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Hepatocyte growth factor (HGF/SF) is a muscle-derived survival factor for a subpopulation of embryonic motoneurons

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SUMMARY

Muscle-derived factors are known to be important for the survival of developing spinal motoneurons, but the molecules involved have not been characterized. Hepatocyte growth factor/scatter factor (HGF/SF) plays an important role in muscle development and motoneuron axon outgrowth. We show that HGF/SF has potent neurotrophic activity (EC_{50}=2 pM) for a subpopulation (40%) of purified embryonic rat motoneurons. Moreover, HGF/SF is an essential component of muscle-derived support for motoneurons, since blocking antibodies to HGF/SF specifically inhibited 65% of the trophic activity of media conditioned by C2/C7 skeletal myotubes, but did not inhibit the trophic activity secreted by Schwann cell lines. High levels of expression of the HGF/SF receptor *c-Met* in the spinal cord are restricted to subsets of motoneurons, mainly in limb-innervating segments. Consistent with this distribution, cultured motoneurons from limb-innervating brachial and lumbar segments showed a more potent response to HGF/SF than did thoracic motoneurons. By the end of the period of motoneuron cell death, levels of *c-Met* mRNA in motoneurons were markedly reduced, suggesting that the effects of HGF/SF may be limited to the period of motoneuron cell death. HGF/SF may play an important role during motoneuron development as a muscle-derived survival factor for a subpopulation of limb-innervating motoneurons.

Key words: hepatocyte growth factor, motoneuron, neurotrophic, subpopulation, rat

INTRODUCTION

One of the oldest paradigms in experimental neurobiology is founded on the observations of Hamburger that spinal motoneurons in chicken embryos die following limb removal at early stages (Hamburger, 1934, 1977; Oppenheim, 1991). His conclusion that developing motoneurons require trophic support from their target muscle has not been seriously questioned over the intervening period. However, the precise nature of the factors involved has proved difficult to determine.

Using a variety of systems in which motoneuron survival may be quantified in vitro and in vivo, several groups have identified factors that are experimentally able to rescue motoneurons from death, at least for a certain period. It is striking that not only is the number of candidates thus identified quite large, but they belong to several different gene families: neurotrophins (brain-derived neurotrophic factor, BDNF; neurotrophin-3, NT-3; neurotrophin-4/5, NT-4/5), cytokines (ciliary neurotrophic factor, CNTF; leukæmia inhibitory factor, LIF; cardiotrophin-1, CT-1), transforming growth factor-beta (TGFβ) family members (glial cell line-derived neurotrophic factor, GDNF; TGFβ-1 and TGFβ-3), fibroblast growth factors (FGF-1, FGF-2 and FGF-5) and other growth factors (insulin-like growth factor-1, IGF-1) (reviewed by Henderson, 1996; Oppenheim, 1996).

Given the plethora of candidates, one may reasonably ask whether it is useful to search for other motoneuron trophic factors. The major argument for doing so is that very little data is available to demonstrate that the candidates cited do indeed play a role in regulating motoneuron numbers in vivo. Indeed, apart from limb ablation, in no case has experimental trophic deprivation been shown to lead to loss of all motoneurons. Of all the strains of knockout mice for neurotrophic factors or their receptors, only three have been reported to show motoneuron loss at birth, and the losses are always fractional: *cntfr*$^{-/-}$ (40% loss of motoneurons), *lifr*$^{-/-}$ (40% loss) and *gdnf*$^{-/-}$ (20-30% loss) (DeChiara et al., 1995; Li et al., 1995; Moore et al., 1996; Sanchez et al., 1996). Therefore, even if these losses prove to be additive in double-knockout mice, it is unlikely that the whole motoneuron population will have been accounted for.

Another reason for searching for new candidates is that the survival-promoting actions of such factors may be more complex than initially thought. It is known that the group of neurons collectively referred to as spinal motoneurons in fact show great molecular and functional diversity, both in the embryo and at adult stages, for instance in terms of

expression of different members of the Islet family of LIM homeoproteins (Tsuchida et al., 1994). Different subsets of motoneurons may therefore require different neurotrophic factors (Pennica et al., 1996). Furthermore, studies on sensory neuron development suggest that neurotrophic factors may play important roles outside the period of programmed cell death (Davies, 1994). Motoneurons may thus require different neurotrophic factors at different stages of their development.

In this article, we have studied the potential role of a member of yet another family, hepatocyte growth factor/scatter factor (HGF/SF), a large (90 kDa) heterodimeric heparin-binding factor that is structurally related to plasminogen, but does not have enzymatic activity (Nakamura et al., 1989; Weidner et al., 1991). Like plasminogen, HGF/SF is secreted as a pro-protein, which is cleaved by plasminogen activators or HGF/SF activator to generate the biologically active two-chain form (reviewed by Rosen et al., 1996). As its complete name suggests, HGF/SF can affect mitosis or motility of a variety of cell types, and can induce formation of branching tubules and gland-like structures in epithelial cells from several tissues (reviewed by Gherardi and Stoker, 1991; Matsumoto and Nakamura, 1996). All these actions are mediated by the receptor tyrosine kinase MET, the product of the protoncogene *c-Met* (Naldini et al., 1991; Bottaro et al., 1991; Weidner et al., 1993). HGF/SF is thought to play a role in early development as it is expressed during neurulation in chicken embryos, and during gastrulation and organogenesis in mouse (Streit et al., 1995; Andermarcher et al., 1996). During organogenesis, HGF/SF acts in systems as diverse as angiogenesis, and development of mammary gland, kidney, liver and placenta (reviewed by Zarnegar and Michalopoulos, 1995). Typically HGF/SF is produced by mesenchymal cells, and acts on adjacent epithelia (Sonnenberg et al., 1993). Knockout mice for *Hgf/Sf* and *c-Met* both die during embryogenesis between E13 and E16, apparently because of major placental and liver defects (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). However, the HGF/SF-MET system also appears to play an important role in muscle development, as *c-Met* knockout mice and transgenic mice defective for the MET signalling pathway show absence of migration of limb-muscle precursor myoblasts (Bladt et al., 1995; Maina et al., 1996).

Actions of HGF/SF are not limited to the periphery, and there is evidence for HGF/SF-MET signalling in neurons of the central nervous system (Jung et al., 1994; Honda et al., 1995). In particular, several observations suggest that, in addition to its role during axon outgrowth (Ebens et al., 1996), the HGF/SF-MET system might play a role in motoneuron development at stages corresponding to the period of naturally occurring cell death. First, the *c-Met* receptor is expressed at high levels by ventral spinal cord cells at E11-E15 in the mouse (Sonnenberg et al., 1993; Ebens et al., 1996). Secondly, HGF/SF is expressed in limb at appropriate stages (E11-E15 in mouse) for a candidate trophic factor (Sonnenberg et al., 1993). Lastly, HGF/SF can keep embryonic spinal motoneurons alive in culture (Ebens et al., 1996; Wong et al., 1997) and can act synergistically with CNTF to stimulate choline acetyltransferase activity in mixed cultures of spinal cord (Wong et al., 1997).

