preipp counts/mg" in Tables I-1 and I-2). After immunoprecipitation (ipp), the tissue homogenate supernatants were counted as immunoprecipitable activity ("postipp/ml"), which represents the amount of radioactivity of XNF peptides that could be recognized by an anti-neurotrophin polyclonal antibody. Figures I-1 through I-4 present a graphical comparison among the tissues.

The s.c. injection site tissue exhibited the greatest total tissue activity (cpm/mg) at both 2 hours and 6 hours (Tables I-1 and I-2). Although the highest value of preipp counts/mg was plasma followed by the injection site, the highest values of ipp at both timepoints (except kidney at 6 hr) further reveal that most XNF protein materials at the injection site remained minimally degraded, unlike plasma, where immunoprecipitable activity (postipp) did not rise significantly relative to preipp values. Since some XNF should have diffused beyond the excised injection site, the actual total s.c. XNF activity was probably even greater than was measured here.

Due to its large volume, plasma contained the largest portion of radioactivity, 32% of total dose at 2 hours and 9.14% at 6 hours (Tables I-1 and I-2, Figures I-5 and I-6), indicating that it contained the largest pool of radioactivity during this period. It appears that a considerable amount of XNF activity in plasma was derived from free ^{125}I and degraded XNF molecules that could not be precipitated by the antibody, which was a polyclonal antibody that is able to precipitate several neurotrophin molecules.

It was not surprising that inguinal nodes, which drain the injection site, displayed one of the strongest preipp and postipp activities both at 2 hours and 6 hours (Tables I-1 and I-2, Figures I-1 through I-4). However, the mesenteric nodes, which drain the gut and do not drain the injection site, also exhibited moderate activity (post-ipp). This result suggests that the XNF molecules circulated between the lymphatic system and blood very quickly and efficiently. Other lymphatic organs like the spleen and thymus did not show significant accumulation (post-ipp).

High perfusion organs like the liver, pancreas, and heart exhibited low to moderate uptake both at 2 hours and 6 hours as expected from non-neuronal tissues. The accumulation in liver as assessed by organ (preipp/organ) was significant among the tissues, due primarily to its large size. It did not have positive results in postipp activity, consistent with clipping of iodine in hepatic tissue. On the other hand, the stomach showed significant uptake and accumulation of XNF. It had dose recoveries comparable to that of the injection site at both timepoints. This observation for the stomach has been observed previously (personal communication, Dr. E. Escandon, Genentech), and is difficult to explain.

The nervous system including the brain and spinal cord exhibited low levels of uptake and accumulation. Because of the blood-brain barrier, theoretically there should be no significant uptake of peripherally administered ^{125}I -XNF.

The overall preipp and postipp activities of these tissues decreased between 2 hours and 6 hours. This was consistent with the pharmacokinetic data for XNF (see Introduction), where, following i.v. injection, the XNF activity in plasma decreased rapidly.

SDS-PAGE at 2 hours (Figure I-7) and 6 hours (Figure I-8) show the ipp activity bands. The densest bands at the bottom of the gel (far left of the pictures) represent the XNF monomer (around 13 kDa). The bands at about 26 kDa represent the XNF dimer. The results indicate that the disulfide bonds in most XNF molecules dissociated during immunoprecipitation procedure, due to boiling or because of reducing conditions. The band densities are generally consistent with their postipp activities. Some tissues like the injection site, inguinal nodes and plasma have high molecular weight complexes of ~80–120 kDa. These could be the ligand-receptor complexes, which are about 96 kDa (XNF dimer is 26 kDa, trk receptors are about 70 kDa, and p75 is 75 kDa). Alternatively, this could be aggregation of XNF molecules. There is no significant amount of degradation products that are visible following immunoprecipitation (would be present as bands <13 kDa, in relative densities that exceed that of the XNF control).

In this study, there is an important assumption that the uptake by tissues other than those we examined, such as bone or thyroid, is negligible. The fifteen tissues examined here are commonly considered to be important for the metabolism of protein drugs following s.c. administration (personal communication, Dr. E. Escandon, Genentech).

Many tissues displayed high XNF accumulation such as liver, kidney and heart (cpm/organ) but low levels of tissue activity (cpm/mg) whereas other tissues such as lymph nodes displayed high tissue activity but negligible total activity due to their small mass. Only the injection site exhibited consistent high profiles of both measures, which rendered it as the main target tissue for our further study of local disposition of XNF. That XNF injected s.c. may not be able to readily leave the skin is a reasonable explanation for the low s.c. bioavailability of XNF observed in pharmacokinetics studies.

It is quite interesting that few degradation bands can be detected from SDS-PAGE. This observation may be explainable as due to the antibody specificity. Several lines of evidence show that NT-Y, NT-Z and XNF, in addition to being structurally similar proteins, are immunologically related (Acheson 1991). Antibodies raised against one of these neurotrophins (used here) react with all neurotrophins in ELISAs carried out at neutral pH. In nondissociating buffers, XNF was slightly less reactive than intact NT-Y (personal communication with Dr. V. Hale at Genentech). We could not find evidence of degraded XNF on these gels and it is possible that such unfolded fragments would not be recognized immunologically.

The SDS-PAGE of plasma is not shown due to its extremely low activity after immunoprecipitation. The autoradiography of other half organs was not performed also due to minimally detectable activities.

II. BINDING STUDY

There are three groups in this study. Group 1 was the control. Group 2, in which 100-fold excess unlabeled XNF was added, was to examine whether there was significant binding to trkX. Group 3, in which 100-fold excess unlabeled NT-Y was added, was to examine whether there was significant binding to the p75 low-affinity receptor. Injection site tissue and plasma were collected in the study. Analysis methods used in the Distribution Study were followed except that TCA precipitation was performed to examine the total protein recovery in the samples.

