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Hepatocyte growth factor mediates MSCs stimulated functional recovery in animal models of MS

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Abstract

Mesenchymal stem cells have emerged as a potential therapy for a range of neural insults. In animal models of multiple sclerosis, an autoimmune disease that targets oligodendrocytes and myelin, treatment with human MSCs results in functional improvement that reflects both modulation of the immune response and myelin repair. Here we demonstrate that conditioned medium (CM) from human MSCs reduces functional deficits in mouse $MOG_{35–55}$-induced EAE and promotes the development of oligodendrocytes and neurons. Functional assays identify a critical role for Hepatocyte Growth Factor (HGF) and its primary receptor cMet in MSCs stimulated recovery in EAE, neural cell development and remyelination. Active MSC-CM contains HGF and exogenously supplied HGF promotes recovery in EAE while cMet and anti-HGF antibodies block the functional recovery mediated by HGF and MSC-CM. Systemic treatment with HGF dramatically accelerated remyelination in lysolecithin-induced rat dorsal spinal cord lesions and in slice cultures. Together these data strongly implicate HGF in mediating MSC-stimulated functional recovery in animal models of multiple sclerosis.

Keywords

Mesenchymal stem cells; Hepatocyte growth factor; cMet; Stem cells

Introduction

Multiple sclerosis (MS) is an autoimmune disease that results in progressive functional deficits. The initial course of the disease is characterized by relapsing remitting episodes that

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frequently progress to a more chronic functional loss[1]. The target of immunological attack appears to be myelin sheaths and the loss of myelin, and death of myelinating oligodendrocytes leaves axons functional compromised and vulnerable to damage[2]. The majority of current therapies for MS are directed toward suppression of the immune response, however long-term functional recovery will likely depend on the ability of the CNS to replace lost oligodendrocytes and repair myelin sheaths damaged by disease. The realization that the adult CNS retains a population of oligodendrocyte precursors[3] as well as more multipotent neural stem cells[4] has encouraged the development of therapies oriented toward myelin repair.

The development of novel therapeutic approaches for MS as well as our understanding of the pathobiology of demyelinating diseases has been facilitated by the use of distinct animal models such as Experimental Allergic Encephalitis (EAE). Induction of EAE by immunization with peptides of Myelin Oligodendrocyte Glycoprotein (MOG) results in chronic functional deficits that are correlated with areas of demyelination and inflammation in white matter tracts, particularly in the spinal cord. Likewise, the details of myelin repair have been illuminated by the use of local chemically induced demyelination such as results from the injection of lysolecithin or ethidium bromide.

Cell based therapies are emerging as an important approach to the treatment of MS[5,6]. While the majority of current therapies are directed toward modulation of the immune system, cell based therapies offer the possibility of localized multifaceted influences that promote effective remyelination during the course of the disease concomitant with modulation of immunological attack[6]. Several stem cell populations have shown therapeutic promise in the setting of different neural insults. For example, neural stem cells promote repair in models of MS[5,7], while adult oligodendrocyte progenitors or NG2+ cells enhance axonal regeneration after spinal cord injury[8]. One of the most intensively studied stem cell populations in the context of tissue regeneration are mesenchymal stem cells (MSCs)[9]. Initial work identified MSCs as a powerful regulator of graft-versus-host disease following bone marrow stem cell transplants[10] and more recently they are emerging as a promising approach for cell-based therapies for a number of neurological disorders including stroke[11] spinal cord injury[12], MS[6,13,14] and other demyelinating diseases[15].

In animal models of MS, the efficacy of MSCs to enhance functional recovery appears to reflect their ability to modulate both the immune system and neural cell responses[13,16]. In EAE, treatment with MSCs results in a biasing of the immune response from proinflammatory TH1 to anti-inflammatory TH2 based responses[13]. In addition, MSCs localize to the areas of demyelination in the CNS and promote functional recovery. Although MSCs have been proposed to have the capacity to give rise to neural cells[17] in the setting of demyelination, their primary role appears to be the promotion of endogenous repair mechanisms[13] that we show can be recapitulated by conditioned medium, suggesting it reflects the release of soluble factors.

The molecular mechanisms responsible for functional recovery in EAE following treatment with MSCs have not been identified. Here we show that the effects of MSCs reside in a fraction of MSC conditioned medium a critical component of which is Hepatocyte Growth

Factor (HGF). Hepatocyte growth factor is a pleiotropic cytokine primarily made by cells of mesenchymal origin. Originally described as a major mitogen for hepatocytes[18], HGF has been identified in multiple different tissues including the CNS[19]. In general HGF is thought to promote angiogenesis and cell survival[20,21] and injection of HGF has been demonstrated to enhance kidney and liver regeneration as well as protect against the onset of renal failure[22]. The effects of HGF are not restricted to liver and kidney and it has been shown to prolong the survival of cardiac allografts and improve myocardial function following myocardial infarction[23].

The biological effects of HGF are primarily mediated by the tyrosine kinase transmembrane receptor cMet[24]. During development, cMet is expressed in several different tissues including the CNS[25] and liver. Expression in liver is retained in normal adult and activated during parenchymal regeneration in adult animals[26], consistent with a role for this pathway in tissue regeneration.

In this study we identify a central role for the HGF/cMet signaling pathway in mediating functional recovery and remyelination stimulated by MSCs. Human MSCs in growth conditions secrete HGF and their capacity to promote recovery in EAE depends upon signaling through the cMet receptor. The beneficial effects of both MSC-CM and HGF in EAE are selectively blocked by treatment with function blocking cMet and anti-HGF antibodies. Systemic treatment with HGF dramatically accelerates the rate of remyelination in a non-immune mediated spinal cord LPC lesion model of demyelination and in cerebellum slice cultures. These data support the notion that a major role of MSCs is to provide a source of trophic factors[14] including HGF that modulate the inflammatory environment in demyelinating diseases and stimulate endogenous neural cell remyelination to enhance functional recovery.

Results

MSC -CM modulates neural development and disease in EAE

Long-term functional recovery in demyelinating disease depends on myelin repair and suppression of pathogenesis both of which appear to be influenced by MSCs. To determine whether the neural responses and functional recovery seen after treatment of animals with infused MSCs reflected release of soluble factors, the effects of MSC conditioned medium (MSC-CM) on neural cell development and functional recovery were assayed. Exposure of neurosphere cultures to MSC-CM resulted in a reduction in the proportion of astrocytes and an increase in the proportion of A2B5+ oligodendrocyte precursor cells (OPCs), oligodendrocytes and neurons that developed (Fig. 1a). In control cultures after 3 days in vitro, approximately 30% of cells were GFAP+ astrocytes and A2B5+ OPCs while 25% were O4+ cells and less than 5% neurons. By contrast after 72 hrs in MSC-CM, the proportion of astrocytes was reduced to 20%, the proportion of O4+ cells (50%) and neurons (25%) increased significantly (Fig. 1b)($p<0.05$).

Injection of MSC-CM significantly reduced disease load in EAE animals (Fig. 1c). Injection of MSC-CM 17 days after $MOG_{35–55}$ immunization, at the peak of disease resulted in a rapid reduction in functional deficits in 11/ 11 animals. While control animals maintained a

clinical score between 2.5 and 3, all animals that received MSC-CM showed some functional improvement. Four of the 11 animals improved to a clinical score of 1 and 2 other animals appeared completely normal within 5 days. This functional recovery was correlated with a reduction in the extent of regions of demyelination seen in spinal cord sections stained with Luxol Fast Blue (Fig. 1d).

MSC-CM stimulated recovery depends on a 50–100Kd activity

Size fractionation studies demonstrated the disease modifying activity of MSC-CM required a fraction with a molecular mass between 50–100kD. Fractions with masses 1–50kD did not significantly modify disease progression in $MOG_{35–55}$-EAE in vivo (data not shown). By contrast, 1–100kD ($MSC\text{-}CM_{100kD}$) fractions reduced disease burden in the EAE model (n=11) (Fig. 2a) and increased the proportion of oligodendrocytes and neurons from neurosphere cultures (Fig. 2b). This functional improvement was correlated with improvements in the histology of the spinal cord that were comparable to that seen with complete MSC-CM. Injection of control medium or heat inactivated CM (n=8) had no obvious effect on either functional outcome or histology.

