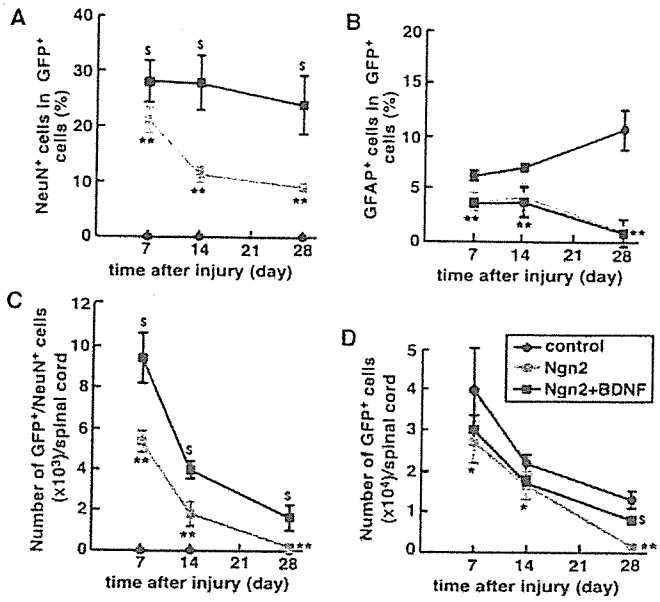


Figure 7. Survival of newly generated neurons in injured spinal cords. ***A–D***, Percentages of NeuN$^+$ (***A***) and GFAP$^+$ (***B***) cells among total GFP$^+$ cells, and estimated numbers of GFP$^+$/NeuN$^+$ (***C***) and total GFP$^+$ (***D***) cells were quantified at various time points after injury. Injured spinal cords were treated with GFs and control viruses (red lines), GFs and Ngn2 viruses (green), and GFs, BDNF, and Ngn2 viruses (blue). All data are mean ± SD (3–12 independent experiments; $^*p < 0.05$ and $^{**}p < 0.01$ compared with control virus infection; $^{\$}p < 0.01$ compared with Ngn2 virus infection alone).

example, GFP$^+$/NeuN$^+$ cells detected in the anterior horn were scattered within a cluster of large motor neurons and smaller interneurons, but their soma size (10–19 μm in diameter; 14.4 ± 3.3 μm; $n = 6$) was similar to that of the latter subtype (14.5 ± 3.7 μm; $n = 8$) (Fig. 6*F*). However, the morphology and location of individual GFP$^+$/NeuN$^+$ cells were highly variable depending on their relative distance from the lesion epicenter and also among treated animals. Moreover, none of these neurons expressed subtype-specific molecular markers examined such as HB9, Islet1, Lim1, and Lim3 (Yamamoto et al., 2001b and references therein), and therefore whether they differentiated into specific neuronal subtypes remained undetermined.

The coadministration of BDNF with GFs neither increased the percentage of GFP$^+$/HuC/D$^+$ cells compared with GF treatment alone, nor induced GFP$^+$/NeuN$^+$ cells in control virus-infected animals (no GFP$^+$/NeuN$^+$ cells among 652 GFP$^+$ cells examined). When combined with Ngn2 and GFs, however, BDNF significantly increased the percentage of GFP$^+$/NeuN$^+$ cells among total GFP$^+$ cells (28.2 ± 3.4%; $n = 3$ animals; $p < 0.01$ compared with animals without BDNF treatment) (Fig. 7*A*). Concomitant with this increase, the percentage of GFP$^+$/GFAP$^+$ cells was significantly lower in both Ngn2/GF- and Ngn2/GF/BDNF-treated animals compared with the control level (3.8 ± 0.9 and 3.7 ± 0.4% vs 6.3 ± 0.5%; $p < 0.01$) (Fig. 7*B*). This decrease alone, however, could not fully account for the much larger increase of GFP$^+$/NeuN$^+$ cells, suggesting that Ngn2 and BDNF did not simply inhibit gliogenesis, but rather actively promoted generation of neurons.