Injection site tissue analysis

A comparison of XNF total tissue activity (cpm/mg), lysate activity (cpm/ml), TCA-precipitable activity (any XNF peptides), and immunoprecipitable activity (antibody recognizable XNF peptides) at the injection site among all timepoints (Table II-1, Figures II-1 to II-8) yielded no significant difference among three treatments groups (Student *t* test, $p > 0.05$) ($p < 0.01$ is highly significant, $p < 0.05$ is significant). At late timepoints (6 and 24 hr), groups 2 and 3 had consistently greater values by all assessments than the control group. The ipp recovery, calculated as the percentage of immunoprecipitable activity relative to total lysate activity was two- to three-fold lower than the control group at earlier times (10 to 30 min) (Table II-2). No difference was found in TCA recovery (Table II-2) at any time. Overall, these data suggest that there is not significant specific binding of XNF at the s.c. injection site to either trkX or p75.

Surprisingly, XNF activity dropped to ~40–50% of initial activity at injection site within 10 minutes in all three treatment groups. This result assumes that 100% of the administered

dose of XNF was placed into the injection site initially (Figure II-5). XNF activity remaining at the injection site was calculated as the percentage of total injection site tissue activity relative to total administered XNF corrected for syringe loss. Table II-2 shows that the three treatment groups display similar values except at 24 hours when approximately 10-fold more XNF activity remained at the injection site for groups 2 and 3. This data suggests that at very late times, specific binding may lead to retention of XNF at the injection site, but the magnitude of effect is minimal for plasma recovery of drug.

Plasma analysis

Like the injection site data, total activity and TCA precipitable activity in plasma do not differ from the control group ($p=0.95-0.13$). They reached their peaks at 90 minutes, remained at plateau until 360 minutes, and subsequently dropped to minimum levels at 24 hours (Table II-3 and Figure II-6). Most immunoprecipitable activities of XNF in plasma were below 500 cpm/100 μ l, which was the minimally detectable amount of ^{125}I . Therefore, the recovered cpm/ml of ipp activity indicates the minimum ipp recovery. The data in Table II-4 show that the ipp recoveries were superior in groups 2 and 3 at earlier timepoints (from 10 to 30 min) relative to control, consistent with time-dependent degradation events in plasma (due to the minimum value of plasma counts at 24 hours, the high recoveries may be considered artifacts).

The dose recovery, calculated as percentage of the total XNF activity in plasma (volume of plasma in mouse: Davis 1993) relative to the total dose administered corrected for syringe loss (no more than 3.2%), was similar to that observed in rats (~9–38%, see Distribution Study, Tables I-1 and I-2).

The SDS-PAGE profiles of injection site lysate supernatant including total activity (Figure II-10) and ipp activity (Figure II-11) display similar pattern to those in Distribution Study. Most of the recovered XNF molecules existed as a form of the monomer and minor portions as dimer. High molecular weight bands were visible in each lane.

When samples of control and study groups were juxtaposed in SDS-PAGE, no apparent differences were detected between them for dimer, monomer, and high molecular weight complex bands. These results are consistent with all count variants discussed above. These data suggests that the high-affinity receptor binding to trkX and low-affinity binding to p75 do not significantly contribute to the injection site disposition of XNF in mice. No signal was detected in dermis, which is most closely identifiable with the subcutaneous space. p75 distribution data in non-neuronal tissues and trkX in mouse skin is not available at this time. However, with an assumption that the 100-fold excess unlabeled XNF or NT-Y were able to saturate these receptors, we propose that there was not a significant number of receptors present in the mouse injection site. Otherwise, the excess amount of unlabeled XNF and NT-Y would have been able to displace ^{125}I -XNF from trkX and p75 respectively and result in more absorption of ^{125}I -XNF into blood.

The degraded bands seen in the immunoprecipitation gel are of too low a density to be considered as the result of significant proteolysis *in vivo*. Alternatively, the result could be derived from the immunoprecipitation procedure since similar bands were not observed from the total activity gel (Figure II-10).

III. PROTEOLYSIS STUDY

The effect of a protease inhibitor (PI) cocktail including aprotinin, leupeptin, and AEBSF on the recovery of XNF from the injection site and plasma was used to determine the significance of proteolysis on the fate of XNF.

Aprotinin is a competitive reversible inhibitor of proteolytic and esterolytic activity (Calbiochem Product Data Sheet). As a relative heat- and acid-stable serine protease inhibitor (Robert 1996), it can form a tight complex, blocking the active site of the enzymes. It can inhibit several proteases, including coagulation factors in the prephase of blood clotting, kallikrein, plasmin, tissue and leukocytic proteinases (Dubin 1994), chymotrypsin, and trypsin.

AEBSF is a specific irreversible inhibitor of serine proteases (Calbiochem Product Data Sheet) (Megyeri 1995). It reacts covalently with a component of the active site. It forms sulfonyl derivatives of the active serine residue. It can inhibit similar targets to Aprotinin such as kallikrein, plasmin, and chymotrypsin. It is a stable and nontoxic alternative to PMSF, which can not be use *in vivo*.

The *in vivo* study has shown that Leupeptin can reduce proteolytic activity of cysteine protease (cathepsin B from lysosomal extract) (Banga 1990). Leupeptin is also a stable and non-toxic protease inhibitor.

Injection site tissue analysis

The total tissue counts (Table III-1 and Figure III-1) of the PI group was similar to that of the control group until 1.5 hours, after which, however, the groups diverged. By 6 hours, the PI group was five-fold elevated and was ten-fold elevated by 24 hours ($p < 0.05$). The differences were consistent in total tissue lysate activity (Figure III-2), which suggests that the tissue lysis supernatant activity reflects true tissue activity before homogenization.

The TCA precipitable activity of the PI group began to diverge from the control by 30 minutes and the difference was maintained through 24 hours ($p < 0.01$) (Figure III-3). The immunoprecipitable activity exceeded that of the control as early as 10 minutes (Figure III-4).

TCA and immunoprecipitation recoveries did not display time-dependent changes (Table III-2). The mean TCA recovery of the study groups was about 80% compared with about 54% for control group whereas immunoprecipitation recovery was almost double that of the control group. These results indicate that many more XNF molecules exited in the subcutaneous space as intact or minimally degraded forms when protease inhibitors were added.