Isolation of mononuclear cells from the spinal cords of control and $MSC\text{-}CM_{100kD}$ treated EAE animals demonstrated alterations in the relative levels of inflammatory cytokines (Fig. 2c). The level of the pro-inflammatory cytokines interferon-γ (IFN-γ), (IL)-17, tumor necrosis factor- α (TNF-α), IL-2, and IL-12p70 were decreased in animals that received $MSC\text{-}CM_{100kD}$ while levels of anti-inflammatory cytokines such as IL-10 and IL-4 were increased at day 35 after EAE onset.

Recombinant HGF modulates disease progression in EAE

Based on the size fractionation studies, known MSC expression data[27,28] and previously described biological properties[29,30] hepatocyte growth factor (HGF) was identified as a potential candidate molecule within the $MSC\text{-}CM_{100kD}$ fraction that could modulate disease in EAE. Western blot analyses of active $MSC\text{-}CM_{100kD}$ identified HGF (Mwt 62kD alpha chain + 34kD beta chain) at a concentration of between 600–800ng/ml in all active samples (n= 6) but not in controls (Fig. 3a). To determine whether recombinant HGF treatment could promote functional recovery in EAE, animals at the peak of disease (clinical score=3) received injections of either PBS (n=10) or HGF at two different concentrations (50 and 100ng/animal) (n=10) every other day over a five-day period (days1, 3 and 5) and their clinical scores monitored for 30 days post immunization. The clinical score of control animals remained relatively constant between 3–3.5 while those that received HGF improved (Fig. 3b). The higher dose of HGF appeared to provide slightly better functional recovery although both doses reduced the mean functional score below 1.5 (Fig. 3b). Compared to IV injections the functional improvement of animals following IP injection of HGF (100ng) was reduced. For example, at 30 days post immunization the mean score of IP treated animals was approximately 2.4 compared to 1.5 for IV treated animals (Fig. 3b). All subsequent studies utilized IV delivery.

The HGF-induced functional improvement was closely correlated with histological improvement. In control EAE animals, Luxol Fast Blue staining revealed extensive regions

of white matter pathology along the length of the spinal cord 30 days after immunization (Fig. 3c). In HGF treated animals at the same stage, the degree of demyelination was dramatically reduced and the majority of white matter appeared relatively normal (Fig. 3c). The level of inflammatory cell infiltration in EAE animals was also reduced in HGF treated animals. In control EAE animals, H & E staining revealed white matter regions with a high density of infiltrated cells (Fig. 3c) and this was confirmed by labeling with anti-CD3 antibodies (Fig. 3c). Both the extent of the infiltrate and the density of cells were reduced in animals treated with HGF. Myelin integrity was confirmed in 1um Epon sections labeled with Toluidine Blue and by ultrastuctural analyses (Fig. 3c) that demonstrated a decrease in the number of unmyelinated axons. In lesion areas of EAE animals a majority of axons were unmyelinated (80±20%) while in lesion areas of HGF treated EAE animals a minority (10±4% p=0.05) were unmyelinated. Analyses of myelin thickness in lesions areas showed that HGF treatment resulted in substantially thicker myelin sheaths around small diameter axons, as well as an increase in the relative number of small diameter (>1μm) axons (Fig. 3d). Together these studies suggest that treatment of EAE animals with HGF results in significant functional improvement and an enhancement in the extent of spinal cord remyelination. To assess the timing of HGF-induced histological improvement, animals were sacrificed at the time of treatment initiation and 3, 11 and 17 days after treatment initiation and their histology assessed on 1um sections. Spinal cords had extensive demyelinated lesions and cellular infiltrates at initiation of treatment that were still apparent after 3 days of treatment (Sup. Fig. 1). After a further 8 days, the lesions were largely resolved although there was a residual elevated cellularity. By 17 days after initiation of treatment, light microscopy (Fig. 3c) and ultrastructural analyses (Fig. 3c) reveal extensive remyelination in HGF treated animals with essentially all fibers including small diameter fibers extensively myelinated. These data suggest HGF both reduces pathological pressure and enhances or facilitates myelin repair.

HGF and MSC-CM stimulated recovery is mediated by cMet

In other systems the biological properties of HGF are mediated through the tyrosine receptor kinase cMet, the best characterized receptor for HGF[31]. To determine if the functional benefits of HGF in EAE were also mediated by cMet, EAE animals were treated with a function blocking cMet antibody [32,33] (2× 250ng/animal IV 24hr prior to HGF treatment). In animals that received control IgG followed by 3 injections of HGF at the peak of disease (n=8), functional improvements were evident after 24 hrs that plateaued after 5–7 days (Fig. 4a). By contrast, animals treated with cMet antibodies (n=10) failed to show any significant improvement over the subsequent 14 days. Histologically the lesions in animals treated with cMet antibodies appeared similar to controls with only limited remyelination (data not shown).

The capacity of HGF to modulate the immune response in EAE animals was also negated by treatment with cMet antibodies. For example, compared to untreated EAE animals relatively low levels of the proinflammatory cytokines IFN-γ, IL-17, TNFα, and IL-2 were detected in HGF treated animals (Fig. 4c) and these were significantly elevated in animals that received cMet antibodies. Likewise the level of anti-inflammatory cytokine IL-10 and IL-4 elevated in HFG treated animals were reduced following treatment with cMet antibodies (Fig. 4c).

ELISPOT assays demonstrated that HGF treatment resulted in a reduction in the frequency of spinal cord derived MOG35– -specific proinflammatory cytokine producing cells (IFNγ IL17) and an increase in IL-10 producing cells that was reversed by cMet antibody treatment (Fig. 4e). These results confirm that in the setting of EAE, the biological activity of HGF is mediated through cMet.

Treatment with cMet antibodies blocked MSC-CM mediated functional recovery. In control IgG treated animals (2X injections, 24hr prior to treatment), MSC-CM stimulated a rapid and sustained functional recovery (Fig. 4b) (n=9). By contrast, animals treated with cMet antibodies showed little or no functional improvement over the subsequent 15 days (Fig. 4b) (n=11). Treatment of MSC-CM with function blocking anti-HGF for 2 hrs prior to injection also inhibited its capacity to reduce functional deficits in EAE (Fig. 4b, black line). Cytokine analyses revealed that the decrease in pro-inflammatory cytokines and the increase in anti-inflammatory cytokines associated with MSC-CM were largely reversed by prior treatment with cMet antibodies (Fig. 4d) as were the alterations in the frequencies of antigen specific IFNγ, IL17 and IL-10 secreting cells shown by ELISPOT analysis (Fig. 4f). These data strongly suggest that HGF signaling in MSC-CM is critical for mediating functional recovery and promoting histological improvement in MOG induced EAE.

cMet mediates HGF/MSC-CM stimulation of neural development

To assess the role of HGF in MSC-C_{M100kD} induced neural cell development the relative generation of the different classes of neural cells from postnatal neurospheres and the dependence on cMet signaling was compared between HGF and MSC-CM_{100kD}. In control neurosphere-derived cultures grown for 7 days, the proportion of A2B5+ OPCs, O4+ oligodendrocyte lineage cells and beta-tubulin+ neurons was below 10% while the proportion of GFAP+ astrocytes was greater than 60% (Fig. 5a–b). By contrast, in the presence of MSC-CM_{100kD} the proportion of A2B5+ OPCs was increased to approximately 25%, the proportion of O4+ cells was greater than 30% and the proportion of beta-tubulin+ neurons was greater than 40% while the proportion of astrocytes was dramatically reduced (approx. 10%). In both cases, cells tended to form clusters although this was more prevalent in controls and total cell numbers were not significantly different in either condition. Similar changes in cell proportions were seen in cultures treated with HGF (45ng/3×10^5 cells) although the reduction in GFAP+ astrocytes was less pronounced with HGF than with MSC-CM_{100kD}. Blocking cMet negated the effects of both MSC-CM100kD and HGF and the cellular composition of the cultures reverted to that of controls (Fig. 5a–b). Similarly, incubation of $CM_{1–100kD}$ with function blocking anti-HGF antibodies negated its effects on neural cell development consistent with a major role for MSC-CM-derived HGF in the regulation of neural cell development (Fig. 5a–b).