Here, we demonstrate using blocking antibodies that HGF/SF is a major essential component of the trophic activity of myotube-conditioned medium for motoneurons. Furthermore, we show that HGF/SF acts preferentially on a subset of limb-innervating motoneurons, in accordance with the pattern of *c-Met* expression at different rostrocaudal levels in the embryo. This subset of motoneurons are precisely those that potentially have access to HGF/SF, which is expressed at sites of muscle development in limb but not trunk. Lastly, in contrast to most other receptors for neurotrophic factors, *c-Met* levels in motoneurons markedly decrease at the end of the period of motoneuron cell death, suggesting that the HGF/SF-MET system may be involved in timing of the trophic dependence of motoneurons on muscle.

MATERIALS AND METHODS

Motoneuron purification and culture

Motoneurons were purified from ventral spinal cord of E14 rat embryos by a combination of metrizamide density-gradient centrifugation and immunopanning on dishes coated with the 192 antibody (Camu et al., 1993; Henderson et al., 1995). This antibody recognizes the rat low-affinity neurotrophin receptor, $p75^{NTR}$, and specifically labels motoneurons in the ventral spinal cord at these stages (Chandler et al., 1984; Yan and Johnson, 1988). Typical yields were 10-15 000 large motoneurons per E14 spinal cord. Motoneurons were seeded on polyornithine-laminin-coated substrata at a density of 2000 cells per 35 mm dish, or 800 cells per 16 mm well. Unless otherwise indicated, complete culture medium was L15 medium with the following supplements: 50 ng/ml insulin, 16 μg/ml putrescine, 100 μg/ml conalbumin, 5 ng/ml sodium selenite ('IPCS supplement') and 2% horse serum, 0.0625% sodium bicarbonate, 6 ng/ml progesterone. Where indicated, the medium was Neurobasal, supplemented with B27, glutamate, β-mercaptoethanol and 2% horse serum following the supplier's instructions (Gibco-BRL).

Motoneuron survival was quantified by counting in duplicate or triplicate dishes the number of large phase-bright neurons with long axonal processes; these are known from other experiments to stain positively for the presence of Islet-1/2 using the 4D5 monoclonal antibody (not shown; Henderson et al., 1993). In some experiments (e.g. Fig. 4) in which motoneurons from different spinal cord regions were compared, survival values were normalized to the value for the same region in basal medium (taken as 0%) and the number of motoneurons from the same region surviving in optimal concentrations of BDNF (1 ng/ml), taken as 100%.

Culture of motoneurons from brachial, thoracic and lumbar regions

Rat embryos (E14) were decapitated and, using a scalpel, transverse cuts were perfomed slightly rostral to the rostral limit of the forelimbs and at the level of the caudal limit of the forelimbs: this region was referred to as 'brachial spinal cord'. A short piece of spinal cord caudal to the forelimbs was discarded, to avoid contamination of thoracic motoneurons by limb-innervating motoneurons. Subsequently, a portion of the trunk of the embryo that was comparable in length to the brachial region was removed: this was referred to as 'thoracic spinal cord'. This region always ended at a point that was clearly rostral to the rostral limit of the hindlimbs. 'Lumbar spinal cord' was a portion that extended from just rostral to the extent of the hindlimbs to a point that represented their caudal limit.

Ventral spinal cord was dissected from each region and purification of brachial, thoracic and, where indicated, lumbar motoneurons by metrizamide-panning was carried out in parallel. The yields of brachial and thoracic motoneurons were similar; absolute values ranged from 2500-3000 motoneurons per region. Yields from

lumbar spinal cord were higher (*ca*. 5000 per embryo). Since this difference was not apparent until the immunopanning step, it is probable that it reflects the stronger expression of $p75^{NTR}$ on lumbar motoneurons compared to brachial motoneurons at this stage (Yan and Johnson, 1988). After cell counting, motoneurons from each region were seeded at the same nominal densities (see above) and their survival was quantified and expressed as described in the legend to Fig. 4.

Neurotrophic factors

Recombinant neurotrophic factors were generously provided by Dr R. Schwall, Dept. of Molecular Oncology, Genentech, Inc. (recombinant human HGF/SF), Dr A. Rosenthal, Department of Neuroscience, Genentech, Inc. (recombinant rat GDNF) and Dr J. Winslow, Department of Neuroscience, Genentech, Inc. (recombinant rat BDNF). They were stored at −70°C in small aliquots at a concentration of 10 μg/ml in PBS/10% horse serum.

Conditioned media

The mouse C2/C7 muscle cell line (Catala et al., 1995) was expanded as myoblasts in DMEM with 20% fetal calf serum until confluent, and then allowed to differentiate into myotubes in DMEM with 2% horse serum. Motoneuron culture medium (Neurobasal with either IPCS or B27 supplements) was then conditioned by the myotubes over a period of 3 days. Several batches of conditioned medium were harvested and pooled, and then stored as aliquots at −80°C until use. Cultures of mouse Schwann cell line MSC80 (Boutry et al., 1992) were grown to 70-80% confluence prior to addition of motoneuron culture medium for a conditioning period of 3 days (as above).

cDNA preparation

Total RNA was isolated using the guanidium acid/phenol/chloroform method (Chomczynski and Sacchi, 1987), and treated with RNase-free DNase I. For cDNA preparation from cultured motoneurons, total RNA from cultured motoneurons (approximately 10,000 cells per point) purified from E14.5 rat embryos was resuspended in 18 μl of reverse transcription buffer (Gibco-BRL) containing 500 μM dNTP mix (Pharmacia), 10 mM DTT, 20 pmol of random hexanucleotides and incubated with 100 U Superscript II (Gibco-BRL) for 50 minutes at 42˚C. For the analysis of mRNA expression in the spinal cord, total RNA was isolated from ventral spinal cords of E14, E18, P0 and P20 rats, and cDNA synthesis was performed according to the manufacturer's specification in a 40 μl reaction volume containing 3 μg of total RNA, 1 mM dNTP mix, 10 mM DTT, 130 pmol of random hexanucleotides and 100 U of Expand™ Reverse Transcriptase (Boehringer Mannheim). cDNA preparation from cell lines was performed as described (Pennica et al., 1996).

PCR analysis

cDNA concentration was carefully adjusted using PCR with *Gapdh* primers as described (Henderson et al., 1994). PCR reactions were performed in a final volume of 30 μl PCR buffer (Promega) containing 10 pmol of each primer, 200 μM dNTP mix, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase (Promega), and the cDNA. The PCR profile was: 95˚C for 30 seconds, 55˚C for 30 seconds, 72˚C for 45 seconds (26 cycles for *Gapdh*, 35 cycles for *c-Met* and *Hgf/Sf*), followed by incubation for 5 minutes at 72˚C. PCR products were analysed on 2% NuSieve GTG agarose gels (FMC BioProducts), transferred to Hybond N^+ membranes (Amersham), and identified using ^{32}P end-labelled oligonucleotide probes complementary to the amplified sequence.

The following oligonucleotides were used: *c-Met* (nucleotides 1088 to 1107 for sense primer, 1344 to 1362 for antisense primer, and 1155 to 1178 for internal probe; GenBank accession number Y00671); *Gapdh* (nucleotides 60 to 93 for sense primer, 421 to 444 for antisense primer, and 370 to 393 for internal probe; GenBank accession number M17701); *Hgf/Sf* (nucleotides 927 to 945 for sense primer, 1372 to 1391 for antisense primer, and 1097 to 1125 for internal probe; GenBank accession number D10213). *Gapdh* PCR primers were designed to encompass an intron; for *c-Met* and *Hgf/Sf*, controls in which reverse-transcriptase had been omitted (RT–) were always performed to exclude contamination by genomic DNA.