The differences in XNF activity as a function of protease inhibition were reflected by the density of bands in SDS-PAGE profiles at the later timepoints (Figures III-8 and III-9). It is of interest to note that there are no apparent degradation bands detected in SDS gels without the immunoprecipitation procedure. Immunoprecipitation was required to show evidence of protease activity (protein fragments).

Plasma analysis

The plasma data are presented in Tables III-3 and III-4 and shown in Figures III-5 and III-6. Through 6 hours, the total and TCA activities in plasma were significantly lower than control ($p < 0.05$); this result is not as obvious in immunoprecipitable activity (most $p > 0.05$). The difference gradually reduced over time until the crossover of data around 6 hours (Figure III-5). After 6 hours, the XNF level in the control group continued to decline through 24 hours whereas the PI group remained fairly constant to the end of experiment. These data suggest that inhibiting proteases have minimal effect on early plasma concentrations of XNF.

Different from the injection site, the overall TCA and immunoprecipitation recoveries in plasma were not improved in the PI group (Table III-4).

In order to exclude the possibility of competitive binding of endogenous free immunoglobulin G (IgG) to Protein A with the anti-neurotrophin antibody, we conducted a "Preclearance Step." In the experimental group, we pre-incubated extra protein A to saturate and remove free IgG in plasma. The same ipp procedure was then applied to the plasma in both experimental and control group. This "preclearance step did not exhibit apparent improvement over the conventional method of immunoprecipitation recovery (data not shown). It can be concluded that the very low TCA and immunoprecipitation recoveries from plasma in both binding and proteolysis studies can not be explained by endogenous IgG.

Degradation at the injection site

In the last several years, growing evidence has accumulated that the skin is not only a barrier for passive diffusion, but also constitutes an enzymatic barrier. The composition of enzymes and the spectrum of metabolic reactions in the skin are similar to those in the liver but possesses only about 10% of the metabolic activity detected in the liver (Merkle 1989).

There are several types of enzymes responsible for the degradation (Banga 1994). One is ectopeptidases, which are located at the surfaces of a wide variety of mammalian cells. They are integral proteins of the plasma membrane, asymmetrically oriented, with the catalytic site exposed at the extracytoplasmic surfaces. Their catalytic site faces the extracellular space where it can act on circulating peptides. However, the substrate specificity of ectopeptidases is limited to small peptides, up to a maximum of approximately 30 residues.

In contrast, intracellular hydrolases degrade all types of biological polymers, including proteins, polysaccharides, lipids, DNA, and RNA (Banga 1994). Most intracellular hydrolases including serine and cysteine proteases, are located in endosomes and lysosomes of several cells such as macrophages, lymphocytes, and dendritic cells.

In the XNF proteolysis study, by the coadministration of protease inhibitors (Aprotinin, Leupeptin and AEBSF), the XNF disposition profile at the injection site was very different from the control group, suggesting an important role of proteases in the local disposition of XNF. However, concomitant increases in plasma XNF concentrations were not observed until late. These data suggest that proteases have some importance for degradation of XNF, but are not rate-limiting for absorption into blood.

There is supporting evidence that there was significant protection of XNF from degradation at the injection site with protease inhibitors. First, TCA recoveries, which reflect the XNF peptide concentration, were significantly improved throughout the experiment except at 24 hours (Table III-2). Although the immunoprecipitation recoveries are not as obvious as TCA recoveries, the immunoprecipitable activity, which represents more intact XNF than TCA-precipitable peptides, are greater ($p < 0.01$, Table III-1) than those in the control group. Second, in pre-ipp SDS-PAGE (Figure III-9), the free ^{125}I (bands at the bottom of the gel) was clearly observed in the lanes of the control but not in the group with protease inhibitors present. The free ^{125}I is probably more easily cleaved from XNF molecules when degraded.

However, in pre-ipp SDS-PAGE, no degradation bands were observed in either group, which otherwise would provide the most powerful evidence for the protection of XNF molecules from proteases. Although it is possible that the clipping of ^{125}I from degraded XNF obviated its identification in the gel, the immunoprecipitation gel early reveals some bands of degradation. Another possibility could be that the immunoprecipitation concentrated these weak amount of degraded peptides and made them visible in the gel, but this assumption undermines the magnitude of the protease-mediated degradation at the injection site.

Non-specific binding or another form of degradation

Theoretically, by protection from protease inhibitors, more XNF could be absorbed into blood and subsequently improve the AUC in plasma. Table III-5 provides discouraging data showing the AUC in the PI group was only equivalent to that of the control group.

The pI (isoelectric point) of XNF is above 9 (see Introduction), which renders the molecule positively charged and potentially aggregable at neutral pH in the subcutaneous space (personal communication with Dr. V. Hale, Genentech). If aggregated, those molecules that are not reversible should be considered degraded.

Also XNF, having a positive charge, tends to bind to the negatively charged proteoglycans of the extracellular matrix (personal communication with G. DeGuzman, Genentech). Strong experimental evidence supports the concept that growth factors stored in the extracellular matrix and subsequently released during matrix degradation are major mediators in a variety of developmental processes such as regulation of organogenesis and angiogenesis (Matrisian 1990). Vascular endothelial growth factor (VEGF) has been found to have several spliced molecular species that are differentially localized to heparan sulfate containing proteoglycans or released as diffusible proteins (Keyt 1996).

Either aggregation or non-specific binding or both could explain the retention of XNF at the s.c. injection site. Both pathways are potentially reversible, at least in part. Therefore, it is reasonable to propose that at late timepoints (from 1.5 hours to 24 hours), XNF molecules were de-aggregated and/or released from non-specific binding to blood circulation resulting in the constant activity in plasma in the protease inhibitor group (Figures III-5 and III-6)

Rapid clearance in plasma

The plasma concentration profile following i.v. administration in the pilot study (see "Introduction") shows very rapid disappearance of this molecule from blood. In the Distribution Study of rats, by 2 hours and 6 hours, plasma concentrations were reduced by more than 60% and 90%, respectively (Figures I-5 and I-6). Similarly in the Binding and

Proteolysis studies in mice, almost 50% of dosed XNF was cleared from the injection site within 10 minutes (Figure II-7 and Figure III-5). This suggests that XNF was cleared very quickly, degraded in plasma followed by clipping of ^{125}I or cleared by the kidney or liver.