Repopulation of demyelinated lesions depends on OPCs migration that can be stimulated by MSCs[34]. To assess if EAE pathology based responses were dependent on cMet, neurospheres were established from the subventricular zone of animals with ongoing EAE and cell migration compared in the presence or absence of MSC-CM_{100kD} or HGF±cMet supplemented medium. Cell migration was enhanced in the presence of HGF and this was inhibited by cMet antibodies (Fig. 5c). Similarly, cell migration from EAE-derived

neurospheres was enhanced by MSC-CM_{100kD} and this effect was blocked by anti-cMet or anti-HGF antibodies (Fig. 5d). The majority of the migratory cells gave rise to either neurons (Fig. 5c–d) or oligodendrocytes (Fig. 5d) and very few generated astrocytes (data not shown). Consistent with HGF mobilizing OPCs and enhancing remyelination, in vivo EAE lesions of animals treated with HGF contained greater than twice as many EGFP-PLP+ oligodendrocytes (3.1±0.3/200μm^2)than lesions of control animals (1.8±0.2/200μm^2) (Fig. 5e). The average number of lesions was also significantly reduced in animals treated with HGF compared to controls. For example, in control EAE animals the average section of spinal cord had 6±0.4 areas of lesion that was reduced to 3.4±0.6 in animals treated with HGF($p<0.05$).

HGF promotes repair in non-immune mediated demyelination

The restitution of histological integrity seen in EAE following treatment with HGF is likely a combination of suppression of the immune response and the promotion of myelin repair. To determine whether HGF promoted myelin repair in non-immune mediated demyelination in vivo, spinal cord LPC lesion and slice culture models were employed. In animals injected iv with HGF (0.8μ on days 4, 6 and 11 after an LPC lesion and examined on 14 or 28 days post lesion (dpl), a 30% reduction in overall lesion volume was seen by Luxol Fast Blue staining compared to saline controls (Fig. 6a–b). Labeling of frozen sections with antibodies to myelin basic protein (MBP) demonstrated only limited expression in control lesions while in the HGF treated lesions significant MBP staining was seen throughout the lesion at 14 dpl (Fig. 6c–d). Similarly the density of NG2+ cells was increased approximately 8 fold in HGF treated lesions (4±2 to 33±5 /unit area)($p<0.005$) (Fig. 6e,f and p) and greater than 25% of these cells were proliferating as shown by Ki67 double labeling (data not shown). By contrast, while control lesions showed an extensive astrocyte response with intense GFAP labeling around the lesion perimeter this was significantly attenuated in HGF treated animals (Fig. 6g–h).

The increase in NG2+ and MBP+ labeling in the HGF treated lesions was accompanied by a dramatic increase in the extent of remyelination (Fig. 6i–m). In control lesions, (n=9) few myelinated axons were present in lesions, even at the lesion edge (Fig. 6i) while in HGF treated animals (n=8) myelinated axons were distributed throughout the lesion interspersed with cells full of myelin debris (Fig. 6j). Ultrastructural analyses showed that control lesions contained largely unmyelinated axons some of which were ensheathed in glial processes (Fig. 6k) while in HGF treated animals lesions contained axons of different sizes at different stages of remyelination with some relatively thick myelin sheaths (Fig. 6l) but the majority with thin myelin (Fig. 6m). Calculation of G ratios confirmed the abundance of thinly myelinated axons in HGF treated lesions (Fig. 6n) and showed a greater than 5 fold increase in the density of remyelinated axons (18± 2 to 130±6/unit area) ($p<0.0002$). Treatment of animals with 0.4μl HGF resulted in increased but less profound enhancement of remyelination and by day 28dpl some limited remyelination was apparent in controls although the beneficial effects of HGF were still evident (data not shown).

Consistent with the HGF induced functional recovery in LPC lesions, treatment of LPC demyelinated slice cultures with HGF enhanced recovery. Slices of P7 cerebellum myelinate

extensively in vitro over a 7day period (Sup. Fig. 2A,a–b) and treatment with LPC induces a rapid and sustained demyelination (Sup. Fig. 2A, c–d). Exposure of LPC treated slices to 50ng/ml HGF promoted re-ensheathment by oligodendrocytes and reestablished the cytoarchitecture of the slice (Sup. Fig. 2A,e–f). Lower doses of HGF (10ng/ml) had little effect while higher doses (90ng/ml) resulted in marginal additional improvement. A similar HGF-induced recovery of MBP profiles was seen in slice cultures from cerebral cortex treated with HGF (Sup. Fig. 2A,g–i.). Quantification of the extent of myelination/ oligodendrocyte demonstrated a 2.5 fold decrease after LPC treatment that was largely reversed by HGF treatment (Sup. Fig. 2b). This HGF induced functional recovery was negated by exposure to anti-cMet antibodies (Sup. Fig. 2b). This recovery likely reflects stimulation of oligodendrocyte development since growth of P0 spinal cord dissociated cell cultures in 50ng/ml HGF significantly increased the proportion of O4+ and O1+ cells (Sup. Fig. 2c–d) from approximately 3% (O4+) and 1% (O1+) in control cultures to greater than 15% (O4+) and 23% (O1+) after 3 days in HGF. To confirm the expression of cMet on OPCs, cells were double labeled with A2B5 and anti-cMet in vitro after 3 days in culture. Virtually all OPCs in culture expressed cMet (Sup Fig. 3a–c). By contrast in vivo in naïve animals very few cells of the oligodendrocyte lineage expressed detectable cMet while in the setting of demyelinating lesions virtually all cells of the oligodendrocyte lineage expressed high levels of cMet (sup. Fig. 3d–f).

Taken together these studies suggest that the ability of MSCs to reduce disease burden in models of EAE is dependent on their promotion of HGF signaling that regulates both immunomodulation and enhances remyelination in the setting of demyelinating disease.

Discussion

Recent studies suggest that stem cell based approaches are promising therapies for the treatment of demyelinating diseases such as Multiple Sclerosis (MS). Mesenchymal stem cells have emerged as an attractive candidate for the treatment of neurological pathologies based on their effectiveness, relative accessibility, ease of expansion and expression of trophic factors [9]. Previous studies indicated that bone marrow derived MSCs promoted functional recovery and reduced lesion load in animal models of EAE [6,13,35] and these observations have led to the development of a number of clinical trials using MSCs for the treatment of MS[36].

Here we show that in EAE, an animal model of MS, human MSC growth conditioned medium is effective at promoting functional recovery through a combination of immune suppression and promotion of myelination. Several lines of evidence indicate that these MSC activities are dependent on the secretion of hepatocyte growth factor (HGF). For example, fractionation studies indicate the activity of MSC-CM mediating functional recovery in EAE is dependent on a 50–100kD fraction consistent with HGF with a molecular mass of approximately 62–65kD for the alpha and 34kD for the beta chain[37]. Treatment of animals after the onset of EAE disease with HGF reduced the severity of disease and the effects of both HGF and MSC-CM were blocked by pre-treatment with cMet antibodies while the effects of MSC-CM are blocked by pre-treatment with anti-HGF. The cellular responses to MSC-CM and HGF were similar. Both stimulated the development and

migration of oligodendrocytes and neurons and reduced the emergence of astrocytes from neurosphere cultures. Likewise, both MSC-CM and HGF reduced proinflammatory cytokine expression and enhanced anti-inflammatory cytokine expression while HGF dramatically enhanced remyelination in LPC-induced spinal cord demyelination and promoted ensheathment following demyelination in slice cultures of cerebellum and cortex.

