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Actions of CNTF and neurotrophins on degenerating motoneurons: preclinical studies and clinical implications

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Abstract

Spinal motoneurons innervating skeletal muscle were amongst the first neurons shown to require the presence of their target cells to develop appropriately. Isolated embryonic chick and rat motoneurons have been used to identify neurotrophic factors and cytokines capable of supporting the survival of developing motoneurons. Such factors include ciliary neurotrophic factor (CNTF), which is present physiologically in high amounts in myelinating Schwann cells of peripheral nerves, and brain-derived neurotrophic factor (BDNF) which is synthesized in skeletal muscle and, after peripheral nerve lesion, in Schwann cells. These factors have been further analyzed for their physiological significance in maintaining motoneuron function in vivo, and for their potential therapeutic usefulness in degenerative motoneuron disease. Both CNTF and BDNF are capable of rescuing injured facial motoneurons in newborn rats. Furthermore, CNTF prolongs survival and improves motor function of pmn mice, an animal model for degenerative motoneuron disease, by preventing degeneration of motoneuron axons and somata. Thus treatment of human motoneuron disease with neurotrophic factors should be possible, provided that rational means for application of these factors can be established considering also the appearance of potential side effects.

Key words: Motor neuron disease; Ciliary neurotrophic factor; Brain-derived neurotrophic factor; Animal models; Neurotrophic factors

1. Introduction

Neuromuscular disorders involving spinal motoneurons are still a major challenge to both clinical and basic sciences as the mechanisms leading to these diseases are far from understood. Different types of spinal muscular atrophy and inherited forms of amyotrophic lateral sclerosis have been the topic of genetic analysis and significant progress has been made in the search for the genes underlying these disorders. For example, the gene defect responsible for X-linked bulbo-spinal muscular atrophy has been identified (La Spada et al. 1991) and very recently, defects in the Cu^{2+}/Zn^{2+} dependent superoxide dismutase gene have been found to be associated with familial cases of ALS (Rosen et al. 1993).

However, it is still unclear whether these gene defects are also responsible for the majority of sporadic cases of ALS. Differences in histological and pathophysiological appearance suggest that the mechanisms leading to motoneuron degeneration might differ for familial and sporadic ALS (Williams 1992).

Neurotrophic factors are known to play a major role in supporting the survival of many neuronal populations including spinal motoneurons during development (for a review see Oppenheim 1981). Moreover, lesion of motoneurons during the early postnatal period leads to almost 100% cell death of the affected motoneurons, which can be successfully prevented by the addition of neurotrophic factors such as CNTF (Sendtner et al. 1990) and brain derived neurotrophic factor (Sendtner et al. 1992b; Oppenheim et al. 1992b; Yan et al. 1992). These findings, together with other results indicating a physiological role of these factors in the development and maintenance of function of motoneurons, provides important information for the potential treatment of degenerative motoneuron disorders.

2. Results

2.1. Effects of neurotrophic factors on embryonic motoneurons

During the period of developmental cell death many types of neurons including motoneurons are known to depend on survival factors which are produced in the target tissues, i.e. in the case of motoneurons the skeletal muscle. Skeletal muscle extracts are capable of supporting most of the motoneurons which would die in vitro (Arakawa 1990) or in vivo (Oppenheim 1988). However, at-

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tempts to identify motoneuron survival factors from these extracts have been of limited success.

Of the known factors, CNTF was first identified as being highly active in supporting the survival of embryonic chick and rat motoneurons in culture (Arakawa et al. 1990; Magal et al. 1991). At concentrations of 1.5 ng/ml more than 60% of the originally plated neurons survived (EC_{50} 0.023 ng/ml). A subsequent study has shown that CNTF was also active in vivo in rescuing motoneurons from cell death when supplied daily onto the chorioallantoic membrane of chick embryos from day 5 to 9 of development (Oppenheim 1991). Basic FGF (bFGF) was shown to support about 50% of embryonic day 6 motoneurons in culture, although the concentrations necessary for maximal effects were about ten times higher than those of CNTF. Also insulin-like growth factor I (IGF-I) showed a significant survival effect at very high concentrations. Interestingly, when motoneurons from 15-day-old rat embryos were isolated and kept in culture under serum-free conditions, IGF-I was able to support more than 50% of the originally plated neurons at concentrations between 10 and 100 ng/ml) (Hughes et al., 1993). In comparison, embryonic day 6 chick motoneurons need at least 1 μg/ml of IGF-I for maximal survival rates of 15–20%. A possible explanation for this discrepancy could be that the IGF-I effect on the cultured chick motoneurons is partially blocked by IGF-binding proteins present in horse serum (Prosser and McLaren 1992), which was used as a supplement (10%) to the culture medium of the chick motoneurons.