IV. MICROAUTORADIOGRAPHY STUDY

This study was performed to visualize XNF at the injection site in a relevant animal model, so as to potentially better define disposition. All the slides were processed and evaluated by Noël Dybdal in the Biopathology Department of Genentech.

In the subcutaneous space associated with the injection site, the signal is very strong at the 30- and 90-minute timepoints (best evaluated at the shorter exposure times), this very strong signal decreases significantly at 6 hours and is present at a very low level at 24 hours (best evaluated at the longest exposure times).

The right thigh muscle exhibits a similar pattern of signal intensity over the superficial areas immediately adjacent to the subcutaneous injection space (i.e., not extending uniformly throughout the muscle). The right popliteal and inguinal lymph nodes exhibit a very low signal at the longest exposure times (5 weeks was best), and these tissues were negative at the shorter exposure times. The skin, muscle, and nodes collected from the contralateral (control) side of the animals are negative at all timepoints and for all exposure lengths. The liver and spleen are similarly negative for radioactivity (pictures not shown).

At the injection site, the positive signal shows a strong cellular association (See Figure IV-1, and Figures V-2 and V-3). The signal is strongest in perivascular areas associated with

cells, perhaps tissue macrophages and mast cells. Some cells which may be fibroblasts also have a slight increase in signal. Sections adjacent to those utilized for autoradiography were stained with Toluidine blue for mast cell detection or ED2 (Serotec, MCA 342; rat macrophage antibody, IgG1) for immunohistochemical detection of macrophages. Both mast cells and macrophages are present in moderate to large numbers primarily in the areas coinciding with areas in the autoradiography sections in which cells have a prominent silver grain accumulation. The pattern is generally dense enough even at short exposure times to obscure underlying cellular detail. There is also a small increase in signal on the superficial or surface regions of collagen fibers within these subcutaneous regions.

Right popliteal and inguinal lymph nodes exhibit minimal accumulation of silver grains above background multifocally within the subcapsular sinuses and on scattered macrophages (very small numbers of silver grains) (Figure V-4). This increase in signal is detectable only at the 6-hour (5 weeks of exposure) and 24-hour (4 and 5 weeks of exposure) timepoints (pictures not shown).

Electron microscopy was performed so as to better identify the apparent cellular localization of XNF. Figure IV-5 shows the transmission electronic microscopy of a macrophage at the injection site of a rat following s.c. injection of ^{125}I -XNF. The XNF associated radioactive material is predominantly distributed into lysosomes and endosomes, which further suggests the important role of macrophages in intracellular disposition of XNF at the injection site. This result is also consistent with the aggregation hypothesis presented in Proteolysis Study.

CONCLUSIONS

The subcutaneous bioavailability of XNF is significantly lower than that of most protein therapeutics. The XNF which is absorbed into blood is cleared extremely rapidly from the circulation.

Following subcutaneous injection, there is a significant amount of XNF remaining at the injection site. Otherwise, its tissue distribution is unremarkable and consistent with specific neuronal or receptor uptake.

The high-affinity binding to trkX and low-affinity binding to p75 do not apparently contribute to the disposition of XNF at the injection site.

Proteolysis in the skin might be important in the local disposition of XNF at the injection site; however, proteolytic byproducts are not readily recoverable from the skin.

XNF aggregation and/or non-specific binding to extracellular matrix components might play a role in the local disposition of XNF.

XNF is quickly associated with cells within the skin (macrophages and mast cells) following subcutaneous injection to rodents.

FURTHER WORK

- To further study the *in vivo* degradation of XNF at the subcutaneous injection site using a cardiac arrest animal model.

This rodent model was employed to study the degradation of insulin at the s.c. injection site several years ago (Hori 1989). Pentobarbital was directly injected into the heart to stop the systemic circulation, so that absorption from the injection site would be negligible. This model not only helps concentrate the degradation products of XNF, but

also helps distinguish whether the initial fast disappearance of XNF from the injection site is due to absorption or to experimental artifacts.

- To study the *in vitro* degradation (proteolysis) of XNF via incubation of homogenate or lysosome extract of skin.

Microautoradiography has suggested the critical roles of phagocytosis of XNF into lysosomes and endosomes of macrophages at the s.c. injection site. Homogenization of injection site tissue involved severe mechanic shearing and strong chemical solubilization (EDTA, Triton-100) that would compromise any cell membrane including lysosomes and endosomes. However, few degradation bands could be detected in pre-ipp SDS-PAGE. *in vitro* incubation directly with s.c. tissue homogenate extract would help to define the contribution of proteolysis in XNF degradation.

- To study the role of macrophages *in vivo* with a macrophage-deletion and macrophage-induction model.

Macrophages can be depleted (inactivated) by pretreatment of rodents with liposome-mediated technique (Van Rooijen 1989). Alternatively, macrophages can be induced by pretreatment with gamma-interferon (Mordenti 1993). The objective of this experiment would be to assess the bioavailability of XNF in rats who are either macrophage depleted or induced, relative to controls. This experiment would provide direct evidence of the role of macrophages in the local disposition of XNF

- To explore the stability of XNF in plasma.

Extremely low radioactivity levels of XNF in plasma resulted from minimum immunoprecipitation recovery in ipp SDS-PAGE. One potential explanation is that plasma contains factors that degrade XNF such that any protein which is absorbed is quickly cleared. *in vitro* incubation with plasma provides a quick experimental assessment of stability.