Antibody blocking experiments

Blocking antibodies to HGF/SF (goat anti-human HGF/SF neutralizing antibody; purified IgG) and GDNF (monoclonal mouse anti-human GDNF neutralizing antibody; purified IgG) were purchased from R&D Systems, and reconstituted according to the supplier's instructions. Although raised against human GDNF, the anti-GDNF antibody also inhibited recombinant rat GDNF (Fig. 2B2). Its ability to inhibit mouse GDNF could not be directly tested, but this antibody also strongly inhibits the trophic activity of media conditioned by mouse Schwann cells (not shown), which are known to be an important source of GDNF (Henderson et al., 1994). The appropriate quantities of conditioned media, recombinant neurotrophic factors, and blocking antibodies for 0.5 ml were incubated together in 16 mm laminin-coated culture wells in a total volume of 250 μl of complete culture medium. After 1 hour in the CO_2 incubator at 37°C, 250 μl of a suspension of motoneurons (800 cells) in complete culture medium were added. The contents of each well were aspirated once into a blue micropipette tip and then gently expelled, in order to ensure uniform distribution of motoneurons. To evaluate motoneuron survival, wells were filled to the brim with warm L15 medium, and the cover of the dish replaced; this provided good phase contrast optics over the whole surface. The number of surviving motoneurons was counted across the diameter of the well, in two perpendicular directions.

In situ hybridization

cDNAs encoding mouse *c-Met* were provided by E. Audero and C. Ponzetto (University of Turin, Italy). A 1.3 kb fragment (nucleotides 304-1576; Chan et al., 1988) of this cDNA was obtained following digestion with *Pst*I, and inserted into pBluescript II (Stratagene). cDNAs corresponding to rat *Islet-1* (1.5 kb fragment)) and *Islet-2* (1.6 kb fragment) were provided by T. Jessell (Columbia University, NY) and S. Pfaff (Salk Institute, San Diego). Digoxigenin-labeled probes were prepared according to the manufacturer's instructions (Boehringer Mannheim). Optimal probe concentrations were determined for each probe by serial dilution. Corresponding sense probes were used as controls.

Rat E14.5 and E19.5 embryos (Sprague-Dawley) were fixed overnight at 4˚C in PBS, pH 7.4, containing 4% paraformaldehyde, and then cryoprotected overnight at 4˚C in PBS containing 20% sucrose. They were embedded in O.C.T. compound (Miles) and frozen rapidly in dry ice. Serial sections (16 μm) were cut at −24˚C and stored at −20˚C in the presence of desiccant until use. Sections were overlaid overnight at 60˚C with hybridization buffer (50% deionized formamide, 0.6 M NaCl, 20 mM Tris-HCl pH 8.0, 10% dextran sulfate, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA, 5 mM EDTA pH 8.0, 500 μg/ml yeast tRNA) containing digoxigenin-labelled probes. The following day, the sections were washed twice in 2× SSC, 50% formamide at 65˚C for 30 minutes, and blocked for 1 hour at room temperature in buffer 1 (100 mM Tris pH 7.5, 150 mM NaCl) containing 1.5% blocking reagent (Boehringer Mannheim). The blocking solution was then replaced with buffer 1 containing a 1:1000 dilution of anti-DIG-AP conjugate (Boehringer Mannheim) and the slides were incubated for 1 hour at room temperature. The slides were washed in buffer 1 for 10 minutes four times at room temperature, twice in buffer 2 (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$), and incubated overnight in buffer 2 containing 450 μg/ml NBT and 175 μg/ml BCIP (Boehringer Mannheim) at room temperature.

RESULTS

HGF/SF has survival activity for a fraction of embryonic rat motoneurons

Recombinant human HGF/SF was added to low-density cultures of motoneurons purified by the metrizamide-immunopanning method from E14 rat embryos (see Methods). In these conditions, motoneurons rapidly developed neurites and, by 3 days in culture, showed typical multipolar morphology (Fig. 1A). After 3 days in culture, the number of motoneurons surviving was counted and expressed as a percentage of the number of motoneurons that developed after 15 hours in culture in optimal concentrations of GDNF (100 pg/ml), taken as 100%. The effect of HGF/SF was dose-dependent, with an EC_{50} of 0.2 ng/ml, or 2 pM (Fig. 1B). At concentrations higher than 100 ng/ml, the survival response decreased (not shown). This dose-response curve is very similar to that published by Ebens et al. (1996) using motoneurons seeded at approximately 40-fold higher densities. However, our results differ from theirs in one important respect. As we reported previously (Henderson et al., 1993, 1994), optimal concentrations of BDNF (1 ng/ml) or GDNF (100 pg/ml) were capable of maintaining nearly all motoneurons for 3 days in these conditions (not shown). In contrast, optimal concentrations of HGF/SF (10-100 ng/ml) saved on average 39±10% (mean±s.e.m.; n=6) of total purified spinal motoneurons. Although highly potent, HGF/SF is therefore less efficacious than BDNF and GDNF, and seems to maintain a specific fraction of motoneurons. It is likely that HGF/SF was signalling through the MET receptor in these neurons, as significant levels of *c-Met* mRNA were detected by RT-PCR in purified motoneurons (Fig. 1C).

HGF/SF is an essential component of the neurotrophic activity of muscle-conditioned media

These results raised the possibility that HGF/SF might play a physiological role as a muscle-derived trophic factor for developing motoneurons. Unfortunately, it is not possible at present to confirm this hypothesis directly in vivo, since most null mutant mice for *Hgf/Sf* and *c-Met* die before the start of programmed cell death of motoneurons and, in any case, show muscle defects that complicate the interpretation of any motoneuron phenotype (see Discussion). We decided therefore to use an in vitro approach to determine whether HGF/SF is an important component of the trophic activity secreted by skeletal muscle. Since primary cultures of rodent myotubes are unavoidably contaminated with fibroblasts and Schwann cells, which produce high levels of *Gdnf* (Henderson et al., 1994), we used cultures of the mouse C2/C7 muscle line, which is able to fuse into myotubes and form neuromuscular junctions in vitro (Jo et al., 1995).

C2/C7 myoblasts were allowed to fuse and were then used to condition motoneuron culture medium for 3 days. The resulting conditioned medium ('C2-CM') was tested for its survival activity at different dilutions (not shown). The concentration chosen for these experiments (dilution 1:20) was at the start of the plateau of the dose-response curve, so that reduction of trophic activity should be reflected in a decrease in the number of surviving motoneurons. We confirmed by RT-PCR and Southern blotting that these cells expressed significant levels of mRNA for *Hgf/Sf* (Fig. 2A, samples 4 and 5) but only low levels of GDNF mRNA (not shown).

