Hepatocyte growth factor (HGF) has been shown to function as a potent mitogen for a variety of cells, transmitting its signal through the c-met tyrosine kinase receptor. Ciliary neurotrophic factor (CNTF) is a cytokine that has been shown to promote survival of motor neurons. We show here that c-met mRNA is present in the embryonic rat spinal cord. Peak expression of c-met (at E14) coincides with the period of naturally occurring cell death in motor neurons, suggesting a possible role of HGF in the regulation of this process. Utilizing a neuron-enriched culture system, we established that HGF, like CNTF, stimulates choline acetyltransferase (CAT) activity in motor neurons. When co-administered to motor neuron cultures, saturating concentrations of HGF and CNTF produced a synergistic increase in CAT levels. We show that this synergy reflects enhanced motor neuron survival. Exposure of motor neuron cultures to the cytokstatic agent vincristine markedly decreased CAT levels; co-treatment with HGF and CNTF (but not either factor alone) restored CAT activity to control levels. Our findings indicate that HGF is a survival factor for motor neurons, that it acts synergistically with CNTF, and that HGF and CNTF can together be neuroprotective in the face of vincristine toxicity.

Hepatocyte growth factor (HGF) was originally identified in the serum of partially hepatocemtomized rats as a potent mitogen for cultured rat hepatocytes (1, 2). Also known as scatter factor, HGF stimulates dissociation of epithelial cell colonies in monolayer culture (3–5). The biologically active HGF molecule is a heterodimer that consists of two disulfide-linked, glycosylated polypeptide chains (6). Until recently, HGF was considered to have a narrow target cell specificity and to act primarily as a humoral mediator of liver regeneration after partial hepatectomy or hepatic injury (for review, see Ref. 7). Increasing evidence has shown that HGF is a multifunctional polypeptide with actions on a wide variety of cell types, including microglia (8), skeletal muscle (9), Schwann cells (10), and central nervous system neurons (11–13).

The HGF receptor has been identified as a transmembrane tyrosine kinase receptor, encoded by the proto-oncogene c-met (14, 15). c-met is expressed in normal epithelium of almost every tissue; however, other cell types, such as endothelial cells, microglial cells, hemopoietic cells, and neurons, have been shown to express this receptor (for review, see Ref. 16). The first indication of a possible role of HGF in neural development was suggested by Stern and Ireland (17). They showed that HGF induced cultured chick ectodermal cells to express a neural rather than an epidermal phenotype, and they speculated that HGF could be a neural-inducing signal during the early development of vertebrate embryos. Disruption of the HGF gene resulted in embryonic lethality at day 15 of gestation, primarily due to defects in development of the liver and placenta (18, 19). Other tissues, including motor neurons and muscle, appeared normal at the time of death. With regard to motor neuron or muscle development, these findings may indicate that either HGF is not essential for the early stages of embryogenesis, or that compensation or redundancy exists in the HGF signaling network. On the other hand, it has been reported that, in the HGF transgenic mice, ectopic expression of HGF in the adjacent neural tube induced inappropriate formation of skeletal muscle in the central nervous system (20). In mice carrying c-met null mutations, myogenic precursor cells failed to migrate, preventing the normal development of limb and body wall muscle (21). In addition, transient expression of c-met and HGF in limb mesenchyme and c-met in motor neurons were also detected (12, 22). Taken together, these observations suggest possible roles of HGF in the development of the neuromuscular system.

In the present study, we observed a developmentally regulated pattern of c-met expression in the embryonic rat spinal cord. The coincidence of maximal expression of the HGF receptor with the peak period of natural occurring cell death in motor neuron suggested that HGF-c-met signaling might play an important role in the regulation of this process. Thus, we sought to determine the physiological effects of HGF on motor neurons using a motor neuron-enriched culture system. Our initial experiments demonstrated that HGF promoted motor neuron survival in vitro (12). Interestingly, HGF shows synergy with ciliary neurotrophic factor (CNTF) both in the stimulation of cholinergic phenotype and in the survival of cultured motor neurons. Furthermore, when motor neuron cultures were exposed to the cytotoxic drug vincristine, a common anti-tumor agent, co-treatment with HGF and CNTF, but not either factor alone, restored cholinergic expression and function to near normal. Taken together, our findings suggest that HGF may play an important role in motor neuron development, and that in pharmacological doses it may have potential as a neuroprotectant.