Another difference between embryonic chick and rat motoneurons in culture is the responsiveness to neurotrophins. Embryonic day 6 chick motoneurons do not respond to BDNF and NT-3 under the same culture conditions where CNTF and bFGF are active. However, in cultures of embryonic day 15 rat motoneurons, BDNF and to a lesser extent also NT-3 show significant survival effects (Hughes et al., 1993). Also in vivo, these two factors are capable of rescuing motoneurons of newborn rats after peripheral lesion (Sendtner et al. 1992b; Yan et al. 1992). Similarly, the number of spinal motoneurons surviving the developmental period of naturally occurring cell death in developing chick embryos could be increased by addition of brain-derived neurotrophic factor onto the chorioallantoic membrane (Oppenheim et al. 1992b). This finding contrasts with the negative effect of BDNF in supporting isolated embryonic chick motoneurons in culture, indicating that chick motoneurons show different responsiveness to BDNF in vivo and in vitro under the given experimental conditions.

Such a differential responsiveness in vivo and in vitro was also observed with bFGF. Although this factor is active in supporting both motoneurons from chick and rat in vitro (Arakawa et al. 1990; Unsicker et al. 1987; Martinou et al. 1992), bFGF did not show any significant survival effect on facial motoneurons after nerve lesion in newborn rats (M.S., unpublished observations) or on spinal motoneurons of developing chick embryos after daily injections onto the chorioallantoic membrane (Oppenheim et al. 1992a). These results, which are contradictory to other reports (Dreyer et al. 1989), leave it open whether bFGF exerts a physiological function for spinal motoneurons in vivo.

2.2. The regulation of CNTF gene expression after peripheral nerve lesion: comparison with other neurotrophic factors

CNTF is not expressed in the rat embryo during the period of naturally occurring cell death. In the adult rat, CNTF protein is found in the cytosol of myelinating Schwann cells of peripheral nerves and a subpopulation of glial cells within the central nervous system (Stöckli et al. 1991). In contrast, CNTF is not expressed in skeletal muscle, the target tissue of spinal motoneurons (Stöckli et al. 1989). The immunohistological location of this factor in the cytosol of synthesizing cells (Sendtner et al. 1991) and the absence of a hydrophobic leader sequence usually present in secretory molecules suggest that this molecule might act as a lesion factor in adult animals. In contrast, BDNF exhibits the structural features typical of a secretory molecule (Leibrock et al. 1989). Furthermore, it is expressed in muscle during development when naturally occurring motoneuron cell death occurs and thus fulfills the criteria for a target-derived neurotrophic factor (Barde 1989) for spinal motoneurons in analogy to the physiological role of NGF for sympathetic and dorsal root sensory neurons (Johnson et al. 1986).

After facial nerve lesion in newborn rats, both BDNF and CNTF can rescue motoneurons from degeneration. However, the differences in structure and regulation of expression suggest that these two factors might have different functions on motoneurons after peripheral nerve lesion in adult rats. The levels of CNTF in the unlesioned peripheral nerves of the rat are extremely high (Table 1). Sciatic nerves from adult rats contain at least 1 μg CNTF per g wet weight (Sendtner, unpublished results), whereas the concentration of NGF is at least 10 000 times lower. Given the high biological activity (60 pg CNTF per ml medium are sufficient to support maximal survival of cultured chick ciliary neurons or motoneurons), it can be concluded that only an extremely small proportion of available CNTF need be released from Schwann cells to influence responsive neurons. Under pathophysiological conditions, when peripheral nerves are lesioned, such low quantities of CNTF might become available to the axons. Semiquantitative estimations (Table 1) show that the quantities of biologically active CNTF in the distal part of a sciatic nerve one week after lesion are still 1000 times higher than these of NGF, a neurotrophic factor whose expression is upregulated in the lesioned nerve (Heumann et al. 1987). Interestingly, BDNF synthesis is also increased in the lesioned nerve, but with a different

Table 1

Comparison of the levels of CNTF and NGF biological activity (expressed as trophic units/mg protein) in the sciatic nerve of the adult rat after lesion. Data are modified according to Heumann et al. (1987) and Sendtner et al. (1992a).