The observations that HGF is required for MSC-CM mediated functional recovery in EAE is consistent with the observations that MSCs release a variety of growth factors[9] including HGF and that this can be enhanced by stimulation with EGF or TNFα[28]. In multiple different conditions the biological effects of MSCs and HGF are similar. For example, in graft-versus-host disease, a major complication that follows transplantation of allogenic bone marrow MSCs provide robust benefit, reducing the incidence of GVHD[38], while in a murine model of GVHD, transfection of human HGF into skeletal muscle was found to inhibit damage to the gut and liver and enhance survival[39]. Similarly, while MSCs appear to show promise for the treatment of myocardial infarcts, transfection of HGF directly into the myocardium in a murine model of autoimmune myocarditis reduced disease burden through a reduction in cardiomyocyte apoptosis and induction of TH2 anti-inflammatory cytokines[40]. Finally, a recent study demonstrated that animals in which expression of HGF was driven off a neuronal specific promoter were resistant to EAE due to tolerization of dendritic cells and induction of TH_2 cytokines[41], consistent with previous studies using MSC treatments in EAE[13,42].

Mesenchymal stem cells and HGF influence similar cellular targets in both the CNS and immune system. In the CNS, HGF is expressed both during development and in the adult where is has been suggested to act as a neurotrophic factor[19]. Consistent with this hypothesis, cMet is expressed on a range of neuronal populations[19] and cMet stimulation promotes axon outgrowth and neuronal differentiation in multiple neuronal populations[29]. The expression of cMet and the effects of HGF are not restricted to neurons. Astrocytes, microglia and oligodendrocyte precursors have been shown to express cMet[30,43]. The expression of cMet on oligodendrocyte lineage cells appears to be environmentally regulated. While the majority of OPC are cMet+ in vitro and in lesions of the CNS they are cMet− in naïve tissue. In humans the expression of cMet is less well characterized but it has been described on some CNS tumor cells and microglia[8,43]. Which of these cellular targets are critical for myelin repair seen in HGF/MSC-CM treated animals is currently unknown. Likewise in the immune system dendritic cells express cMet and HGF induces tolerance in this cell population[41]. Both MSC-CM and HGF bias T cells responses away from TH_1 and toward TH_2 as well as regulating the expression of IL-10[41]. The relative contributions of HGF mediated modulation of immune response and local stimulation of myelin repair are currently unclear. However, the rapid widespread remyelination of spinal cord LPC lesions seen following HGF treatment suggests that HGF promotes myelin repair in the absence of modulating the immune system. For example, although LPC injection induces an immune response it results in a rapid but brief influx of immune cells between 6–48h[44]. In the current studies treatment with HGF was delayed until 5 days post lesion, a time when the lesion has mostly developed. In general remyelination follows recruitment of OPCs to the lesion area and occurs over a 3–5 week period[45]. By contrast in HGF treated animals robust remyelination was apparent throughout the lesion by 14 days post lesion and only 9 days

after the commencement of treatment. This rapid repair suggests that HGF both recruits OPCs as well as enhances remyelination.

The beneficial effects of transient HGF treatment are sustained in EAE. The reduced EAE disease burden in animals that constitutively express HGF off the neuronal-specific enolase (NSE) promoter may reflect the continuous expression of elevated HGF. In the current studies however, a series of 3 injections of HGF at the peak of disease resulted in functional and histological recovery that was sustained for up to two weeks, the longest time examined. Although HGF is known to passage across the blood brain barrier[46], given that its half-life is thought to be relatively short following injection[46], the mechanisms that results in this long term benefit are unclear. It may be that a short period of stimulated recovery is sufficient to inhibit further pathogenesis by permanently shifting the balance of pathological pressure and in favor of repair[47]. Alternatively, in other systems it has been demonstrated that elevation of exogenous HGF levels result in sustained elevation of endogenous HGF levels through a proposed positive feedback auto loop[48]. Finally, the effect of HGF may be to permanently alter the balance of pro- and anti-inflammatory T cells in EAE possibly by influencing dendritic cell secretion of IL-23. If such a mechanism were operative, it could explain persistent recovery.

A functional role for HGF in MS has not been defined. Previous studies suggest that in MS patients as well as those with other neurological diseases, the levels of HGF are elevated in CSF. While this has been proposed to correlate with pathology, particularly demyelinating pathology, our studies suggest it is more likely a reflection of an endogenous repair process stimulated by the disease. Indeed, it may be that susceptibility or disease progression in MS is in part, a reflection of the capacity of MS patient MSCs to release HGF. Consistent with this hypothesis, HGF gene expression decreases with age in murine MSCs and while MSCs from MS patients have similar proliferative, differentiative and cell surface properties to control MSCs, they differ in their cytokine profiles.

In conclusion, we show that the therapeutic efficacy of MSCs in EAE is a result of secreted signals that are found in growth-conditioned medium. The biological effects of the conditioned medium are dependent on the presence of HGF and sustained functional recovery in EAE follows a transient treatment with exogenously supplied HGF. The effects of both HGF and MSC-CM are mediated through the tyrosine kinase receptor cMet and mediate enhanced myelin repair as well as immunomodulation. These studies raise the possibility that the HGF/cMet pathway may provide novel therapeutic opportunities for the treatment of MS.

METHODS

Immunization

All studies were approved by Case Western Reserve University School of Medicine's IACUC and animals were maintained in a NIH/AAALAC-approved facility. Chronic EAE was induced in 8–12 week old female C57BL/6 mice by subcutaneous immunization with 200μL of 200μg $MOG_{35–55}$ with complete Freund's adjuvant. Pertussis toxin (500ng) (List Biological Laboratories, Campbell, CA) was injected IP at 0 and 2 days. Animals were

sacrificed at Day 35 by transcardial perfusion with 4% paraformaldehyde. Animals were graded according to a standard clinical index: 1=limp tail; 2=hind limb weakness; 3=one limb plegic; 4=plegia of 2 limbs; 5=moribund or dead..

Induction of local demyelination and HGF treatment

LPC-induced demyelination of the spinal cord was performed as described previously[49]. Animals were anesthetized with 0.001 mL per kg of body weight of 3 ml ketamine (Fort Dodge), 3 ml xylazine (Lloyd Laboratories), 1 mL acepromazine (Webster Vet). A T10 laminectomy was performed and 1.5 μl of 1% freshly prepared LPC (Sigma) solution was infused at 15 ml h^{-1}. Rats received either 0.4, 0.8μg/Kg HGF or vehicle control at on d5, d6 and d11 by tail vein. Animals were sacrificed at d14 and 28.

Preparation of Human mesenchymal stem cells conditioned medium and treatment protocols

Human mesenchymal stem cells (hMSCs) were isolated from bone marrow aspirates and grown in DMEM-low glucose supplemented with 10% FBS. This growth medium was collected between days 11–14 (MSCs-CM). The MSCs used to prepare conditioned medium were derived from 5 separate donors to insure that the results were not donor specific. Similar medium lacking conditioning by hMSC was collected as control medium (Ctrl-CM)[50]. Conditioned growth medium was concentrated 10-fold through YM-3 centrifugal filter devices with a YM-100 (100,000MW) devices (Millipore, Billerica, MA). This 100kD fraction was termed MSC-CM_{100kD}. Protein concentrations were estimated using the BCA Protein Assay Kit (Pierce, Rockford, IL) and 0.5mg/protein was used for cell culture and in vivo studies. Recombinant HGF (HGF), cMet and anti-HGF antibodies were purchased from R&D Systems (Minneapolis, MN). Two doses of rHGF (50ng and 100ng) were injected intravenously on day 14 at peak disease every other day for a total of three injections. For cMet antibody treatment, animals were injected 24 hours prior to treatment. Conditioned media was incubated with function blocking anti-HGF for 1hr prior to use. In culture, HGF was used at 45ng/2×10^5 cells and anti cMet at 50ng/2×10^5 cells added 1hr prior to HGF.