**EXPERIMENTAL PROCEDURES**

**Motor Neuron-enriched Culture**— Cultures enriched for motor neurons were prepared from E14 rat embryos as described previously (23).

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated from cultures by the GTC extraction method as described (24). Total
RNA from rat spinal cords was isolated by the LiCl method (25). Ten micrograms of total RNA was electrophoresed in 1% agarose, 2.2 M formaldehyde gels, transferred to nylon membranes in 6×SSC, and UV cross-linked (0.1 joule). Blots were prehybridized, hybridized, and washed as described previously (25). A c-met probe encoding the tyrosine kinase domain was derived from the xho-sac fragment of the mouse c-met gene. The plasmid insert was isolated after restriction endonuclease digestion, separated by agarose gel electrophoresis, and purified by electroelution. 32P-Labeled cDNA probe with specific activity of 2–5×10^9 cpm/μg was prepared by primer extension with random hexamers using a Prime-a-Gene kit (Promega) according to the manufacturer’s instructions.

Choline Acetyltransferase Assay—Cultures were harvested and assayed for CAT activity as described previously (23).

Immunocytochemical Staining for p75 LNGFR—Cultures were washed twice with phosphate-buffered saline and fixed with 4% paraformaldehyde for 20 min. Nonspecific protein binding was blocked by incubating cultures in 10% normal goat serum in phosphate-buffered saline (pH 7.4) for 1 h. The cultures were then incubated with a rabbit polyclonal antibody against a peptide fragment of LNGFR (kindly provided by Dr. P. DiStefano, Regeneron) at a dilution of 1:15,000 for 48 h at 4°C. The bound rabbit immunoglobulin (Ig) was detected using a biotinylated goat-anti-rabbit IgG (1:200, Vector), followed by peroxidase-conjugated avidin (1:500; VectorAB Kit). Immunoreactivity was visualized using diaminobenzidine as the substrate for the bound peroxidase enzyme, followed by intensification with nickel sulfate. Cell counts were performed under a 32× objective lens, with the aid of a 0.45-mm grid.

Trophic Factors and Vincristine—Recombinant rat CNTF was prepared as described (26). HGF and vincristine were purchased from R&D Systems and Sigma, respectively.

RESULTS

Expression of c-met mRNA in Spinal Cord and Cultured Motor Neurons—To explore the possibility that developing motor neurons might be responsive to HGF, we first examined the developmental time course of expression of c-met mRNA in the embryonic rat spinal cord. Although detectable at all embryonic ages examined, there was a clear peak of expression of c-met mRNA in spinal cord between E14 and E16 (Fig. 1A). Levels of expression were already lower by birth and essentially undetectable by P19 (Fig. 1A). Following dissection of E14 rat spinal cord into dorsal and ventral halves, we detected substantially higher levels of c-met mRNA in the ventral portion (Fig. 1B), suggestive of a predominant localization in motor neurons. This was substantiated by the fact that c-met mRNA was clearly detectable in motor neuron-enriched cultures derived from E14 rat spinal cord (Fig. 1B).

HGF Is a Neurotrophic Factor for Motor Neurons

Many neurotrophic factors, such as CNTF, BDNF, NT-3, and NT-4, have been shown to stimulate the cholinergic phenotype and/or survival of cultured motor neurons (23, 27). To assess the effects of HGF on cholinergic expression, we measured the activity of choline acetyltransferase (CAT), the rate-limiting enzyme for acetylcholine synthesis. Motor neuron cultures were treated with HGF, CNTF, or both, on the day of plating. HGF increased CAT activity in cultured motor neurons in a dose-dependent manner, but rather modestly, attaining a maximal stimulation (2-fold) at about 10 ng/ml (Fig. 2A). CNTF, on the other hand, stimulated a 5-fold increase in CAT activity at 1 ng/ml (Fig. 2B). Interestingly, in the presence of a fixed maximal dose of HGF (10 ng/ml), the dose-response curve to...
CNTF did not shift with regard to potency (i.e., still saturated at 1 ng/ml) but did increase markedly in magnitude (more than a 10-fold stimulation). Thus, the combination of HGF and CNTF produced a maximal increase in CAT activity that was almost double that achieved by simply adding the effects of the two factors when used alone (Fig. 2B). This synergistic up-regulation of cholinergic phenotype by two growth factors from two different families, CNTF and HGF, is reminiscent of that observed previously with CNTF and BDNF (23).