	NGF	CNTF
Unlesioned sciatic nerve	0.6 ± 0.2 *	17 500 ± 2 500 **
Distal sciatic nerve 7 days after lesion	2.5 ± 0.6 *	3 150 ± 620 **

* One trophic unit of NGF corresponds to about 75 pg/ml, which is the amount necessary for stimulating half maximal survival of embryonic day 8 chick dorsal root sensory neurons in culture.

** One trophic unit of CNTF corresponds to about 30 pg/ml, the amount which supports half maximal survival of embryonic day 8 chick ciliary neurons in culture.

time course (Meyer et al. 1992). An increase in BDNF mRNA is not detectable until 3 days post-lesion, but during the following weeks, BDNF mRNA expression is continuously increased up to levels which are at least 10 times higher than those of NGF. By this means BDNF might come into play at a time when lesioned motoneurons are regrowing towards their original targets, i.e. skeletal muscle. Under pathophysiological conditions, the role of CNTF for lesioned motoneurons might be confined to the first days after lesion when early regulatory events in the motoneuron cell bodies are detectable. Thus at least two factors, CNTF in an early phase and BDNF at later stages might interact in regulating the regeneration of motoneurons after lesion.

Furthermore, under normal, physiological conditions minute quantities of CNTF might be released from the cytoplasm of Schwann cells (e.g. by membrane leakage due to microtrauma of the cell membranes) and these might act in concert with BDNF, the latter being produced in very small amounts by skeletal muscle.

2.3. Action of CNTF in animal models for human degenerative motoneuron disease

A prerequisite for the potential use of neurotrophic factor for treatment of neurodegenerative diseases is a positive effect of these molecules on motoneuron survival in vivo. Many agents which have been clinically tested in ALS patients in the past did not fulfill this criteria, making the lack of effect of these substances not surprising (Rowland 1991).

We have used the pmn mouse mutant as a model for testing the effects of CNTF on degenerating neurons. This mouse mutant was first described in 1991 by Schmalbruch et al. (1991). In animals homozygous for the gene defect, the disease starts at the endplates. The first clinical signs are detectable as weakness of the hindlimbs during the third postnatal week. By the 5th postnatal week, the disorder has already resulted in the degeneration of many peripheral motor axons (more than 70% of the total number of phrenic nerve axons are lost at postnatal day 28), and at late stages of the disease (day 40–42) a significant loss of motoneuron cell bodies is detectable, as measured by the number of facial motoneurons in the brain stem of these mice (Sendtner et al. 1992a). All mice die before postnatal week 7–8, probably because of the loss of functional innervation of the skeletal musculature, in particular the diaphragm.

We have injected embryonic stem (ES) cells stably transfected with a CNTF construct containing an N-terminal hydrophobic leader sequence to allow secretion of CNTF protein from the cells. These cells formed solid tumors after intraperitoneal injection into pmn mice and continuously produced high levels of CNTF protein which could be measured in the blood of these mice. pmn/pmn mice treated in this way showed a marked reduction of progression of the disease: At postnatal day 40, when more than 50% of untreated control mice were dead, 7 out of 8 CNTF treated mice were still alive. Moreover, these mice showed a much better motor performance than their untreated litter mates. This was reflected by a significant increase in the numbers of myelinated axons in the phrenic nerve and motoneurons in the facial nucleus. A recent report on the effects of CNTF on sprouting of motoneurons from endplates and nodes of Ranvier (Gurney et al. 1992) is in agreement with these observations. These results indicate that CNTF not only leads to survival of the motoneuron cell bodies in the spinal cord and brain stem but also prevents axonal and motor endplate degeneration of these neurons thus leading to the improvement of motor function of the treated pmn mice.