Table I-1 Distribution Study
Tissue distribution of XNF activity 2 hr. after
sc injection to rats (N=2)

Organ/Tissue	preipp ¹ (cpm/mg)	Source	preipp ² (cpm/org.)	Distribution %dose ³	postipp ³ (cpm/ml)
adrenal gland	459	ref.	32,100	0.01	2,950
brain	184	ref.	464,000	0.12	768
plasma	11,800	ref.	129,000,000	32.3	5,010
heart	330	ref.	462,000	0.12	306
inguinal node	13,500	x4	2,740,000	0.69	32,700
kidney	1,090	ref.	3,050,000	0.76	472
liver	555	ref.	7,770,000	1.94	441
lung	394	ref.	828,000	0.21	16,100
mesenteric	723	x10	46,900	0.01	18,400
pancreas	1,430	x2	1,010,000	0.25	668
spinal cord	166	x6	69,100	0.02	779
spleen	1,020	ref.	1,080,000	0.27	2,020
stomach	3,530	ref.	6,690,000	1.67	4,200
SC tissue	18,900	x4	21,500,000	5.36	125,000
thymus	249	x2	134,000	0.03	1,380

¹preipp/mg: total XNF activity per mg

²cpm/org: The organ/tissue weight was calculated based on the reference (Davis et al., 1993, *Pharm. Res.* 10(7): 1093.
or based on the estimated percentage of cut tissue/organ of total tissue/organ.

³%dose: percentage of total dosed XNF

⁴postipp: immunoprecipitable XNF activity

Table I-2 Distribution Study
Tissue distribution of XNF activity 6 hr. after s.c.
injection to rats (N=2)

Organ/Tissue	preipp/mg ¹ (cpm)		preipp/organ ² (cpm)	distribution %dose ³	postipp ⁴ (cpm/m)
adrenal gland	699	ref	49,000	0.01	2,700
brain	85	ref	215,000	0.05	775
plasma	3,350	ref	36,560,000	9.14	811
heart	173	ref	242,000	0.06	251
inguinal node	2,930	x4	235,000	0.06	20,200
kidney	678	ref	1,900,000	0.47	43,500
liver	435	ref	6,090,000	1.52	276
lung	245	ref	515,000	0.13	1,370
mesenteric	413	x10	102,000	0.03	5,460
pancreas	1,550	x2	836,000	0.21	305
spinal cord	159	x6	101,000	0.03	398
spleen	828	ref	870,000	0.22	729
stomach	5,230	ref	9,410,000	2.35	4,400
SC tissue	7,370	x5	9,530,000	2.38	34,500
thymus	203	x2	48,800	0.01	812

¹preipp/mg: total XNF activity per mg

²cpm/organ: The organ/tissue weight was calculated based on the reference (Davis et al., 1993, *Pharm. Res.* 10(7): 1093.
or based on the estimated percentage of cut tissue/organ of total tissue/organ.

³%dose: percentage of total dosed XNF

⁴postipp: immunoprecipitable XNF activity

Figure I-1 The tissue distribution of total activity of XNF 2 hr after s.c. injection to rats (N=2)

Figure I-2 The tissue distribution of total activity of XNF 6 hr after s.c. injection to rats (N=2)

Figure I-3 The immunoprecipitable activity of XNF 2 hr after s.c. injection to rats (N=2)

Figure I-4 The immunoprecipitable activity of XNF 6 hr after s.c. injection to rats (N=2)

Fig. I-5 Tissue distribution of dose recovery of XNF 2 hr after s.c. injection to rats (N=2)

Fig. I-6 Tissue distribution of dose recovery of XNF 6 hr after s.c. injection to rats (N=2)

**Fig. I-7 Distribution - 2 hr
SDS-PAGE of immunoprecipitable activity
of XNF 2 hr after s.c. injection to rats (N=2)**

**Fig. I-8 Distribution - 6 hr
SDS-PAGE of immunoprecipitable activity of
XNF 6 hr after s.c. injection to rats (N=2)**

Table II-1 Binding Study - tissue counts
Total tissue activity, tissue lysate activity, TCA precipitable activity, and immunoprecipitable activity of XNF at injection site after s.c. injection to mice (N=3)

Group	Time (min)	Total tissue activity (cpm /mg)	Total lysate activity ¹ (cpm /ml)	TCA activity ² (cpm /ml)	ipp activity ³ (cpm / ml)
XNF	10	13,300 ± 11,200	444,000 ± 377,000	205,000 ± 180,000	167,000 ± 189,839
	20	13,700 ± 10,400	828,000 ± 511,000	166,000 ± 162,000	107,000 ± 67,444
	30	18,200 ± 6,550	606,000 ± 206,000	183,000 ± 147,000	127,000 ± 86,370
	90	4,250 ± 5,140	116,000 ± 127,000	49,900 ± 61,200	210,00 ± 16,595
	360	618 ± 93	28,800 ± 405	9,080 ± 6,970	6,510 ± 1,634
	1440	165 ± 10	6,660 ± 1,630	3,550 ± 3,570	1,230 ± 423
+	10	20,300 ± 3,500	515,000 ± 115,000	259,000 ± 255,000	41,000 ± 24,500
	20	15,300 ± 3,910	327,000 ± 63,900	146,000 ± 119,000	28,900 ± 17,400
	30	19,200 ± 839	514,000 ± 87,300	203,000 ± 172,000	41,500 ± 9,320
	90	10,300 ± 1,220	260,000 ± 41,200	104,000 ± 90,000	27,300 ± 19,400
	360	664 ± 43.0	30,0900 ± 5,2000	9,880 ± 7,920	5,850 ± 1,840
	1440	511 ± 295	12,200 ± 6,590	6,410 ± 5,950	3,300 ± 1,690
+	10	19,500 ± 9,650	481,000 ± 198,000	220,000 ± 198,000	25,900 ± 21,900
	20	20,000 ± 14,00	504,000 ± 60,300	175,000 ± 133,000	27,800 ± 18,700
	30	16,500 ± 1,160	543,000 ± 38,900	173,000 ± 146,000	26,000 ± 23,100
	90	9,340 ± 334	238,000 ± 42,100	75,800 ± 62,000	30,300 ± 32,400
	360	1,200 ± 58.0	48,400 ± 8,730	19,700 ± 18,600	13,600 ± 4,540
	1440	481 ± 97.0	9,030 ± 2,220	4,370 ± 3,530	3,320 ± 2,400

For all tables and graphs listed here on:

¹total lysate activity: total tissue homogenate supernatant activity

²TCA activity: TCA precipitable activity of tissue lysate supernatant

³IPP activity: Immunoprecipitable activity of tissue lysate supernatant

Note: Student t test was applied for the statistical analysis.