**HGF Promotes Survival of Motor Neurons**—Increased CAT activity could be due to the rescue of cholinergic motor neurons which would otherwise degenerate in culture in the absence of appropriate growth factors or to the induction of the cholinergic phenotype, or both. Henderson et al. (28) have shown that p75LNGFR is an accurate marker for developing motor neurons, whereby they successfully established highly purified motor neuron cultures with an immunospanning technique using a monoclonal antibody to p75LNGFR. Thus, we determined the number of motor neurons in our cultures using p75LNGFR as a marker for motor neuron survival. After 2 days in vitro, cultures treated with CNTF or HGF showed enhanced survival of p75LNGFR-positive neurons as compared to control (Fig. 3). When HGF and CNTF were co-administered to the cultures, survival was greater than with either factor alone, and the motor neurons exhibited a highly differentiated phenotype, i.e., larger somal diameter and thicker neurites (Fig. 3). Upon cell counting, we found that HGF was as effective as CNTF in supporting survival of p75LNGFR positive neurons, resulting in a 1.5-fold increase in cell number (Fig. 4). As shown in Fig. 2B, CNTF stimulated CAT activity 5-fold; however, survival of p75LNGFR-positive neurons was only increased by 1.5-fold, suggesting that CNTF simultaneously promotes survival and cholinergic differentiation of cultured motor neurons. Unlike CNTF, HGF predominantly affected motor neuron survival with no apparent additional effect on cholinergic phenotype, as reflected by the relatively similar increase (2-fold) in either CAT activity or the number of p75LNGFR-positive neurons. When HGF and CNTF were co-administered, a 4-fold increase in p75LNGFR-positive neurons was observed (Fig. 4). Since the effect was greater than the sum of either factor added alone (4-fold versus 3-fold), this suggests that HGF and CNTF act synergistically to enhance motor neuron survival as well. Based on visual examination, the estimated total number of phase bright cells in cultures treated with HGF and/or CNTF was clearly greater than that in control cultures (data not shown). This argues against the possibility that HGF and/or CNTF simply up-regulated p75LNGFR expression rather than by enhancing motor neuron survival.

**Vincristine Toxicity in Cultured Motor Neurons**—Motor neurons are vulnerable to many neurotoxins. The vinca alkaloid vincristine sulfate is a chemotherapeutic agent widely used to treat a variety of neoplasms. Its use is, however, limited by a dose-related toxic neuropathy affecting both motor and sensory nerves (29). In the present study, we found that motor neurons in vitro were also susceptible to vincristine toxicity, as demonstrated by the dose-dependent loss of CAT activity in cultures exposed to vincristine (Fig. 5A). In the presence of 1 nM vincristine, motor neurons degenerated within 48 h, as reflected by a nearly 10-fold decrease of CAT activity. Co-treatment of HGF and CNTF Protects Motor Neurons from Vincristine Toxicity: Preservation of Cholinergic Phenotype—In the presence of vincristine (1 nM), control and CNTF-treated cultures showed few phase bright cells with abundant floating debris. However, when HGF or HGF + CNTF were co-administered with vincristine, cultures were clearly healthier with many phase bright cells, especially in the HGF + CNTF group, and this was reflected in the levels of CAT activity. In cultures treated with HGF, CAT levels were restored to about one-third of that of control cultures not exposed to vincristine (Fig. 5B). Although CNTF alone did not reverse the loss...
treated with 1 nM vincristine with or without trophic factors (CNTF, 0 and assayed for CAT activity on day 2 (41). In addition, electrophysiological studies performed on pa-

In the last few years a surprisingly large number of growth factors have been shown to synergize with BDNF to support motor neuron survival and differentiation in vitro (32). HGF and stem cell factor also show synergy in stimulating the formation of multipotent CFU-GEMM colonies (33). In the nervous system, CNTF has been shown to interact synergistically with BDNF to support motor neuron survival and differentiation in vitro (23) and in vivo (34), as well as with either fibroblast growth factor or NGF to promote neuronal differentiation (35, 36). Thus, it is not entirely surprising that co-administration of HGF and CNTF enhanced motor neuron survival and differentiation and protected motor neurons from vincristine neurotoxicity to a greater degree than that achieved with either factor alone.