2.4. Pharmacokinetics of CNTF after intravenous injection in adult rats

First attempts with daily subcutaneous injection of pmn/pmn mice with 5 μg of CNTF did not show significant effects on the course of the disease. In order to investigate whether this lack of effect was due to rapid elimination of CNTF from the circulation, we examined the kinetics of ^{125}I-radiolabelled CNTF after intravenous injection into adult rats. Most of the radioactivity was eliminated from the circulation within a few minutes (Dittrich et al., 1994). The same result was obtained when unlabelled recombinant rat CNTF was injected into the tail vein of adult rats. At different time intervals after intravenous injection, the rats were deeply anaesthetized and blood was taken by cardial puncture. The blood levels of CNTF, measured by a two-site ELISA, declined rapidly within the first 15 min after injection (Fig. 1). At 1 min after injection, only 143 ± 3 ng of CNTF per ml blood were measured, indicating that less than 30% of the originally injected 5 μg of CNTF were detectable in the

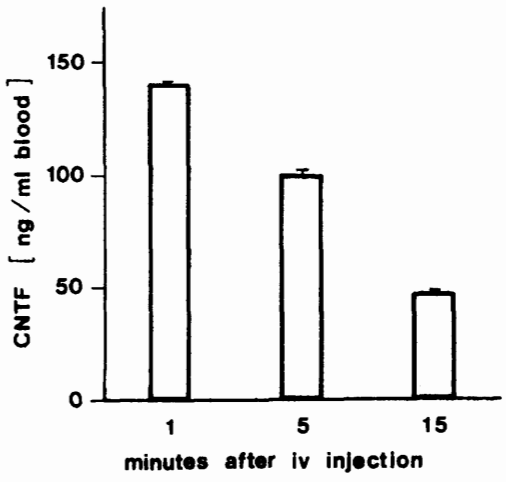

Fig. 1. Pharmacokinetics of CNTF after intravenous injection in rats. 5 μg of recombinant rat CNTF was injected into the tail vein of adult male Wistar rats (200 g body weight). Blood was taken after deep ether narcosis by cardiac puncture at 1, 5 and 15 min after CNTF injection. Heparin was added, and after centrifugation (10 min at 1000 g) the levels of CNTF were determined by a 2 site (sandwich) ELISA using two non-competing monoclonal antibodies against rat CNTF, one of them being coupled to β-galactosidase. Values shown represent mean ± SD from triplicate determinations of blood samples from 3 rats per time point. Recombinant rat CNTF was used as a standard. Control blood samples from non-injected rats did not show significant levels of CNTF (detection limit: 5 ng/ml blood).

circulation (10 ml). After an additional 14 min, CNTF concentration per ml of blood declined to 46.7 ± 11 ng, which is 32.6% of the level at 1 min after intravenous injection. Thus, half-life of CNTF in the blood was 9.4 min, using the concentration measured at 1 minute postinjection as if a reference. CNTF is rapidly removed from the blood, probably by binding to specific receptors in the liver (Dittrich et al., 1994).

2.5. Theoretical CNTF dose requirements for treatment of degenerative diseases of motoneurons

At present, no data are available as to what dose of CNTF would be able to support motoneuron survival and function in human degenerative motoneuron disease. The diffusion of proteins from the circulation to the endplates, which represent the putative uptake site of proteins from the blood into motoneurons (Fishman et al. 1991) depends on size and other characteristics of proteins, and it is not clear whether data obtained from rodent animals models are representative for the uptake of proteins into motoneurons from the circulation in diseased human motoneurons.

In pmn mice, a serum level of about 500 trophic units (TU) (one trophic unit corresponds to about 20 pg of CNTF protein per ml; see Fig. 2) are effective in supporting spinal and brainstem motoneurons. These levels are not directly transferable to humans. The biological activity of recombinant human CNTF is at least 10 times less than recombinant rat CNTF. For half-maximal survival of embryonic day 8 chick ciliary neurons about 300–500 pg of human CNTF are necessary in comparison to about 10–20 pg of rat CNTF (Masiakowski et al. 1991; our own results in Fig. 2). Experiments with human neuroblastoma and hepatoma cell lines show a similar ED_{50} for CNTF effects (Huber and Sendtner, unpublished observations), indicating that the human CNTF protein is also less active on human cells. Therefore 500 TU/ml serum in humans would be an equivalent to 150–250 ng/ml of human recombinant factor. Such high serum levels would