The effects of specific blocking antibodies to HGF/SF and

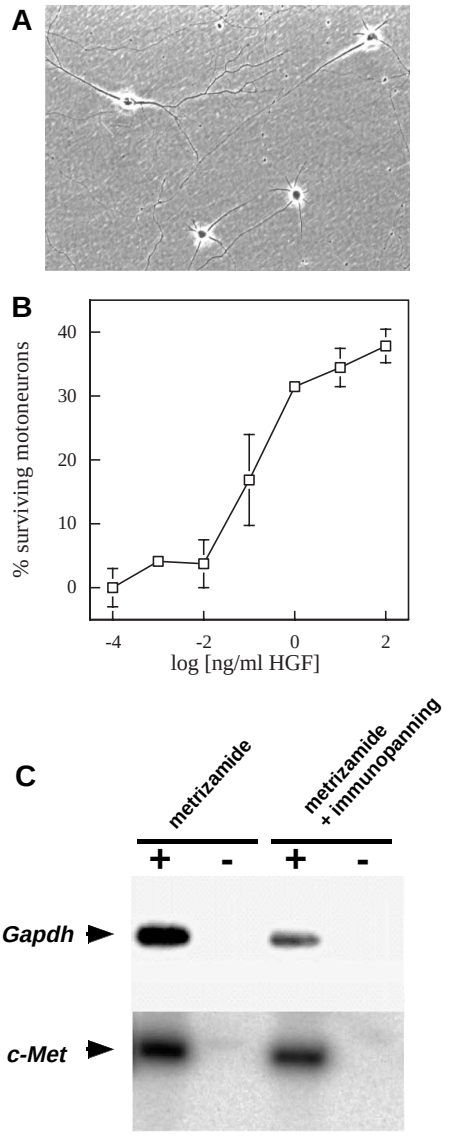

Fig. 1. HGF/SF has survival-promoting activity in vitro for a subpopulation of purified embryonic rat motoneurons. (A) Phase-contrast micrograph of embryonic motoneurons purified from lumbar spinal cord of E14 rat embryos and cultured for 2 days in the presence of HGF/SF (0.2 ng/ml). (B) Dose-response curve for survival of motoneurons purified by metrizamide-panning from total E14 spinal cord and cultured for 3 days in the presence of the indicated concentrations of HGF/SF. The EC_{50} calculated from this curve was 2 pM; note, however, that even at optimal concentrations not all motoneurons are kept alive by HGF/SF. Survival values are corrected for survival in basal medium (taken as 0%) and expressed relative to the number of motoneurons that initially developed in optimal conditions (see text). (C) Cultured motoneurons express *c-Met* mRNA as detected by RT-PCR followed by Southern blotting. RNA was purified from motoneurons purified by metrizamide centrifugation alone, or by metrizamide-panning, and analysed by RT-PCR, followed by Southern blotting with specific probes for *Gapdh* and *c-Met*. PCR was performed on RNA samples that had been incubated with (+) or without (–) reverse transcriptase.

GDNF on the trophic activity of C2-CM were compared. First, the efficacy and specificity of the antibodies were confirmed by testing their ability to inhibit different recombinant neurotrophic factors. At a concentration of 10 μg/ml IgG, blocking antibodies to HGF/SF totally inhibited the activity of human HGF/SF, but did not reduce the number of motoneurons supported by BDNF (Fig. 2B-1). Blocking antibodies to GDNF at 20 μg/ml IgG completely inhibited the trophic activity of rat GDNF, but did not inhibit rat BDNF (Fig. 2B-2). Neither antibody had a significant effect on the number of motoneurons surviving in basal medium. The same antibodies were incubated in culture wells for 1 hour at 37°C with C2-CM before seeding purified motoneurons. Strikingly, the number of motoneurons supported for 2 days by C2-CM was markedly reduced by anti-HGF/SF (Fig. 2C,D), but not significantly affected by anti-GDNF (Fig. 2D). In four independent experiments, HGF/SF antibodies reduced the trophic activity of this dilution of myotube-conditioned medium by 64±5% (mean±s.e.m.; n=4).

The HGF/SF antibodies showed no non-specific toxicity to motoneurons grown in basal medium or in the presence of BDNF (Fig. 2B-1,C). To confirm that they were not nevertheless acting non-specifically by removing a general component of conditioned media, we tested their ability to block the trophic support provided by cultured Schwann cells. We first showed by RT-PCR analysis that cultured Schwann cell lines did not contain detectable levels of HGF/SF mRNA, even when the amplified fragments were analysed by Southern blotting (Fig. 2A, samples 1 to 3); this demonstrates that expression of HGF/SF by C2/C7 cells is not a general response of cell lines to immortalization or culture. Subsequently, we tested the ability of HGF/SF antibodies to inhibit the trophic activity of Schwann cell lines for motoneurons. No significant inhibition was observed (Fig. 2E). The effect of anti-HGF antibodies on C2-conditioned medium thus truly reflected a depletion of HGF/SF. HGF/SF is therefore a major component of the trophic activity secreted by C2/C7 myotubes.