Very often, effects of trophic factors in vitro can be translated to efficacy in vivo. HGF exists as an inactive single chain form in the liver of normal rats; however, after hepatotoxin treatments, HGF production is markedly increased in the liver, and a significant portion of HGF is converted to the active heterodimeric form (37). This conversion is mediated by a serine protease, the activity of which is detected exclusively in the injured liver but not in normal liver (37). Interestingly, although not found in normal adult muscle, HGF has been detected in muscle following injury (9). Such an up-regulation of HGF may provide a retrograde signal to motor neurons, and, thus, HGF may play a role in maintaining the neuromuscular system after injury. Although it is not known whether HGF is up-regulated in muscles after denervation, it is conceivable that this scenario will provide the opportunity for the HGF/ CNTF synergy to occur in vivo. CNTF has been shown to be transiently released by Schwann cells (38) after denervation. If HGF is synthesized in the denervated muscle and CNTF is being released by the terminal Schwann cells, they can interact to signal enhanced motor neuron survival.

To further support the neurotrophic role of HGF in motor neuron survival, we employed a toxic neuropathy model using the vinca alkaloid vincristine sulfate. Vincristine is a widely used cancer chemotherapeutic agent that commonly produces a mixed sensory-motor polyneuropathy, frequently accompanied by muscle weakness. The motor neuropathy in particular may be especially debilitating, sometimes resulting in foot drop and difficulty in ambulation (39). DiGregorio et al. (40) have shown that administration of vincristine to rats induced severe alterations in the mechanical and electrophysiological properties of skeletal muscles. Pathological alterations in skeletal muscle fibers associated with vincristine use have also been reported (41). In addition, electrophysiological studies performed on patients treated with vincristine showed that the drug caused (HGF), has the ability to promote motor neuron survival. Despite the multiplicity of factors that act upon motor neurons, no single neurotrophic factor has been found, so far, that can maintain long term survival of these cells in vitro or in vivo (30, 31). This suggests (i) that all or most motor neurons require multiple factors from diverse sources for sustained survival, (ii) the existence of subpopulations of motor neurons that require different neurotrophic signals for their survival, (iii) the possibility that motor neurons require distinct factors at different stages of development, and (iv) that motor neurons may require certain factors for survival and different factors for regulation of their phenotype. All together, it appears that there are ample reasons to invoke the involvement of multiple neurotrophic factors during motor neuron development to ensure successful formation and maintenance of the neuromuscular system.

Both HGF and CNTF have been demonstrated to synergize with other trophic molecules. In the hematopoietic system, HGF has been shown to synergize with IL-3 and granulocyte macrophage-colony-stimulating factor to stimulate colony formation of hematopoietic progenitor cells in vitro (32). HGF and stem cell factor also show synergy in stimulating the formation of multipotent CFU-GEMM colonies (33). In the nervous system, CNTF has been shown to interact synergistically with BDNF to support motor neuron survival and differentiation in vitro (23) and in vivo (34), as well as with either fibroblast growth factor or NGF to promote neuronal differentiation (35, 36). Thus, it is not entirely surprising that co-administration of HGF and CNTF enhanced motor neuron survival and differentiation and protected motor neurons from vincristine neurotoxicity to a greater degree than that achieved with either factor alone.

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slowing of both motor and sensory conduction in peripheral nerves (42). Thus, the use of vincristine as an anti-cancer drug is limited by such neurotoxicity. One possible way to reduce motor neuron toxicity is to provide motor neurons with additional trophic support prior to or in conjunction with the administration of vincristine. Apfel et al. (39) have reported that some of the abnormalities in motor nerve function after vincristine treatment could be prevented with insulin-like growth factor I. Since HGF andCNTF both play important roles in motor neuron development and muscle regeneration, it is conceivable that co-administration of HGF andCNTF with vincristine may ameliorate or prevent the associated motor neuronopathy.

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Note Added in Proof—While this paper was under review, Ebens et al. (43) reported that hepatocyte growth factor functions as an axonal chemotactrant as well as a neurotrophic factor for spinal motor neurons.

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