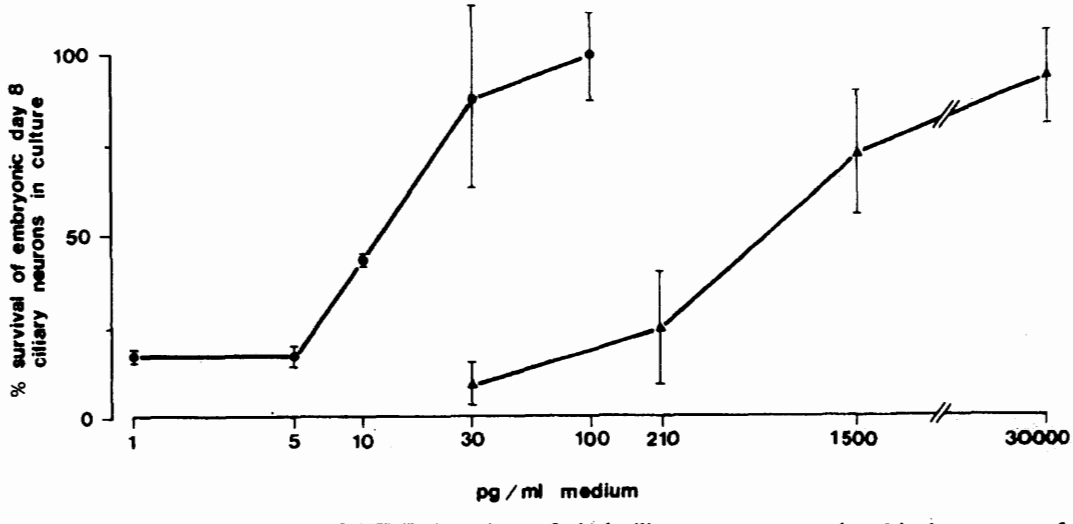

Fig. 2. Dose response curve for human and rat CNTF. Embryonic day 8 chick ciliary neurons were cultured in the presence of recombinant rat (1) and human (s) CNTF. The number of surviving neurons (16.3% of the surface of each dish) were counted under the light microscope after 24 h in culture. 100% represents the number of originally plated cells (about 1000–2000 neurons per well). Values represent mean ± SD of at least two (rat CNTF) or 4 (human CNTF) independent experiments.

only be reached when more than 100 μg of CNTF per kg body weight were to be injected at least once daily into patients.

3. Discussion

Significant progress has been made in the identification of genes which are defective in inherited forms of human X-linked bulbospinal muscular atrophy (La Spada et al. 1991) and familial amyotrophic lateral sclerosis (Rosen et al. 1993). However, it is still open whether the great majority of sporadic ALS cases are caused by similar mechanisms. Differences in clinical and pathological appearance suggest that the cellular defects leading to loss of motoneuron function might be different. This assumption is also supported by findings in rodent animal models: The gene defect of the wobbler mouse, a well-recognized animal model for motoneuron disease, resides on chromosome 11 (Kaupmann et al. 1992); in the mnd mouse, the defect is on chromosome 8 (Messer et al. 1992). It appears very likely that the genetic defect in the pmn mouse is unrelated to those of wobbler and mnd mice.

The CNTF gene resides on chromosome 19 in the mouse (Kaupmann et al. 1991) and on chromosome 11 (Lam et al. 1991) in man. A defect of this gene has not been identified so far as the primary cause of disease in any of the known mouse mutants or inherited forms of human motoneuron disease. In pmn and wobbler mice, biologically active CNTF can be extracted from myelinated peripheral nerves (Kaupmann et al. 1991; Sendtner et al. 1992a). In addition, postmortem tissue of human ALS patients show normal expression of CNTF mRNA and protein, at least in 10 cases which could be analyzed (Sendtner, unpublished observations).

Why should neurotrophic factors such as CNTF be useful for treatment of human motoneuron disease, if their genes are apparently primarily unaffected in these diseases? A large population of motoneurons degenerate during embryonic development (for a review see Oppenheim 1982) and the mechanism by which these cells are eliminated is most probably apoptosis (Raff 1992). If motoneurons from these developmental stages are isolated from chick embryos and brought into culture, then they degenerate in the absence of appropriate survival factors. CNTF, bFGF and IGF-I are amongst the most potent factors in such cultures of chick embryonic day 6 spinal motoneurons (Arakawa et al. 1990). In the case of CNTF, these results were confirmed by in vivo studies when this factor was added onto the chorioallantoic membrane of chick embryos during the same experimental period (Oppenheim 1991). In contrast, bFGF was not active under the same experimental conditions (Oppenheim 1992a), suggesting that the mechanisms by which these two factors act on motoneurons are fundamentally different.