Table II-2 Binding Study - tissue recovery
Immunoprecipitation, TCA recovery, and percentage of XNF remaining at injection site after
s.c. injection to mice (N=3)

Group	Time (min)	IPP recovery ¹ %	TCA recovery ² %	XNF remaining %	at inject. site
XNF	10	28.9 ± 21.1	51.3 ± 3.20	38.0 ± 32.0	
	20	22.1 ± 23.5	48.0 ± 7.00	25.6 ± 15.5	
	30	19.8 ± 9.90	40.0 ± 3.60	42.1 ± 5.90	
	90	22.3 ± 10.4	47.0 ± 4.00	10.3 ± 12.9	
	360	22.6 ± 5.70	47.3 ± 4.70	1.00 ± 0.000	
	1440	18.9 ± 7.00	56.3 ± 3.50	0.130 ± 0.200	
+ excess cold XNF	10	8.50 ± 6.50	65.7 ± 5.70	50.1 ± 0.200	
	20	9.00 ± 5.30	57.0 ± 1.70	30.8 ± 7.80	
	30	8.50 ± 3.10	62.7 ± 10.1	44.3 ± 3.40	
	90	11.0 ± 9.50	54.3 ± 4.00	22.9 ± 7.30	
	360	19.8 ± 8.80	50.3 ± 3.10	1.40 ± 0.500	
	1440	28.3 ± 12.1	65.7 ± 1.50	1.53 ± 0.800	
+ excess cold NT-Y	10	6.20 ± 5.50	61.3 ± 9.50	43.9 ± 14.0	
	20	5.40 ± 3.20	53.0 ± 5.30	41.1 ± 7.20	
	30	4.80 ± 3.70	50.7 ± 18.8	48.3 ± 0.600	
	90	13.1 ± 13.8	45.7 ± 11.0	20.2 ± 2.80	
	360	27.6 ± 5.10	55.0 ± 12.5	3.20 ± 0.700	
	1440	34.1 ± 16.4	59.7 ± 5.50	1.03 ± 0.100	

For all tables and graphs listed here on:

¹ipp recovery = immunoprecipitable activity / total lysate (supernatant) activity

²TCA recovery= TCA precipitable activity / total lysate (supernatant) activity

³XNF remaining at injection site: total injection site tissue activity / total dose administered XNF activity
Note. Total dose administered XNF = syringe counts before dosing-syringe counts after dosing.

Table II-3 Binding Study - plasma counts
Total, TCA precipitable, and immunoprecipitable activity of XNF
in plasma after s.c. injection to mice (N=3)

Group	Time (min)	Total activity (cpm / ml)	TCA activity (cpm / ml)	ipp activity (cpm / ml)
XNF	10	70,400 ± 62,400	53,000 ± 82,500	17,900 ± 25,300
	20	48,100 ± 14,400	20,000 ± 13,100	4,820 ± 3,800
	30	90,800 ± 18,700	26,400 ± 7,190	2,730 ± 745
	90	208,000 ± 56,200	46,900 ± 10,000	3,020 ± 1,570
	360	111,000 ± 103,000	23,800 ± 11,500	1,640 ± 819
	1440	3,400 ± 1,780	805 ± 691	1,130 ± 286
+ excess cold XNF	10	16,600 ± 3,840	3,430 ± 2,520	3,270 ± 2,130
	20	31,500 ± 33,600	21,700 ± 21,300	8,950 ± 8,350
	30	29,200 ± 7,280	21,000 ± 6,480	5,550 ± 1,370
	90	121,000 ± 62,400	40,900 ± 19,200	4,880 ± 749
	360	154,000 ± 21,400	43,400 ± 8,340	7,620 ± 9,590
	1440	39,100 ± 61,400	2,610 ± 231	2,810 ± 1,980
+ excess cold NT-Y	10	18,700 ± 4,490	6,270 ± 3,160	4,390 ± 1,770
	20	28,200 ± 707	10,900 ± 4,920	3,050 ± 1,040
	30	53,900 ± 10,700	14,000 ± 2,300	4,000 ± 691
	90	197,000 ± 44,900	42,100 ± 7,800	2,610 ± 193
	360	161,000 ± 34,200	36,900 ± 781	1,580 ± 131
	1440	3610 ± 2,090	2,110 ± 326	4,150 ± 3,510

Table II-4 Binding Study - plasma recoveries
TCA, immunoprecipitation recoveries, and percentage of XNF
remaining in plasma after s.c. injection to mice (N=3)

Group	Time (min)	ipp recovery %	TCA recovery %	¹ remaining in plasma %
XNF	10	21.6 ± 10.9	39.0 ± 28.9	1.00 ± 0.774
	20	16.2 ± 9.51	29.3 ± 8.50	0.738 ± 0.192
	30	9.80 ± 8.94	18.3 ± 2.08	1.46 ± 0.410
	90	8.40 ± 10.3	18.0 ± 5.29	3.19 ± 0.862
	360	8.90 ± 9.42	16.7 ± 5.51	1.76 ± 1.61
	1440	41.3 ± 10.8	52.3 ± 6.81	0.0545 ± 0.0327
+cold XNF	10	23.7 ± 9.19	27.3 ± 7.23	0.231 ± 0.0457
	20	42.6 ± 36.2	38.3 ± 16.4	0.471 ± 0.494
	30	27.0 ± 11.0	34.7 ± 3.21	0.421 ± 0.136
	90	12.2 ± 10.8	19.0 ± 8.54	1.83 ± 0.910
	360	9.40 ± 7.58	15.7 ± 1.53	2.36 ± 0.335
	1440	39.7 ± 19.9	57.3 ± 13.3	0.585 ± 0.912
+cold NT-Y	10	24.6 ± 4.87	30.3 ± 7.64	0.314 ± 0.0847
	20	19.6 ± 12.2	27.3 ± 9.29	0.456 ± 0.0529
	30	13.7 ± 8.65	22.7 ± 2.08	0.908 ± 0.243
	90	9.40 ± 11.1	19.0 ± 4.58	3.20 ± 1.00
	360	7.30 ± 8.82	15.3 ± 2.89	2.74 ± 0.696
	1440	113 ± 75.9	66.0 ± 14.8	0.0631 ± 0.0375

¹Total volume of plasma of mouse is calculated based on the ratio of 1ml/20g body weight. (Davis et al., 1993, Pharm. Res. 10(7): 1093.