We further followed the survival of GFP$^+$/NeuN$^+$ cells *in vivo*. At DAI7, the estimated number of GFP$^+$/NeuN$^+$ neurons was 5.4 ± 0.5 × 10^3 ($n = 3$) per spinal cord in Ngn2 virus-infected/GF-treated animals (Fig. 7*C*). Their numbers, however, were only 33 and 3% at DAI14 and DAI28, respectively, compared with that detected at DAI7. Although the total number of GFP$^+$ cells decreased during this period (Fig. 7*D*), the percentage of NeuN$^+$ neurons among them also decreased over time (Fig. 7*A*). Thus, GFP$^+$/NeuN$^+$ new neurons appeared to be eliminated faster than other GFP$^+$ cell populations in injured tissue. Silencing of the GFP transgene could partly explain the observed loss of GFP-labeled new neurons (Vroemen et al., 2003). However, a higher percentage (33%) of control virus-infected cells, in which the fraction of new neurons was much smaller, survived up to DAI28. Furthermore, we observed longer survival of Ngn2 virus-infected cells in other parts of the CNS (our unpublished results). Thus, we favor the idea that the observed decrease reflected the actual loss of new neurons in injured spinal cords. Consistent with this idea, when the neurotrophic factor BDNF, which is thought to promote survival of neurons, increased the number of GFP$^+$/NeuN$^+$ cells 1.9-fold in Ngn2/GF-treated animals at DAI7 (9.4 ± 0.2 × 10^3; $n = 4$; $p < 0.001$ in two-tailed unpaired *t* test) (Fig. 7*C*). Moreover, larger numbers of GFP$^+$/NeuN$^+$ cells remained at DAI14 and DAI28 in BDNF-treated animals ($p < 0.0001$) (Fig. 7*C*). However, few GFP$^+$/NeuN$^+$ cells remained detectable at DAI56 or later time points (data not shown). Thus, the long-term survival of newly generated neurons appears to be very limited in the injured spinal cord.

Stimulation of oligodendrogenesis by Mash1

We next tested the effect of another proneural transcription factor, Mash1, which has been implicated in both neurogenesis and oligodendrogenesis during development (Parras et al., 2004). When NPCs were isolated as neurospheres from Mash1 virus-infected tissue, significantly higher percentages of Mash1-expressing cells differentiated into O4$^+$ and GalC$^+$ oligodendrocytes, and conversely, a much smaller fraction became GFAP$^+$ astrocytes compared with control virus-infected cells (Fig. 8*A*). Unlike Ngn2, Mash1 did not change the percentage of TuJ1$^+$ neurons among GFP$^+$ cells. Thus, Mash1 selectively increased oligodendrocytes in culture of adult spinal cord NPCs.

As described above, a substantial fraction of control virus-infected cells were GalC$^+$ *in vivo* (Fig. 4*I*). These results are consistent with previous studies in which production of new oligodendrocytes by NG2$^+$ cells was detected under various insult conditions (McTigue et al., 1998, 2001; Ishii et al., 2001; Watanabe et al., 2002, 2004; Talbott et al., 2005; Zai and Wrathall, 2005; Yang et al., 2006). In line with this, we found that some NG2$^+$ cells in injured tissue expressed endogenous Mash1 (Fig. 8*B*). This is in sharp contrast to endogenous Ngn2; we could not detect any cells expressing Ngn2 at any time point examined after injury (data not shown) (Yamamoto et al., 2001b). Such NG2$^+$/Mash1$^+$ cells, however, were small in number at DAI14, and almost disappeared at DAI28. These results raise the possibility that endogenous Mash1 is involved in the generation of new oligodendrocytes, but its limited expression accounts for their restricted generation and maturation in injured tissue.