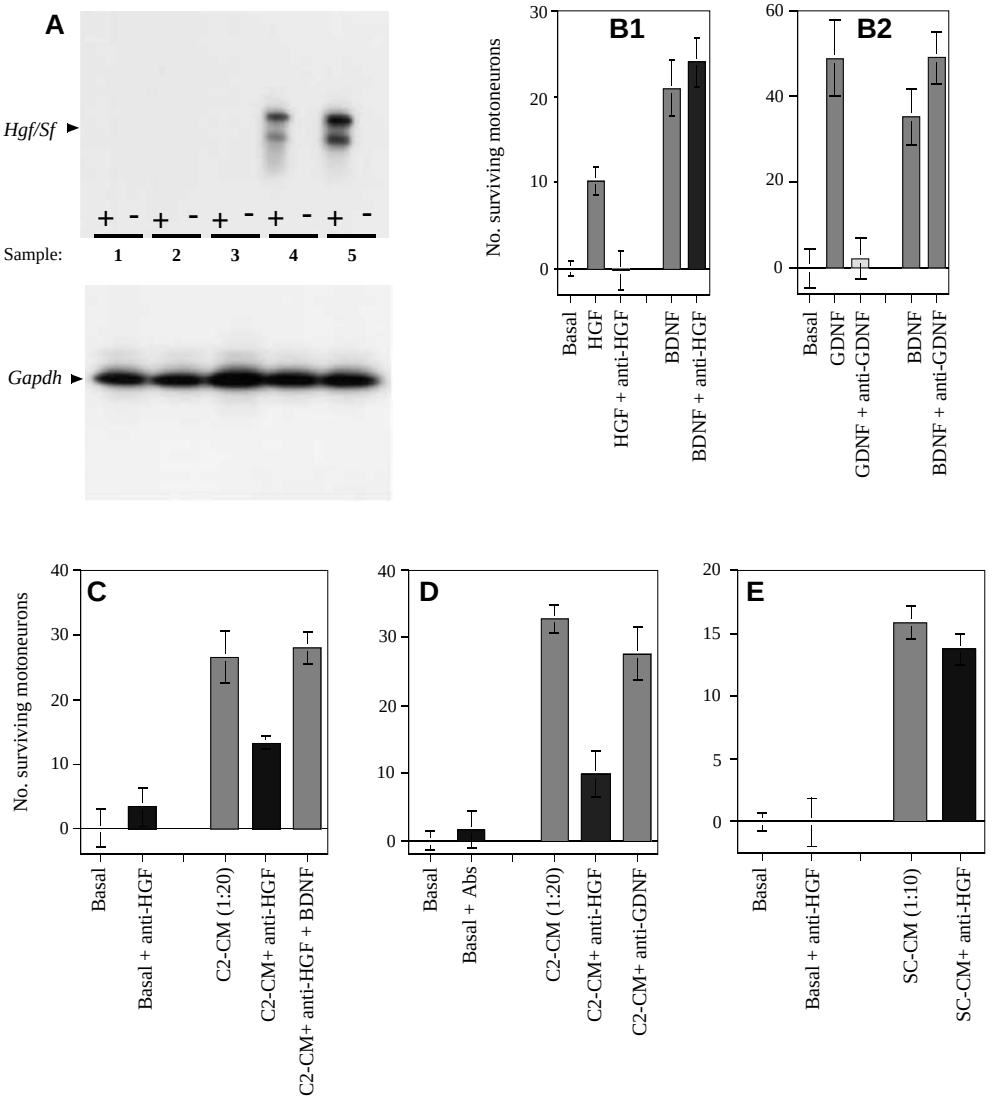

Fig. 2. HGF/SF is a major component of the motoneuron trophic activity of myotube-conditioned medium but not Schwann cell-conditioned medium. (A) RT-PCR showing the presence of *Hgf/Sf* mRNA in C2/C7 myotubes (sample 5) and myoblasts (sample 4), but its absence from Schwann cell lines MSC-80 (samples 3) and TSC-2 (samples 1 and 2; separate cultures). All samples contained equivalent levels of *Gapdh* cDNA. (B1) Anti-HGF/SF (10 μg/ml) completely inhibits physiological concentrations (10 ng/ml) of human HGF/SF but not of rat BDNF (1 ng/ml). (B2) Anti-GDNF (20 μg/ml) completely inhibits rat GDNF (0.1 ng/ml) but not rat BDNF (1 ng/ml). (C) Anti-HGF/SF (10 μg/ml) markedly reduces the trophic activity for motoneurons of mouse C2/C7 myotube-conditioned medium (C2-CM). This is not a toxic effect as these neurons are protected by the addition of BDNF (1 ng/ml) in the same conditions. (D) The effects of anti-HGF/SF are specific: anti-GDNF (20 μg/ml) does not significantly inhibit the trophic activity of C2-CM. Basal + Abs: basal medium with both anti-HGF and anti-GDNF. (E) Anti-HGF/SF is without effect on the motoneuron trophic activity of mouse MSC80 Schwann cell-conditioned medium (SC-CM). Values are expressed as actual motoneuron counts per diameter of a 16 mm well (mean ± s.e.m.; n=4); survival values in basal medium in each experiment have been subtracted. For clarity, results in B-E using anti-HGF/SF are represented by solid bars.

c-Met mRNA is expressed by more motoneurons at limb-innervating levels than at trunk-innervating levels

We sought to characterize further the subpopulation of motoneurons that might be responding to HGF/SF in cultured motoneurons prepared from total E14 spinal cord. We used non-radioactive in situ hybridization to analyse expression of *c-Met* RNA on a cell-by-cell basis in the E14 rat embryo. It has been reported in chick and in mouse that HGF/SF is produced in limb but not trunk at sites of muscle development (Sonnenberg et al., 1993; Yang and Park, 1993; Théry et al., 1995). We therefore focussed our attention on potential differences in *c-Met* expression between limb-innervating and trunk-innervating motoneurons.

E14 rat embryos were fixed and processed for in situ hybridization as described in Methods. In order to ascertain that any expression observed could be attributed to motoneurons, groups of three adjacent sections were collected on different slides and used to analyse the expression of *c-Met*, and that of the motoneuron markers *Islet-1* and *Islet-2*, in parallel. This comparison was performed at several different rostrocaudal levels from cervical to lumbar levels. Typical examples from cervical, brachial (forelimb-innervating) and thoracic (trunk-innervating) spinal cord are shown in Fig. 3. Islet-1 and -2 are LIM-family homeoproteins that are known to be expressed by motoneurons in the spinal cord and rhombencephalon of the chicken embryo (Tsuchida et al., 1994), but their expression patterns in rodents have not been described in detail. It is apparent from inspection of the sections (Fig. 3 and not shown) that most motoneurons seem to express both mRNAs, although the relative levels may vary. This is in apparent contrast to the situation in the chick, where at comparable stages the most lateral column of limb-innervating motoneurons express *Islet-2* but undetectable levels of *Islet-1* (Tsuchida et al., 1994). To be certain of identifying the major pools of somatic motoneurons at each level, we analysed the expression of both *Islet-1* and *Islet-2*.

At all rostrocaudal levels analysed, only a fraction of the Islet-positive motoneurons showed strong hybridization for *c-Met* (Fig. 3). This was the case at brachial levels, where subsets of motoneurons in each of the major groups showed strong labelling (Fig. 2D-I). The number of motoneurons expressing *c-Met* was even lower in cervical (Fig. 2A-C) and thoracic (Fig. 2J-L) spinal cord. Here, only a small group of medially situated motoneurons expressed *c-Met* at high