Fig. II-1 The total tissue activity of XNF at injection site after s.c. injection to mice (N=3)

Figure II-2 Total injection site lysate activity of XNF after s.c. injection to mice (N=3)

For all graphs, preipp: the activity before immunoprecipitation = total activity

Figure II-3 TCA precipitable activity of XNF after injection site after s.c. injection to mice (N=3)

Figure II-4 Immunoprecipitable activity of XNF after injection site after s.c. injection to mice (N=3)

Fig. II-5 The percentage of total XNF activity remaining at injection site after s.c. injection to mice (N=3)

Fig. II-6 TCA precipitable activity of XNF at injection site and plasma after s.c. bolus to mice (N=3)

Fig. II-7 Total plasma activity of XNF after s.c. injection to mice (N=3)

Fig. II-8 TCA precipitable activity of XNF in plasma after s.c. injection to mice (N=3)

Fig. II-9 Immunoprecipitable activity of XNF in plasma after s.c. injection to mice (N=3)

Fig. II-10 Binding
SDS-PAGE of immunoprecipitable activity of XNF
at injection site after s.c. injection to rats (N=3)

10', 20' and 30': 10 min, 20 min and 30 min

Fig. II-11 Binding
SDS-PAGE of total activity of XNF
at injection site after s.c injection to rats (N=3)

Table III-1 Proteolysis Study - injection site
Total tissue, tissue lysate activity, TCA precipitable, and immunoprecipitable activity of XNF at injection site after s.c. bolus to mice (N=3)

Group	Time min.	Tissue activity cpm / mg	t test* p	Tissue lysate cpm / ml	t test p
XNF	10	18,900 ± 3,200	-	687,000 ± 180,000	-
	20	13,800 ± 4,900	-	566,000 ± 140,000	-
	30	18,800 ± 5,500	-	762,000 ± 200,000	-
	90	8,000 ± 4,200	-	284,000 ± 160,000	-
	360	2,400 ± 630	-	54,900 ± 2,000	-
	1440	340 ± 150	-	18,500 ± 11,000	-
+PI	10	16,500 ± 2,300	0.35	626,000 ± 53,000	0.21
	20	12,200 ± 4,500	0.68	408,000 ± 160,000	0.56
	30	16,400 ± 4,500	0.60	571,000 ± 110,000	0.44
	90	9,100 ± 1,500	0.70	407,000 ± 12,000	0.14
	360	13,400 ± 1,100	0.00	359,000 ± 54,000	0.00
	1440	4,100 ± 790	0.00	128,000 ± 12,000	0.00

Group	Time (min)	TCA activity (cpm / ml)	t test p	ipp activity (cpm / cpm)	t test p
XNF	10	509,000 ± 63,000	-	150,000 ± 4,500	-
	20	503,000 ± 83,000	-	100,000 ± 2,800	-
	30	534,000 ± 160,000	-	193,000 ± 25,000	-
	90	183,000 ± 83,000	-	64,200 ± 2,400	-
	360	37,900 ± 2,300	-	12,800 ± 310	-
	1440	10,200 ± 3,700	-	3,670 ± 25	-
+PI	10	653,000 ± 160,000	0.61	187,000 ± 7,400	0.00
	20	428,000 ± 190,000	0.27	205,000 ± 5,000	0.00
	30	636,000 ± 130,000	0.22	288,000 ± 6,200	0.00
	90	784,000 ± 550,000	0.36	179,000 ± 9,700	0.00
	360	412,000 ± 48,000	0.00	169,000 ± 3,000	0.00
	1440	115,000 ± 17,000	0.00	36,900 ± 2,200	0.00

*Student t test was applied in the statistical calculation. p<0.05 is considered to be significant.

Table III-2 Proteolysis - injection site recoveries
TCA, immunoprecipitable recoveries and XNF activity remaining injection site
after s.c. bolus to mice (N=3)

Group	Time (min)	ipp %	recovery	t test p	remaining %	at inj. site ²	t test p	TCA %	recovery	t test p
XNF	10	22.8 ± 5.80		-	44.3 ± 1.20		-	57.9 ± 5.70		-
	20	18.50 ± 5.10		-	38.1 ± 7.00		-	59.8 ± 5.90		-
	30	26.3 ± 5.90		-	36.9 ± 1.30		-	51.5 ± 2.60		-
	90	33.5 ± 29.1		-	15.5 ± 0.600		-	57.5 ± 16.8		-
	360	23.4 ± 1.00		-	3.60 ± 0.900		-	54.5 ± 10.2		-
	1440	25.3 ± 14.1		-	0.800 ± 0.200		-	42.7 ± 32.8		-
+ ¹ PI	10	29.9 ± 2.70		0.13	45.2 ± 2.80		0.62	75.7 ± 1.90		0.01
	20	58.2 ± 30.5		0.09	35.4 ± 11.0		0.74	79.5 ± 1.00		0.00
	30	52.1 ± 12.2		0.03	38.5 ± 4.60		0.58	90.7 ± 7.70		0.00
	90	46.9 ± 13.7		0.51	32.4 ± 1.70		0.00	81.6 ± 3.30		0.07
	360	47.9 ± 7.80		0.01	28.7 ± 4.70		0.00	88.1 ± 2.70		0.01
	1440	29.1 ± 4.40		0.68	9.20 ± 1.10		0.00	70.2 ± 3.20		0.22

¹PI=protease inhibitor cocktail.

²remaining at inj. site=total XNF activity remaining at injection site.