To test this idea, we examined the effect of constitutive overexpression of Mash1 together with GF treatment *in vivo*. Consistent with the results of the above *in vitro* experiments, significantly larger fractions of Mash1 virus-infected cells became GalC$^+$ and GST-π$^+$ oligodendrocytes compared with control virus-infected cells (Fig. 8*C*,*F*). Over one-third (38.9 ± 7.2%; $n =$ 4 animals) of total Mash1-expressing cells were GST-π$^+$ at DAI7 (Fig. 8*F*). Because few GFP$^+$ cells expressed these markers at DAI3, these results suggest that Mash1 stimulated the production of new oligodendrocytes *in situ*. Furthermore, at DAI28, a small but significant fraction of GFP$^+$ cells expressed RIP (Fig. 8*D*) and PLP (Fig. 8*E*), markers for more mature, myelin-forming

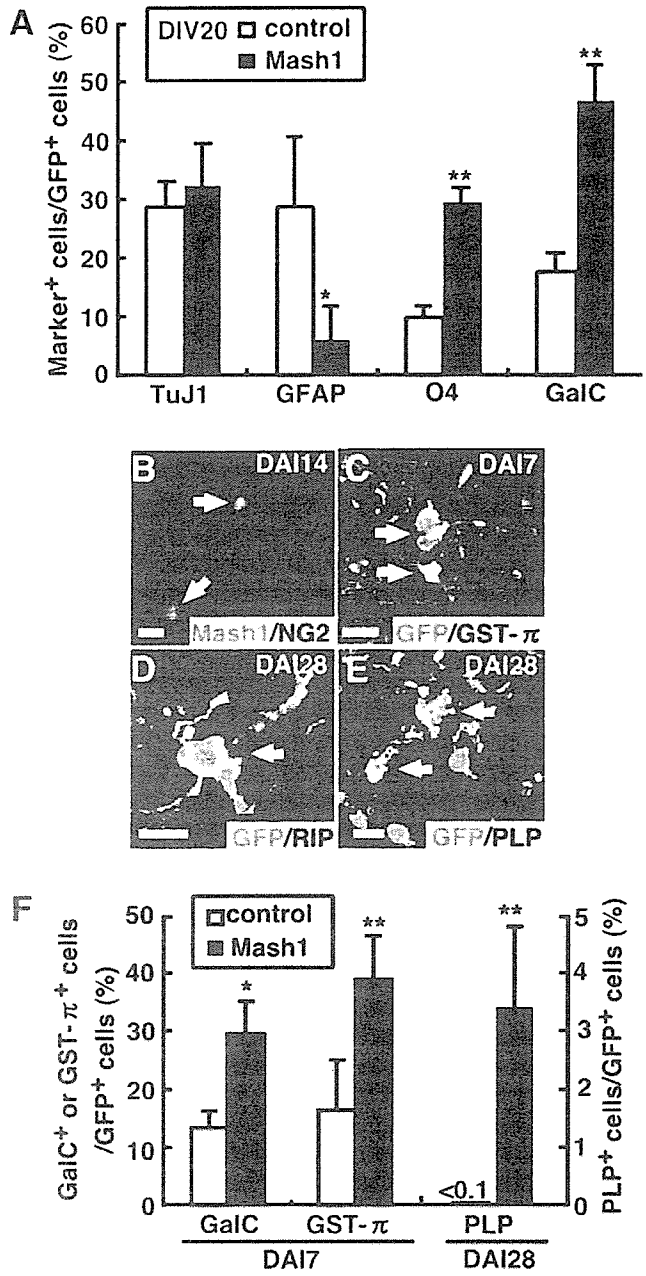


Figure 8. Stimulation of oligodendrocyte generation by Mash1. ***A***, Increased oligodendrocyte differentiation in Mash1-expressing neurosphere cells. Injured spinal cords infected with control (open bars) and Mash1 (filled bars) viruses were subjected to neurosphere culture at DAI3. Neurospheres formed at DIV14 were dissociated into single cells and induced to differentiate in monolayer for 6 d. The percentages of GFP$^+$ cells expressing respective neuronal and glial cell markers were quantified (mean ± SD; $n = 3$–6 animals; $^*p < 0.05$; $^{**}p < 0.01$ compared with control virus-infected cells). ***B***, Expression of endogenous Mash1 (green) in NG2$^+$ cells (red, indicated by arrow) *in vivo*. ***C–E***, Expression of the oligodendrocyte lineage cell markers GST-π (***C***), RIP (***D***), and PLP (***E***) (red) in Mash1 virus-infected, GFP$^+$ cells (green, indicated by arrows). ***F***, Stimulation of oligodendrocyte differentiation by Mash1. The percentages of GFP$^+$ cells expressing oligodendroglial markers in spinal cords infected with control (open bars) and Mash1 (filled bars) viruses were quantified at DAI7 or DAI28. GalC$^+$ cells were examined using dissociated single cells, whereas GST-π$^+$ and PLP$^+$ cells were detected in tissue sections. Data are mean ± SD ($n = 3$ animals; $^*p < 0.05$; $^{**}p < 0.01$ compared with control virus-infected cells). Scale bars: ***B***, ***E***, 20 μm; ***C***, ***D***, 10 μm.