Western blot

Conditioned medium proteins were separated on 10% SDS-Polyacrylamide gels transferred to a polyvinylidene difluoride membrane, and incubated with anti-HGF monoclonal primary antibodies (Cell Signaling Technology, Beverly, MA) and HRP-conjugated secondary antibodies. Visualization was performed with an enhanced chemiluminescence system (ECL, GE Healthcare Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Neuropathology

Sections (4% PFA fixed) were stained with Hematoxylin and Eosin (H&E) for detection of inflammatory infiltrates and Luxol Fast Blue (LFB) for myelin detection. For electron microscopy, animals were perfused with 2.5% glutaraldehyde. CNS tissues were postfixed in 1% osmium tetroxide for 1 hour, dehydrated, and embedded in epoxy resin. One-micron epoxy sections were stained with Toluidine Blue and examined by light microscopy. For

EM, thin sections were stained with uranyl acetate and lead citrate and examined in a Joel 100CX microscope at 80Kv. For LFB, H&E and Toludine blue analysis 3 different animals were assayed with at least 16 sections/animal. For quantification of the proportion of myelinated and unmyelinated axons was taken from multiple sections at least 2 different spinal cord regions from 2 animals. G ratios were determined by analysis of at least 200 axons in each condition. For analysis of lesion load the data was obtained from 10 sections taken from 3 different animals of each control and HGF treated EAE. Data was collected using image j and the units represent cm^2 ± standard deviation. Sample sizes were determined based on power estimates. Lesions were identified as shadow areas in LFB stained sections. All images were taken by an investigator blinded to the treatment of the individual animals.

Neural Cell Cultures

Neurosphere cultures were prepared from newborn mouse cortex and adult SVZ and grown in 10ng/mL EGF. Second passage neurospheres were dissociated and plated at 2×10^5cells/coverslip in the absence or presence of whole (MSC-CM) or fractioned (MSCs-CM 100kD), rHGF ± anti-cMet. (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) for 3 days prior to analyses. Cells were labeled with antibodies to Nestin (rat-401, Developmental Studies Hybridoma Bank, Iowa City, IA), A2B5, O4, β-tubulin III (Sigma Aldrich, St. Louis, MO) and GFAP (R&D Systems, Minneapolis, MN). The proportion of positive cells was determined in 3 randomly selected areas/coverslip in at least three separate experiments derived from 3 independent neurosphere preparations. Data assessed by independent t-test. To determine the effects of MSC CM1-100 and HGF on the migration of neurosphere derived cells, neurospheres were grown for 14 days and replated on poly-L-lysine coated coverslips in the presence of CM_{100kD}, CM_{100kD} + anti-cMet, CM_{100kD} + anti-HGF, and HGF + anti-cMet. Cells were allowed to migrate for 24hrs and the preparations fixed with cold methanol and processed for immunohistochemistry as above. Cerebellar slice cultures were established from 300μm saggital slices of P9 cerebellum cut on a Leica Vibratome. Slices were grown for 3 days in DMEM/BME with N2 and PDGF -AA (20ng/ml) before being switched to media containing 15% horse serum. Slices were treated with Lysolecithin (LPC, Sigma) at a final concentration of 0.5mg/ml for 16 hours rinsed and then incubated in either control media or control media containing 50ng/ml HGF for 3 to 7 days. Oligodendrocyte ensheathment was assayed by MBP expression (Covance, SMI99, Invitrogen Alexa-594). Cortical slices were treated similar but obtained from coronal sections of P4 brain and grown for 4 days prior to LPC treatment.

ELISPOT assay for $MOG_{35–55}$-specific CD4+ T cells in the CNS

Cells were isolated from the spinal cords of animals with EAE following gross manual disruption, filtered, washed and counted. ELISPOT plates (Millipore, Billerica, MA) were coated with IFN-γ antibody (eBioscience, San Diego, CA), anti-IL-10 (eBioscience) or anti-IL-17 (eBioscience) overnight. Cells were stimulated with medium alone or with 10μg/ml autoantigen. Subsequently, biotinylated anti-IFN-γ (Mabtech, Cincinnati, OH), anti-IL-10 or anti-IL-17 (eBioscience) was added, followed by Strepavidin-HRP (Mabtech) and developed with TMB ELISPOT substrate (Carl Zeiss Vision, Hallbergmoos, Germany). Spots were counted by AID ELISPOT plate reader version 4.0. Individuals were designated

as responders if the numbers of spots in the presence or absence of HGF or HGF plus cMet-Ab were significantly altered (t-test) ($P < 0.05$) from controls and the frequency of myelin reactive CD4+ cells calculated.

Cytokines analysis

IL-17, IFN-γ, TNF-α, IL-2, IL-10 and IL-4 were analyzed with the Qiagen LiquiChip System (BioChipNet, Reutlingen, Germany) using anti-mouse monoclonal antibodies with supernatant samples and standards in duplicate. Data represent mean±standard deviation assayed by t-test (n=3). Biotinylated secondary antibodies were added followed by streptavidin-horseradish peroxidase (Strep- HRP) and peroxide-chromagen substrate following the manufacturer's instructions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Prineas, JW. The neuropathology of multiple sclerosis. In: Koestier, JC., editor. Handbook of clinical neurology. Elsevier; Amsterdam: 1985.
2. Trapp BD, et al. Axonal transection in the lesions of multiple sclerosis. New England Journal of Medicine. 1998; 338:278–285. [PubMed: 9445407]
3. Wolswijk G, Noble M. Identification of an adult-specific glial progenitor cell. Development. 1989; 105:387–400. [PubMed: 2680425]
4. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science. 1992; 255:1707–1710. [PubMed: 1553558]
5. Ben-Hur T, Goldman SA. Prospects of cell therapy for disorders of myelin. Annals of the New York Academy of Sciences. 2008; 1142:218–249. [PubMed: 18990129]
6. Uccelli A, Pistoia V, Moretta L. Mesenchymal stem cells: a new strategy for immunosuppression? Trends in immunology. 2007; 28:219–226. [PubMed: 17400510]
7. Pluchino S, et al. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. Nature. 2003; 422:688–694. [PubMed: 12700753]
8. Busch SA, et al. Adult NG2+ cells are permissive to neurite outgrowth and stabilize sensory axons during macrophage-induced axonal dieback after spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2010; 30:255-–265. [PubMed: 20053907]
9. Caplan A, Dennis JE. Mesenchymal stem cell as trophic mediators. J. Cell Biochem. 2006; 98:1076–1084. [PubMed: 16619257]
10. Lazarus HM, et al. Cotransplantation of HLA-identical sibling cultureexpanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2005; 11:389–398.
11. Yoo SW, et al. Mesenchymal stem cells promote proliferation of endogenous neural stem cells and survival of newborn cells in a rat stroke model. Experimental & molecular medicine. 2008; 40:387–397. [PubMed: 18779651]