Table III-3 Proteolysis Study - plasma counts
Total, TCA precipitable, and immunoprecipitable activity of
XNF in plasma after s.c. injection to mice (N=3)

group	time min.	total activity cpm / ml	t test P value	TCA activity cpm / ml	t test p value	IPP activity cpm / ml	t test p value
XNF	10	56,000 ± 11,000	-	6,900 ± 1,000	-	2,700 ± 1,400	-
	20	91,000 ± 15,000	-	12,400 ± 1,600	-	4,000 ± 1,800	-
	30	150,000 ± 7,900	-	19,200 ± 2,000	-	4,550 ± 270	-
	90	218,000 ± 33,000	-	26,000 ± 5,100	-	3,870 ± 880	-
	360	67,200 ± 16,000	-	10,900 ± 600	-	2,300 ± 1,100	-
	1440	7,600 ± 290	-	3,800 ± 430	-	1,030 ± 450	-
+PI	10	27,400 ± 3,400	0.00	2,700 ± 260	0.01	1,960 ± 1,000	0.50
	20	46,200 ± 9,600	0.01	4,700 ± 2,300	0.01	1990 ± 770	0.15
	30	41,800 ± 5,600	0.00	3,900 ± 1,600	0.00	1,870 ± 720	0.00
	90	78,200 ± 12,700	0.01	6,900 ± 4,600	0.00	3,190 ± 2,100	0.63
	360	46,600 ± 6,000	0.32	9,700 ± 1,600	0.11	2,800 ± 1,700	0.70
	1440	41,400 ± 18,000	0.05	10,300 ± 4,000	0.03	2,270 ± 930	0.10

Table III-4 Proteolysis Study- plasma recoveries
TCA, immunoprecipitable recoveries, and XNF remaining in plasma
after s.c. bolus to mice (N=3)

group	time min.	IPP recovery %	T-test P value	TCA recovery %	T-test P value	XNF remaining %	T-test P value
XNF	10	4.80 ± 2.10	-	23.7 ± 4.30	-	0.787 ± 0.232	-
	20	4.40 ± 2.00	-	25.2 ± 0.170	-	1.35 ± 0.191	-
	30	3.00 ± 0.220	-	24.0 ± 1.70	-	2.16 ± 0.180	-
	90	1.80 ± 0.740	-	19.8 ± 1.80	-	2.94 ± 0.300	-
	360	3.50 ± 2.10	-	29.3 ± 6.30	-	0.868 ± 0.220	-
	1440	13.6 ± 6.10	-	93.9 ± 8.50	-	0.0965 ± 0.0100	-
+PI	10	7.40 ± 4.60	0.42	18.9 ± 2.70	0.17	0.383 ± 0.0620	0.45
	20	4.30 ± 1.60	0.96	19.3 ± 2.10	0.01	0.634 ± 0.190	0.81
	30	4.70 ± 2.30	0.29	17.7 ± 2.80	0.03	0.520 ± 0.0550	0.085
	90	3.90 ± 2.20	0.21	17.8 ± 7.10	0.66	1.01 ± 0.0980	0.00
	360	5.90 ± 3.00	0.33	36.5 ± 7.71	0.28	0.604 ± 0.100	0.15
	1440	5.60 ± 0.240	0.084	64.0 ± 2.91	0.01	0.542 ± 0.220	0.014

**Table III-5 Mean AUC (Area Under Curve)
of XNF activity in plasma**

Study group	Time (min)	post-TCA (cpm/ml)	st. dev.	AUC (cpm × min./ml)
XNF control	0	0	0	
	10	6,900	10,800	34,500
	20	12,400	15,300	96,300
	30	19,200	7,920	158,000
	90	26,000	33,400	1,360,000
	360	10,900	16,300	4,980,000
	1440	3,770	290	7,910,000
			AUC=	14,500,000
+PI	10	2,730	3,420	13,700
	20	4,710	9,590	37,200
	30	3,930	5,600	43,200
	90	6,950	12,700	326,000
	360	9,740	6,040	2,250,000
	1440	10,300	18,300	10,800,000
			AUC=	13,500,000

Fig. III-1 Total tissue activity of XNF at injection site after s.c. bolus to mice (N=3)

Fig. III-2 Total tissue lysate supernatant activity of XNF at injection site after s.c. bolus to mice (N=3)

• For all graphs, +PI: protease inhibitors added

Fig. III-3 TCA precipitable activity of XNF at injection site after s.c. bolus to mice (N=3)

Fig. III-4 Immunoprecipitable activity of XNF at injection site after s.c. bolus to mice (N=3)

Fig. III-5 Total, TCA precipitable and immunoprecipitable activity of XNF in plasma after s.c. injection to mice (N=3)

Fig. III-6 TCA precipitable activity of injection site tissue lysate and plasma after s.c. bolus to mice (N=3)

Fig. III-7 The percentage of total XNF activity remaining at injection site after s.c. bolus to mice (N=3)

Fig. III-8 Proteolysis
SDS-PAGE of total XNF activity at
injection site after s.c. injection to mice (N=3)

*PI: protease inhibitors

Fig. III-9 Proteolysis
SDS-PAGE of immunoprecipitable XNF activity at
injection site after s.c. injection to mice (N=3)

• PI: protease inhibitors

Fig. IV-1 The light microscopy of injection site tissue 30 min following s.c. of ^{125}I -XNF to rats. XNF associated radioactive material (black) was dispersed throughout subcutaneous space but showed apparent local densities.

Fig. IV-2 The light microscopy (high magnification) of injection site 30 min after s.c. of ^{125}I -XNF in rats. XNF molecules seem to be localized into circular pattern - perhaps cellular association with macrophages, mast cells and fibroblasts.

Fig. IV-3 The light microscopy (high magnification) of injection site 30 min after s.c. of ^{125}I -XNF to rats. This is the same region as Fig. IV-2 but with macrophage-specific staining (polyclonal antibody). Blue staining for viable cells and black blue for macrophages. This figure helps to show that the distribution pattern of macrophages is similar to that of XNF associated radioactive materials suggesting that the macrophages may be holding XNF.

Fig. IV-4 The light microscopy of inguinal node that drains the injection site 1.5 hr after s.c. injection of ^{125}I -XNF in rats. No radioactive material is visible.

Fig. IV-5 The endocytosis of XNF in macrophage at injection site. The XNF associated radioactive material (black spots) was engulfed by many lysosomes and endosomes in the macrophage.

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