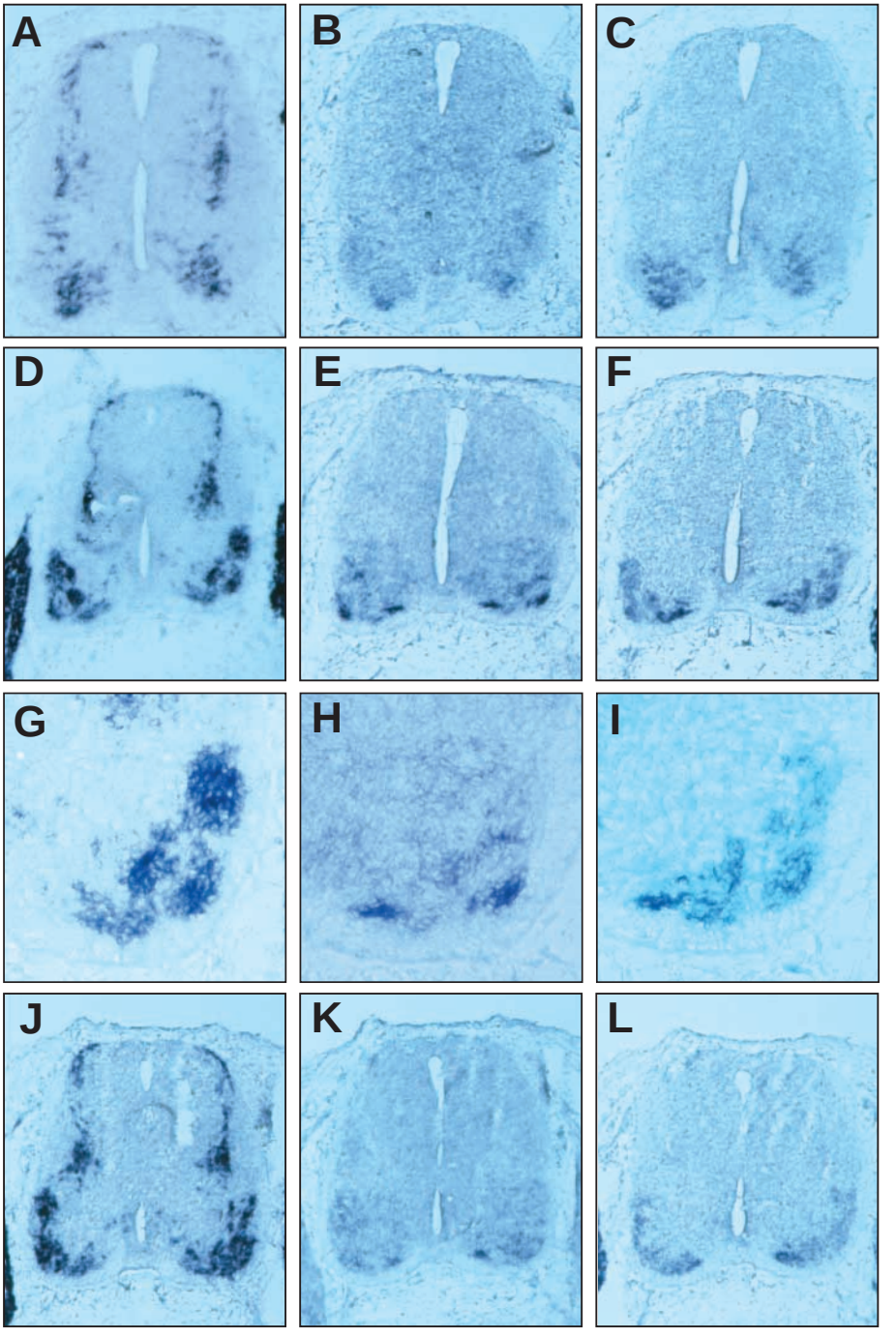

Fig. 3. *c-Met* mRNA is expressed at high levels by a subpopulation of spinal motoneurons at the beginning of the cell death period. Localization of mRNA for *Islet-1* (A,D,G,J), *c-Met* (B,E,H,K) and *Islet-2* (C,F,I,L) by non-radioactive in situ hybridization at different rostrocaudal levels of E14 rat embryos. The three probes were hybridized to immediately adjacent sections at the following rostrocaudal levels: cervical (A-C), brachial (forelimb-innervating) (D-I) and thoracic (trunk-innervating) (J-L). Note that *c-Met*-positive motoneurons are more abundant at limb-innervating levels and that, at all levels, they represent only a fraction of motoneurons identified by *Islet-1* and *Islet-2*. This is true even at limb-innervating levels; compare enlargements of one brachial ventral horn in G (*Islet-1*), H (*c-Met*) and I (*Islet-2*).

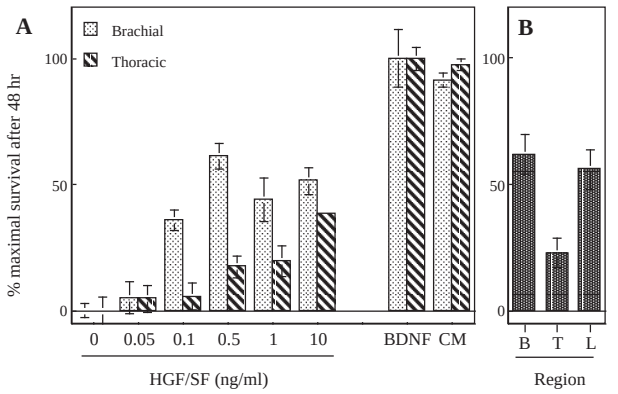

Fig. 4. Limb-innervating motoneurons are more sensitive than thoracic motoneurons to HGF/SF. (A) Comparative dose-response curves to HGF/SF for motoneurons purified from brachial (stippled bars) and thoracic (hatched bars) regions of the E14 spinal cord. At lower concentrations (0.5 ng/ml), only brachial motoneurons respond to HGF/SF to a significant extent. However, at high concentrations (>10 ng/ml), both populations show a partial survival response. Note that motoneurons from brachial and thoracic regions respond to a comparable extent to other neurotrophic factors such as BDNF and C2/C7 myotube-conditioned medium supplemented with BDNF (CM), used here as positive controls. Survival values (mean ± s.e.m.; *n*=4) are normalized for survival in basal medium (defined as 0%; absolute values 2±1 (brachial) and 6±1 (thoracic)) and expressed for each region as a percentage of the number of motoneurons that developed in 1 ng/ml BDNF (defined as 100%; absolute values 19±2 (brachial) and 27±1 (thoracic)). (B) Comparison of the survival response to 0.2 ng/ml HGF/SF of motoneurons purified from brachial (B), thoracic (T) and lumbar (L) regions of E14 spinal cord. Survival values are mean±range (*n*=2).

levels. These motoneurons strongly expressed *Islet-2*, and were only weakly positive for *Islet-1*; it is not however clear to which motoneuron pool they belong. Patterns of in situ hybridization for *c-Met* at lumbar levels were similar to those at brachial levels (not shown), demonstrating that the differences observed do not simply reflect a rostrocaudal gradient in differentiation. Thus, only a fraction of motoneurons express high levels of *c-Met* mRNA at the start of motoneuron cell death, and these motoneurons are preferentially located in spinal segments that are likely to have access to the HGF/SF that is produced by limb, but not axial, muscles.

Brachial motoneurons are more sensitive than thoracic motoneurons to HGF/SF

In order to determine whether the expression pattern of *c-Met* reflected the responsiveness of different motoneuron pools to HGF/SF, we purified motoneurons from different rostrocaudal levels of E14 rat embryos. For technical reasons, we first focussed our attention on a detailed comparison of brachial and thoracic levels. Purified motoneurons from each region were seeded in 16-mm wells at comparable densities. After 2-3 days in three independent experiments, survival of brachial and thoracic motoneurons in optimal concentrations of BDNF or muscle-conditioned medium was indistinguishable, and close to 100% of those that initally developed in optimal conditions (not shown). There was thus no difference in the intrinsic survival capacity of motoneurons from brachial and thoracic levels.

The responses of brachial and thoracic motoneurons to HGF/SF were however markedly different (Fig. 4A). At low concentrations of HGF/SF (0.5 ng/ml), brachial motoneurons showed a significant survival response (53±9% of BDNF-induced survival; *n*=2 independent experiments), whereas survival of thoracic motoneurons (11±3 % survival; *n*=2) was much lower. Thus at low HGF/SF concentrations, as expected from the in situ hybridization data, the proportion of brachial motoneurons capable of responding to HGF/SF was higher than that in total spinal cord motoneurons (39%; Fig. 1), and few thoracic motoneurons were supported.