12. Jung DI, et al. A comparison of autologous and allogenic bone marrowderived mesenchymal stem cell transplantation in canine spinal cord injury. Journal of the neurological sciences. 2009; 285:67–77. [PubMed: 19555980]
13. Bai L, et al. Human bone marrow-derived mesenchymal stem cell induce Th2 polarized immune reponse and promote endogenous repair in animal modesl of multiple sclerosis. Glia. 2009; 57:1192–1203. [PubMed: 19191336]
14. Rafei M, Birman E, Forner K, Galipeau J. Allogeneic mesenchymal stem cells for treatment of experimental autoimmune encephalomyelitis. Molecular therapy : the journal of the American Society of Gene Therapy. 2009; 17:1799–1803. [PubMed: 19602999]
15. Tanaka F, et al. Exogenous administration of mesenchymal stem cells ameliorates dextran sulfate sodium-induced colitis via anti-inflammatory action in damaged tissue in rats. Life sciences. 2008; 83:771–779. [PubMed: 18950645]
16. Barhum Y, et al. Intracerebroventricular transplantation of human mesenchymal stem cells induced to secrete neurotrophic factors attenuates clinical symptoms in a mouse model of multiple sclerosis. Journal of molecular neuroscience : MN. 2010; 41:129–137. [PubMed: 19902385]
17. Krampera M, et al. Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. Bone. 2007; 40:382–390. [PubMed: 17049329]
18. Michalopoulos GK. Liver regeneration: molecular mechanisms of growth control. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1990; 4:176–187. [PubMed: 2404819]
19. Achim CL, et al. Expression of HGF and cMet in the developing and adult brain. *Brain research*. Developmental brain research. 1997; 102:299–303. [PubMed: 9352114]
20. Sulpice E, et al. Cross-talk between the VEGF-A and HGF signalling pathways in endothelial cells. Biology of the cell / under the auspices of the European Cell Biology Organization. 2009; 101:525–539. [PubMed: 19281453]
21. Kitamura K, et al. Hepatocyte growth factor promotes endogenous repair and functional recovery after spinal cord injury. Journal of neuroscience research. 2007; 85:2332–2342. [PubMed: 17549731]
22. Anan F, et al. Predictors for silent cerebral infarction in patients with chronic renal failure undergoing hemodialysis. Metabolism: clinical and experimental. 2007; 56:593–598. [PubMed: 17445532]
23. Deuse T, et al. Hepatocyte growth factor or vascular endothelial growth factor gene transfer maximizes mesenchymal stem cell-based myocardial salvage after acute myocardial infarction. Circulation. 2009; 120:S247–254. [PubMed: 19752375]
24. Sheth PR, Hays JL, Elferink LA, Watowich SJ. Biochemical basis for the functional switch that regulates hepatocyte growth factor receptor tyrosine kinase activation. Biochemistry. 2008; 47:4028–4038. [PubMed: 18324780]
25. Sonnenberg E, Meyer D, Weidner KM, Birchmeier C. Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. The Journal of cell biology. 1993; 123:223–235. [PubMed: 8408200]
26. Ishiki Y, Ohnishi H, Muto Y, Matsumoto K, Nakamura T. Direct evidence that hepatocyte growth factor is a hepatotrophic factor for liver regeneration and has a potent antihepatitis effect in vivo. Hepatology. 1992; 16:1227–1235. [PubMed: 1427661]
27. Polisetti N, et al. Gene expression profile of epithelial cells and mesenchymal cells derived from limbal explant culture. Mol Vis. 2010; 16:1227–1240. [PubMed: 20664697]
28. Wang Y, et al. MEK, p38, and PI-3K mediate cross talk between EGFR and TNFR in enhancing hepatocyte growth factor production from human mesenchymal stem cells. Am J Physiol Cell Physiol. 2009; 297:C1284–1293. [PubMed: 19692652]
29. Ebens A, et al. Hepatocyte growth factor/scatter factor is an axonal chemoattractant and a neurotrophic factor for spinal motor neurons. Neuron. 1996; 17:1157–1172. [PubMed: 8982163]

30. Yan H, Rivkees SA. Hepatocyte growth factor stimulates the proliferation and migration of oligodendrocyte precursor cells. Journal of neuroscience research. 2002; 69:597–606. [PubMed: 12210825]
31. Bottaro DP, et al. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science. 1991; 251:802–804. [PubMed: 1846706]
32. Sun W, Funakoshi H, Nakamura T. Localization and functional role of hepatocyte growth factor (HGF) and its receptor c-met in the rat developing cerebral cortex. Brain Res Mol Brain Res. 2002; 103:36–48. [PubMed: 12106690]
33. van der Horst EH, et al. Discovery of fully human anti-MET monoclonal antibodies with antitumor activity against colon cancer tumor models in vivo. Neoplasia. 2009; 11:355–364. [PubMed: 19308290]
34. Bai L, Caplan A, Lennon D, Miller RH. Mesenchymal stem cell signals regulate neural stem cell fate. Neurocehmistry Research. 2007; 32:353–362.
35. Gerdoni E, et al. Mesenchymal stem cells effectively modulate pathogenic immune response in experimental autoimmune encephalomyelitis. Annals of neurology. 2007; 61:219–227. [PubMed: 17387730]
36. Siatskas C, Payne NL, Short MA, Bernard CC. A Consensus Statement Addressing Mesenchymal Stem Cell Transplantation for Multiple Sclerosis: It's Time! Stem cell reviews. 2010
37. Birchmeier C, Gherardi E. Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. Trends in cell biology. 1998; 8:404–410. [PubMed: 9789329]
38. Koc ON, et al. Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH). Bone marrow transplantation. 2002; 30:215–222. [PubMed: 12203137]
39. Kuroiwa T, et al. Hepatocyte growth factor ameliorates acute graft-versus-host disease and promotes hematopoietic function. The Journal of clinical investigation. 2001; 107:1365–1373. [PubMed: 11390418]
40. Futamatsu H, et al. Hepatocyte growth factor ameliorates the progression of experimental autoimmune myocarditis: a potential role for induction of T helper 2 cytokines. Circ Res. 2005; 96:823–830. [PubMed: 15774858]
41. Benkhoucha M, et al. Hepatocyte growth factor inhibits CNS autoimmunity by inducing tolerogenic dendritic cells and CD25+Foxp3+ regulatory T cells. Proc Natl Acad Sci U S A. 2010; 107:6424–6429. [PubMed: 20332205]
42. Karussis D, Kassis I. The potential use of stem cells in multiple sclerosis: an overview of the preclinical experience. Clinical neurology and neurosurgery. 2008; 110:889–896. [PubMed: 18375051]
43. Di Renzo MF, et al. Selective expression of the Met/HGF receptor in human central nervous system microglia. Oncogene. 1993; 8:219–222. [PubMed: 8380919]
44. Ghasemlou N, Jeong SY, Lacroix S, David S. T cells contribute to lysophosphatidylcholine-induced macrophage activation and demyelination in the CNS. Glia. 2007; 55:294–302. [PubMed: 17096403]
45. Franklin RJ, Ffrench-Constant C. Remyelination in the CNS: from biology to therapy. Nature reviews. Neuroscience. 2008; 9:839–855. [PubMed: 18931697]
46. Pan W, et al. Permeation of hepatocyte growth factor across the blood-brain barrier. Experimental neurology. 2006; 201:99–104. [PubMed: 16733055]
47. Miller RH, Fyffe-Maricich SL. Restoring the balance between disease and repair in multiple sclerosis: insights from mouse models. Disease models & mechanisms. 2010; 3:535–539. [PubMed: 20647413]
48. Hayashi S, et al. Autocrine-paracrine effects of overexpression of hepatocyte growth factor gene on growth of endothelial cells. Biochemical and biophysical research communications. 1996; 220:539–545. [PubMed: 8607801]
49. Kerstetter AE, Padovani-Claudio DA, Bai L, Miller RH. Inhibition of CXCR2 signaling promotes recovery in models of multiple sclerosis. Experimental neurology. 2009; 220:44–56. [PubMed: 19616545]

50. Lennon DP, Caplan AI. Isolation of human marrow-derived mesenchymal stem cells. Experimental hematology. 2006; 34:1604–1605. [PubMed: 17046583]

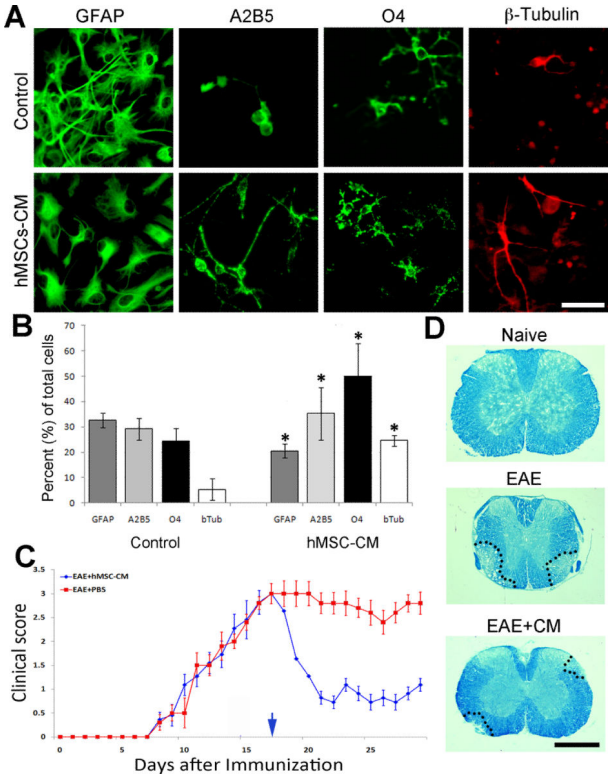

Figure 1.