But also in postnatal animals, when nerves are lesioned, the subsequent cell death of motoneurons can be prevented by the addition of neurotrophic factors such as CNTF (Sendtner et al. 1990), BDNF (Sendtner et al. 1992b; Yan et al. 1992) and, to a lesser extent, NT-3 (Sendtner et al. 1992b). The differential regulation of CNTF and BDNF (Sendtner et al. 1992c; Meyer et al. 1992) after peripheral nerve lesion suggests that the biological function of these different factors are not redundant. Furthermore, the different expression patterns of BDNF (low but significant amounts in skeletal muscle) and CNTF (predominant expression in myelinating Schwann cells) suggest that functional maintenance of motoneurons is regulated by different factors from different cellular sources. The finding that CNTF overexpression in pmn mice leads to significant improvement of motoneuron function (Sendtner et al. 1992a) is an additional indicator that this factor is usually not available in abundance to motoneurons. Therefore the pharmacological application of CNTF would be justified under conditions where motoneurons are degenerating.

Recent discussions have focussed on the question whether the pathological appearance of pmn mice might be representative of human motoneuron disease (Vrbová et al. 1992). In postmortem tissue of ALS patients, a significant loss of spinal motoneurons occurs, the remaining cells appear shrunken, dark, with basophilic cytoplasm and pyknosis of the nuclei. These morphological characteristics very much resemble the changes occurring during apoptosis (for a review see Cohen 1993). Many pieces of evidence, such as the finding that more myelinated nerve fibers are detectable in proximal than in distal parts of motor nerves of ALS patients (Bradley et al. 1983), have led to the model that ALS starts at the endplates, proceeds as degeneration of the axons in a dying back-fashion and finally leads to degeneration of the cell bodies in the spinal cord and brain stem (Chou 1992). This would be consistent with the pathological findings in pmn mice (Schmalbruch et al. 1991). In both cases, clinical symptoms are expected to become apparent at the stage when motoneuron nerve terminals are affected and not after they have died. Based on the effectiveness of CNTF in the pmn mouse mutant, this neurotrophic factor could indeed interfere with the pathophysiological mechanisms leading to functional loss and death of motoneurons in human motoneuron disease.

Nonetheless, several important questions concerning the rational treatment of human motoneuron disease with CNTF and other neurotrophic molecules remain open. Bolus injection of CNTF leads to only very limited availability of the factor for the motoneurons. It appears likely that given the high theoretical doses (more than 100 μg/kg body weight) necessary for reaching sufficient systemic levels of the factor, side effects, in particular in the liver could preclude this approach from the clinic. Liver cells have functional CNTF receptors (Dittrich et al. 1994) and primary cultures of liver cells have been re-

ported to react by induction of acute phase proteins after CNTF addition (Schooltink et al. 1992; Nesbitt et al. 1993). This is not unexpected, as the known acute phase mediators interleukin-6 and leukemia inhibitory factor interact with the same receptor subunit (gp130) as CNTF on responsive cells (Taga and Kishimoto 1992; Ip et al. 1992). It remains to be established whether low doses of CNTF which would not lead to inflammatory responses in liver cells are sufficient to support motoneurons in ALS patients. Alternative pharmacological approaches which would allow increased availability of CNTF for motoneurons and reduced side effects might help to overcome this problem. Such alternative approaches might anyhow be necessary in order to support the upper motoneurons which are involved in many forms of human motoneuron disease. As the neurotrophic requirements of these cells are still not known, it remains open whether clinical improvement could be reached even in the case when maintenance of lower motoneurons would be possible. It remains to be established whether approaches such as intrathecal administration (Kroin and Penn 1989; Ochs and Struppler 1987) of neurotrophic factors can help to overcome these problems or whether new strategies such as recombinant viruses for therapeutic gene transfer (Anderson 1992) are necessary.

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