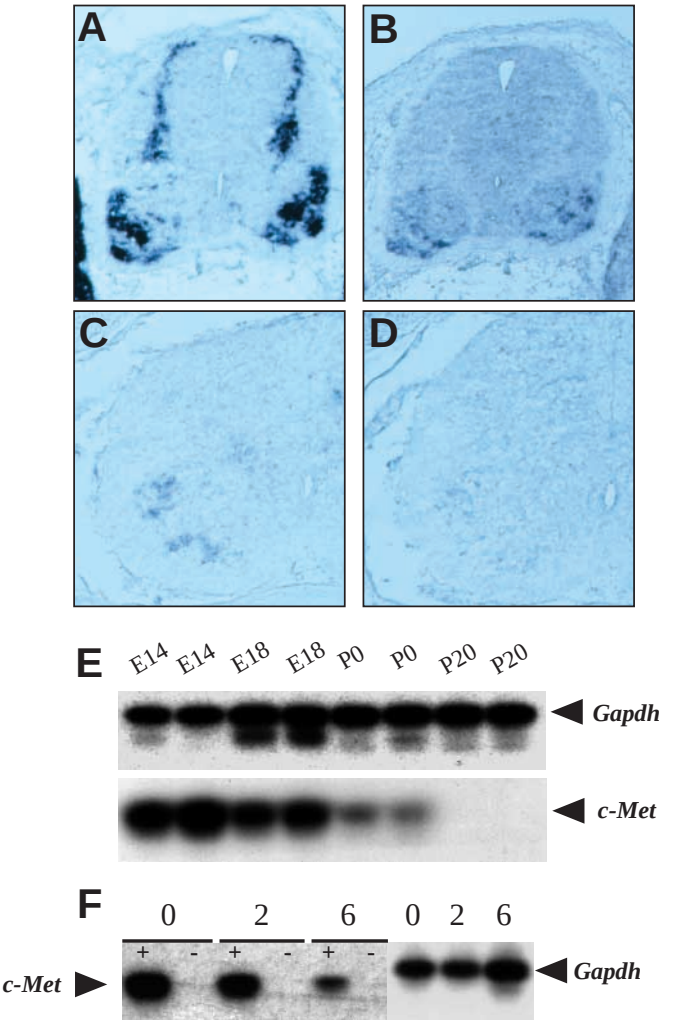

Fig. 5. *c-Met*-expression in motoneurons is strongly down-regulated at the end of the cell death period. Sections from E14 (A,B) and E19 (C,D) brachial spinal cords were hybridized in parallel with probes to *c-Met* (B,D) and *Islet-1* (A,C). Whereas *Islet-1* expression is still distinct at E19, *c-Met* is no longer detectable at this stage. (E) *c-Met* mRNA in spinal cord is down-regulated during motoneuron development. RT-PCR analysis of RNA from ventral spinal cord at the indicated ages. Specific amplified fragments were revealed using radiolabelled oligonucleotides specific for *c-Met* and *Gapdh*. (F) RT-PCR analysis of *c-Met* expression in cultures of motoneurons maintained in optimal conditions (C2-CM plus 10 ng/ml HGF in Neurobasal/B27) for the indicated number of days. The signal for *c-Met* decreased markedly after 6 days in culture, whereas that for *Gapdh* did not. PCR was performed on RNA samples incubated with (+) or without (–) reverse transcriptase.

Unexpectedly, when higher doses of HGF/SF (10-50 ng/ml) were used (Fig. 4A and not shown), the fraction of thoracic motoneurons supported (mean value for three independent experiments: 42±10%) was close to that for brachial motoneurons (59±6%, n=3). Thus, some motoneurons from both regions can respond to HGF/SF, but HGF/SF action on brachial motoneurons is 50- to 100-fold more potent than on thoracic motoneurons. The recruitment of less HGF/SF-sensitive populations at high concentrations of HGF/SF may explain the fact that the dose-response curve for HGF/SF does not reach a true plateau (Fig. 1 and Ebens et al., 1996).

It remained possible that the higher sensitivity of brachial motoneurons to HGF/SF reflected a rostrocaudal gradient of maturity. We therefore compared the response of brachial, thoracic and lumbar motoneurons purified in parallel from the same embryos (Fig. 4B). Survival of motoneurons from different regions was once again not significantly different in the presence of 1 ng/ml BDNF (not shown). However, motoneurons from the limb-innervating brachial and lumbar regions responded significantly better to an intermediate concentration of HGF/SF (0.2 ng/ml) than did thoracic motoneurons (Fig. 4B).

Motoneurons express *c-Met* over a limited period of development

Although neurotrophic factors have long been thought to play an important role during the period of naturally occurring cell death of different neuronal populations, it is now clear that they have important functions both before and after the cell death period. Indeed, receptors such as TrkB, TrkC and CNTFRα are expressed by motoneurons from early embryonic stages and throughout adult life (Henderson et al., 1993; MacLennan et al., 1996). The HGF/SF receptor, *c-Met*, is expressed by early motoneurons, consistent with its role in axon outgrowth (Sonnenberg et al., 1993; Ebens et al., 1996), but few data are available on its later expression.

Sections from E14 and E19 rat embryos, representing the beginning and the end of the motoneuron cell death period, were collected on the same slides and hybridized with the *c-Met* probe (Fig. 5). Strikingly, by the end of the cell death period, *c-Met* was barely detectable in brachial motoneurons (Fig. 5D) or thoracic motoneurons (not shown). Adjacent sections were hybridized with a probe to *Islet-1* (Fig. 5A,C). Specific labelling on motoneurons was still clearly apparent at E19, though decreased in intensity as expected. In accordance with these results, RT-PCR performed on samples prepared from ventral spinal cord of E14 to P20 mice showed that levels of *c-Met* mRNA rapidly decreased at the end of the period of motoneuron cell death (Fig. 5E), as also recently reported by Wong et al. (1997).

Purified motoneurons were cultured for 6 days in Neurobasal medium with B27 supplement, in the presence of HGF/SF (10 ng/ml) and C2-CM (dilution 1:10). Essentially all motoneurons survived in these conditions (not shown). However, levels of *c-Met* mRNA relative to *Gapdh* progressively decreased with time in culture (Fig. 5F), suggesting that motoneuron maturation in these conditions also results in a progressive loss of responsiveness to HGF/SF.

DISCUSSION

Our results suggest that HGF/SF potentially regulates motoneuron survival during development. The list of candidate motoneuron trophic factors is already long and, as for most of the others, conclusive in vivo evidence for the role of HGF/SF is not available. However, we have shown here that blocking antibodies to HGF/SF inhibit 65% of the motoneuron trophic activity secreted by myotubes in culture, suggesting that it is an important muscle-derived factor. In addition, HGF/SF has other features that make it of special interest: it acts preferentially on a subpopulation of limb-innervating motoneurons and probably ceases to play a role by the end of the period of motoneuron cell death.

A major concern of those working in the field of motoneuron trophic factors has been to determine to what extent those factors that can keep motoneurons alive experimentally in vitro or in vivo are indeed involved in regulating motoneuron numbers during development. One obvious approach is to ask whether knockout mice for a given factor or its receptor show a loss of motoneurons by the end of embryogenesis. In fact, few definitive results have been obtained in this manner, either because the null mutant mice lose no motoneurons (BDNF, NT-3, NT-4/5, TrkB, TrkC and corresponding double mutants, IGF-1), or because they show embryonic lethality at early stages (FGFR-1) or perhaps because there is compensation between different members of a family (CNTF, LIF, FGF-5) (for review, see Henderson, 1996). The only two factors that have been implicated as motoneuron survival factors in this manner are GDNF and an unidentified ligand of the CNTFRα-LIFR cytokine receptor complex (DeChiara et al., 1995; Li et al., 1995; Moore et al., 1996; Sanchez et al., 1996). In each case, there is a significant but fractional loss of motoneurons in knockout mice, but even here it is not yet clear whether the null mutations affect parameters other than survival, such as proliferation or differentiation.