Conditioned growth medium from human MSCs biases the development of neurosphere derived cells towards oligodendrocytes and neurons and promotes functional recovery in $MOG_{35–55}$ induced EAE. **A**. In the presence of human MSC-CM the proportion or GFAP+ astrocytes are reduced while the proportion of oligodendrocyte lineage cells and neurons is increased. **B**. Quantitation of relative number of distinct cell types in the presence and absence of MSC-CM. (Control vs. MSC-CM, A2B5 p=<0.01, O4 p=<0.05, β–tubulin p=<0.05, GFAP p=0.005) **C**. Treatment with MSC-CM (0.5mg) (arrow), but not PBS, at peak of disease after $MOG_{35–55}$ immunization results in functional improvement in EAE. **D**. The functional improvement is correlated with a reduction in myelin loss and tissue damage seen with Luxol Fast Blue staining of spinal cord sections. Lesions are outlined in D. Data in B represent the mean±SEM of duplicate preparations taken from 3 independent experiments. Note there is both overlapping expression and non-labeled cells in these preparations. Bar = 50μm in A, 500μm in D.

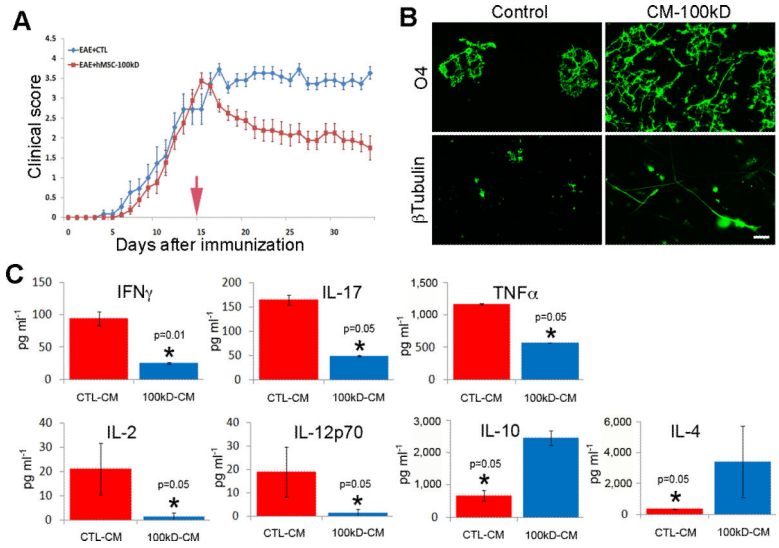

Figure 2.

The activity of MSC-CM to enhance functional recovery in EAE is dependent on a 1–100kD fraction. **A**. Treatment with MSC-CM_{100kD} (0.5mg/animal n=11), but not control CM, enhances functional recovery in animals with EAE. **B**. Growth of neurosphere-derived cultures in CM_{100kD} biases cell development in favor of oligodendrocytes and neurons compared to control CM. **C**. Treatment with CM_{100kD} reduces proinflammatory cytokine expression by spinal cord derived mononuclear cells in EAE animals. Significantly reduced expression of IFNγ, IL-17, TNFα, IL-2 and IL-12p70 and increased expression of IL-10 and IL-4 were seen in animals treated with MSC-CM_{100kD}. Results represent the mean±SEM from three independent experiments.

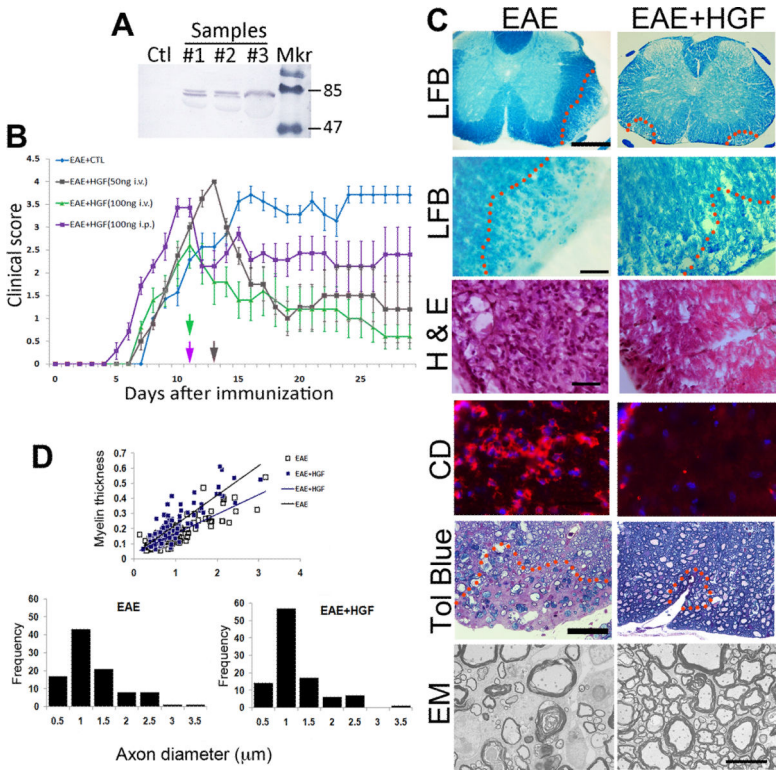

Figure 3.

Human MSC-CM_{100kD} contains HGF and HGF promotes functional and histological recovery in EAE. **A**. Western blot of 3 samples of active MSC-CM_{100kD} show the presence of HGF. **B**. Treatment with HGF (1 injection every other day for a total of 3 over a 5 day period, n=10) results in functional improvement compared to controls. Both dosage and route of delivery influence the efficacy of HGF to modulate disease. Intravenous (IV) injections are more effective than intraperitoneal (IP) injections. Arrows indicate treatment initiation day. **C**. HGF improves tissue histology. Sections from animals following 100ng IV injections at 30 days after immunization with $MOG_{35–55}$. The improvement in myelination is apparent by Luxol Fast Blue staining. The reduction in immune cell infiltrates is evident in H&E and anti-CD3 labeled sections and confirmed in Toluidine Blue stained 1μm sections. Lower panel are representative electron micrographs through spinal cord lesion areas in EAE control and HGF treated animals 17 days after initiation of treatment. **D**. Upper Analysis of myelin thickness vs. axon diameter in lesion areas of control and HGF treated animals demonstrates thicker myelin in HGF treated animals compared to controls. Lower: Comparison of the relative axons diameters in lesion areas of control EAE and HGF treated animals demonstrates a reduction in small diameter fibers and an increase in medium diameter fibers in HGF treated animals. Bars = 500μm in C LFB (top), 50μm in C LFB (2^{nd}panel), H&E, CD3, Tol Blue and 2μm in the electron micrographs.

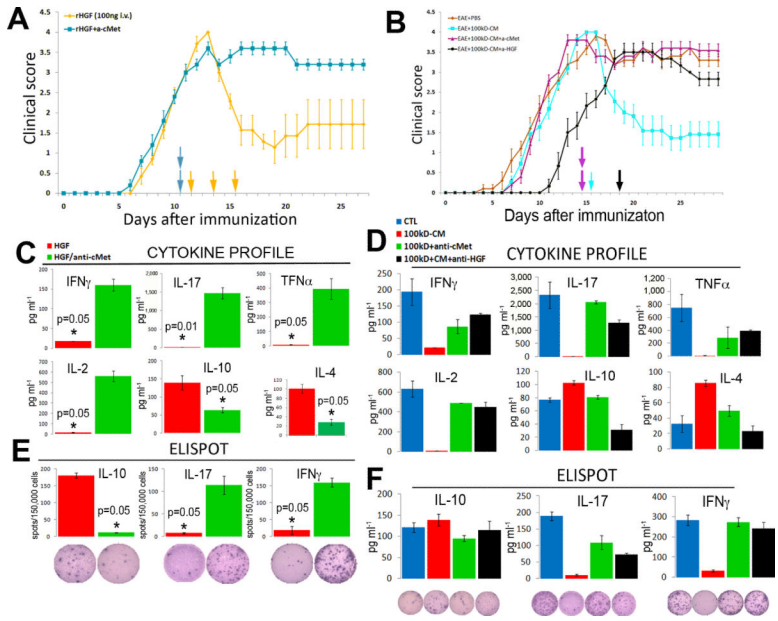

Figure 4.

Inhibition of HGF signaling with cMet antibodies negates the capacity of both HGF and MSC-CM to induce functional recovery and reverses EAE induced changes in cytokine expression. **A**. Two injections of function blocking cMet antibodies 12hrs apart, (green arrows) delivered 1 day prior to initiation of HFG treatment regime (red arrows) inhibits functional recovery. **B**. Treatment with cMet antibodies increases proinflammatory cytokine expression and reduces anti-inflammatory cytokine expression as shown by cytokine profile. **C**. ELISPOT analysis on spinal cord derived cells demonstrates that treatment with cMet antibodies increases the frequencies of TH-1/TH-17 cells compared to HGF treated animals. Similar data (D,E,F) was obtained following cMet inhibition of MSC-CM_{100kD} stimulated recovery. Treatment of MSC-CM_{100kD} with anti-HGF antibodies also blocked its capacity to enhance functional recovery in EAE and inhibited MSC-CM induced reduction in pro-inflammatory cytokine expression. The delay in disease onset reflects the use of a different preparation of $MOG_{35–55}$ peptide and not anti-HGF treatment. The data are representative from triplicate studies and counts represent the mean±standard deviation from one of three experiments.

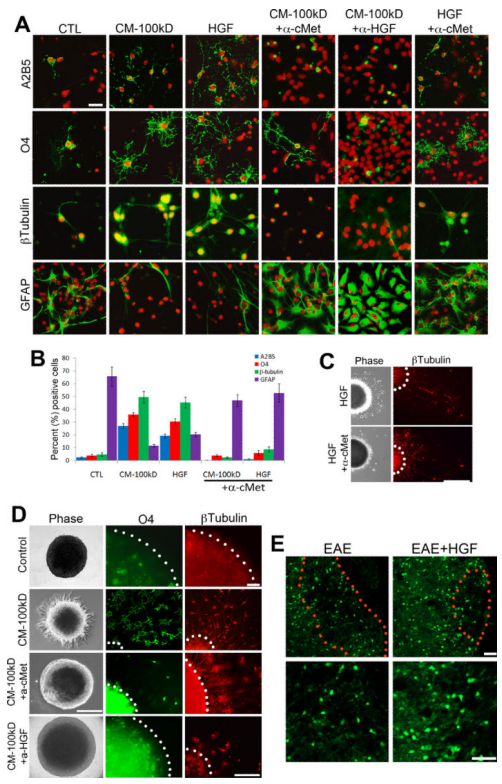

Figure 5.

Inhibition of HGF signaling with cMet or anti-HGF antibodies blocks the ability of MSC-CM_{100kD} and HGF to alter the development and migration of neural cells from neurospheres. **A**. The proportions of A2B5+, O4+, β tubulin + and GFAP+ cells are altered in the presence of MSC-CM_{100kD} and HGF and reversed by cMet and anti-HGF. **B**. Quantification of different cell types in the presence and absence of MSC-CM_{100kD} and HGF± cMet antibodies. Compared to controls the proportion of O4+ and β tubulin + cells is increased by MSC-CM and HGF ($p=<0.05$ for both) and blocked by cMet antibodies ($p=<0.01$ for both) **C**. Treatment with HGF stimulates migration of neuronal precursors from adult SVZ derived neurospheres of EAE animals and is blocked by cMet antibodies. **D**. Treatment with MSC-CM_{100kD} stimulates migration of OPCs and neuronal precursors from adult EAE SVZ derived neurospheres and is blocked by cMet and anti-HGF. **E**. The mobilization of PLP+ OPCs into EAE lesions is enhanced in animals treated with HGF. The number of EGFP-PLP cells which populate lesions increase following HGF treatment.. Bars = 20μm in A and 100μm in C and D. and 50μm in E. Data in B represent the mean±SD of the proportion of individual cell types taken from 5 random fields from at least 2 independent experiments. Lesion areas are outlined in E. Note that in B there is both unlabeled and overlap of antigen expression on individual cells.

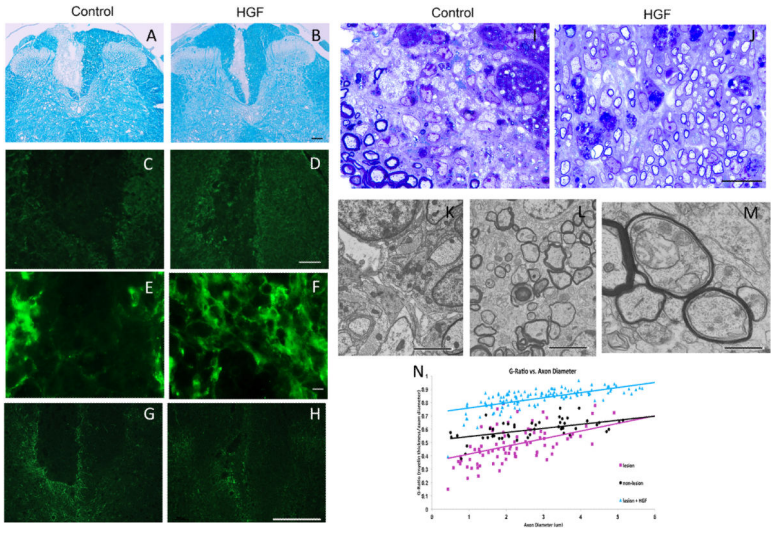

Figure 6.

Systemic HGF treatment stimulates remyelination of rat spinal cord LPC lesions. **A,B** Treatment with 0.8mg/Kg HGF on days 5,9 and 11 post lesion results in 30% smaller demyelinated lesions on 14dpl as shown by Luxol Fast Blue. Lesion volumes were calculated from serial sections through the lesion based on the equation for an elliptical cone (V=pi/3*a*b*h) and location of the lesion midpoint. C–H Immunohistochemical labeling of frozen sections demonstrated an increase in MBP labeling (**C,D**), increase in NG2+ cells (**E,F**) and a decrease in GFAP expression (**G,H**) in lesion areas from HGF treated animals at 14 days post lesion. **I–J** 1μm Toluidine Blue sections show extensive remyelination at 14 dpl in HGF treated animals but not in controls and ultrastructural analyses showed that axons of different caliber had myelin sheaths of different thicknesses indicative of ongoing repair in HGF (**K,L,M**) but not control lesions (**K**). **N** G ratios showing thin myelin sheaths in HGF treated animals.. Bars = A–F =100μm, G,H=50μm, I,J =5μm, K= 1μm, L=2μm, M= 0.5μm.