Direct analysis of the *Hgf/Sf* and *c-Met* mutant mice currently available is unlikely to lead to a clear idea of the role of HGF/SF in motoneuron survival, for two main reasons. First, many null-mutant embryos die at E13, before naturally occurring motoneuron death has begun in the mouse embryo (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). Secondly, even where this is not the case, as with certain MET signalling mutants (Maina et al., 1996), the pronounced muscle phenotype makes it impossible to distinguish between motoneuron defects caused indirectly by lack of muscle, and those due directly to absence of HGF/SF. We therefore took an alternative approach: the use of blocking antibodies to analyse the functional constituents of media conditioned by the cellular partners of the motoneuron. This approach has obvious drawbacks when extrapolating to the in vivo situation. However, it also presents certain advantages. First, there is no possibility of a compensatory reaction to the depletion of neurotrophic factor. Secondly, the cellular source of the factor acting on motoneurons is apparent, which is not the case even when knockout mice do show motoneuron loss. The C2/C7 muscle line has been much studied, and many facets of its development in culture and co-culture faithfully reproduce molecular events in myogenesis and neuromuscular junction formation in vivo (Jo et al., 1995). Furthermore, the fact that C2 myotubes can produce and secrete HGF/SF is consistent with what is known of HGF/SF expression by immature muscle in the embryo (Sonnenberg et al., 1993; Takebayashi et al., 1995; Andermarcher et al., 1996). We were therefore particularly struck by the fact that HGF/SF was responsible for 65%

of the short-term trophic activity secreted by C2 myotubes, suggesting that it may have a major role to play in motoneuron development in vivo. The only other factor for which antibody depletion data have been published, FGF-5, also seems to be responsible for less than 100% of the survival activity of muscle extract (Hughes et al., 1993). It will be of interest to analyse the results of combinations of these antibodies, and their effects on motoneuron populations from different axial levels.

HGF/SF is the first factor for which a tentative correlation may be established between its biological activity on a fraction of motoneurons, and the presence in the embryo of a subpopulation of motoneurons that express high levels of its receptor. However, preferential responsiveness of characteristic motoneuron subpopulations to given neurotrophic factors may be a more general phenomenon. Using long-term cultures, we recently showed that cytokines such as CNTF and CT-1 were able to support about 40% of motoneurons, a figure very close to that for the loss of motoneurons in knockout mice for CNTFRα or LIFR (Pennica et al., 1996). This figure is also comparable to the fraction (45%) of the motoneurons that normally die that can be saved by CNTF administration to chicken embryos during motoneuron PCD (Oppenheim et al., 1991). As another example, optimal concentrations of GDNF were only able to save about 25% of cultured rat motoneurons at survival times greater than 9 days (Pennica et al., 1996); this fraction is very similar to that lost in null mutants for *gdnf* (Sanchez et al., 1996; Moore et al., 1996) and to the proportion of motoneurons saved by in ovo administration of GDNF (Oppenheim et al., 1995). These correlations raise the possibility that different subpopulations of motoneurons preferentially respond to, and require, different trophic factors. However, at least in the case of the GDNF receptor subunits RET and GDNFRα, there is no evidence that their expression is limited to a subpopulation of motoneurons; other elements may therefore determine the requirement of motoneurons for GDNF. Responsiveness to HGF/SF may be limited to subpopulations of motoneurons even at very early stages, since inspection of radioactive in situ hybridization images led Ebens et al. (1996) to conclude that not all motoneurons were *c-Met*-positive even at E10.5 in the mouse.

There were clear differences in the distribution of *c-Met* mRNA between motoneurons in brachial and thoracic segments of the spinal cord (Fig. 3). This matched well with the greater sensitivity of brachial motoneurons to HGF/SF in culture (Fig. 4A). However, we were also struck by the fact that a significant number of thoracic motoneurons were supported by higher concentrations (>10 ng/ml) of HGF/SF. It is possible that the thoracic motoneurons that survived in culture corresponded to the small subset of strongly *c-Met*-positive medial motoneurons shown in Fig. 3K. However, given the comparable yields of brachial and thoracic motoneurons obtained from the purification procedure, it is unlikely that this subset could correspond to 40% of the total thoracic motoneurons. We therefore favour the possibility that some thoracic motoneurons express levels of *c-Met* that are too low to be clearly visualised by in situ hybridization, but nevertheless allow them to respond to higher concentrations of HGF/SF. This would perhaps help to explain the observation by Ebens et al. (1996) that most axons appear to leave ventral explants in response to HGF/SF, although not all show a positive signal for *c-Met* by in situ hybridization. It would also be consistent with the fact that HGF/SF can have effects on sensory neurons (R. Klein, F. Maina, M. Hilton and A. Davies, personal communication), which show no detectable *c-Met* mRNA on our sections (Figs. 3, 5). Another possibility might be that motoneurons from different levels express different HGF receptors. However, the only receptor closely related to MET, the tyrosine kinase RON, is not thought to respond to HGF/SF (Wang et al., 1994).

When and where might HGF/SF be available to motoneurons during the period of programmed cell death? Like another candidate motoneuron trophic factor, CT-1 (Pennica et al., 1996), *Hgf/Sf* is expressed in muscle but not in Schwann cells in culture (Fig. 2A). Strikingly, it is expressed in limb, but not in most axial, muscle precursors in the embryo (Sonnenberg et al., 1993; Théry et al., 1995). Levels of *Hgf/Sf* mRNA are initially high in limb, but decrease during the period of motoneuron cell death (Sonnenberg et al., 1993; Myokai et al., 1995). Although few data are available, the expression of *Hgf/Sf* in limb at later stages is patchy (Jennische et al., 1993; Sonnenberg et al., 1993; Takebayashi et al., 1995). This suggests that only certain muscles may produce HGF/SF and, indeed, some sites of expression may correspond to connective tissue (Jennische et al., 1993; Takebayashi et al., 1995). It is tempting to propose that these sites of expression may correspond to the targets of motoneurons expressing high levels of *c-Met*. In particular, the presence of *Hgf/Sf* in intercostal muscles (Takebayashi et al., 1995) may help to explain why a limited number of thoracic motoneurons express the receptor at high levels. In addition to the expression of the receptor by a subset of motoneurons, the potential action of the HGF/SF-MET system on motoneuron survival is thus further circumscribed by the compartmentalized expression of the HGF/SF ligand itself.

The non-uniform pattern of expression of *Hgf/Sf* at different sites of muscle development is reminiscent of reports concerning several other polypeptides potentially involved in motoneuron survival or plasticity. NT-4/5 is expressed at high levels in adult muscle, but is restricted to slow-twitch Type I fibres (Funakoshi et al., 1993). FGF-5 is strongly expressed in a group of facial muscles (Haub and Goldfarb, 1991). GDNF is apparently expressed in all Schwann cell precursors, but only in a small group of embryonic muscles (Silos-Santiago et al., 1996). Thus, in studying factors involved in muscle-motoneuron interactions, it may be important to define not only the class of motoneuron, but also the specific target muscle.

The timing of expression of *c-Met* is of particular interest with respect to the period of programmed cell death in the motoneuron population, which occurs between approximately E14 and E19 in the rat (Oppenheim, 1986). At the beginning of this period (as in the preceding days), levels of *c-Met* mRNA were high, but by the end of motoneuron cell death only low levels of *c-Met* mRNA could be detected, either by in situ hybridization or by RT-PCR (Fig. 5; Sonnenberg et al., 1993; Wong et al., 1997). This pattern of regulation has not been described for any other receptor for a motoneuron trophic factor. These results suggest that the physiological actions of HGF/SF on motoneurons may be limited to the cell death period, and may indeed be involved in defining this period. Those motoneurons that survive cell death presumably do so through the actions of other neurotrophic factors, whose receptors continue to be expressed by motoneurons. It will be

interesting in this context to see whether HGF/SF can save neonatal motoneurons from cell death induced by axotomy.

Our data provide a further illustration of the pluripotent activities of HGF/SF in regulating mesenchyme-epithelial interactions. They demonstrate that the regulation of motoneuron numbers during development is likely to be complex in terms of the molecules and cell types involved. A full understanding of this aspect of motoneuron development will require both further identification of the factors involved, and a better description of the motoneuron subpopulations present.

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