cells, which were never detected in control virus-infected tissues (Fig. 8*F*). We detected $4.8 \pm 0.7 \times 10^4$ and $1.5 \pm 0.4 \times 10^4$ GFP$^+$ cells at DAI7 and DAI28, respectively, in animals treated with Mash1 viruses and GFs. The estimated number of GFP$^+$/GST-π$^+$ cells at DAI7 was, thus, 1.87×10^4 cells per spinal cord. Despite this relatively large number of immature cells detected early, only 2.7% of them appeared to advance to PLP$^+$ cells at DAI28 (510 GFP$^+$/PLP$^+$ cells per spinal cord). Moreover, GFP$^+$/PLP$^+$ and GFP$^+$/GST-π$^+$ cells were barely detectable at DAI56 and later time points (data not shown). Instead, the majority (50.8 ± 6.3%; $n = 3$ animals) of Mash1-expressing cells remained NG2$^+$ at DAI28. These results suggest that the major limiting step in regeneration of oligodendrocytes is the survival of immature cells and their maturation to myelin-forming cells.

Discussion

Spontaneous tissue regeneration after damage is very limited in the adult spinal cord. Many lines of recent studies have demonstrated that such limitation is attributable to, at least in part, restricted differentiation of endogenous NPCs *in vivo* (for review, see Q. Cao et al., 2002). In this study, we describe strategies to overcome such restriction.

Retrovirus-mediated genetic manipulation of NPCs *in situ*

We used GFP-expressing retroviruses to genetically manipulate proliferative cells in the injured spinal cord. We found that a fraction of virus-infected, GFP$^+$ cells grew as neurospheres and differentiated into neurons and glia in culture, demonstrating that they exhibited the properties of NPCs. Importantly, the majority (>80%) of GFP$^+$ cells that formed neurospheres were Olig2$^+$ and Nkx2.2$^+$, and ~30% of them were also NG2$^+$. Cells expressing these markers were also the predominant cell type among the whole neurosphere-forming cells derived from the injured spinal cord.

NG2$^+$ cells in the adult CNS have originally been thought to be glia-restricted progenitors (Horner et al., 2000; Dawson et al., 2003). Previous studies, however, have revealed that a subpopulation of NG2$^+$ cells in the adult forebrain possesses the ability to produce neurons (Belachew et al., 2003; Nunes et al., 2003). NG2$^+$ cells are also the major proliferative cells in the adult spinal cord (Ishii et al., 2001; McTigue et al., 2001; Watanabe et al., 2002, 2004; Talbott et al., 2005). In particular, Horky et al. (2006) have demonstrated previously that NG2$^+$ cells are the predominant cell type that divides early after injury. Other studies have shown that Olig2$^+$ and Nkx2.2$^+$ cells also comprise a significant fraction of proliferative cells, and that many of them coexpress NG2 (Yamamoto et al., 2001b; Watanabe et al., 2004; Talbott et al., 2005; Kitada and Rowitch, 2006). Consistent with these observations, GFP retroviruses administered immediately after injury preferentially infected Olig2$^+$/Nkx2.2$^+$/NG2$^+$ cells. Horky et al. (2006) also reported a similar result using a different virus construct and injury paradigm. Given the observation that a significant fraction of these cells differentiated into neurons or oligodendrocytes in GF-treated animals, these results suggest that they represent at least a part of endogenous NPCs in the adult spinal cord. We found, however, that only ~40% of NG2$^+$ cells coexpressed Olig2 and Nkx2.2 in injured tissue. Likewise, Olig2$^+$ and Nkx2.2$^+$ cells contain both NG2$^+$ and NG2$^-$ cell populations (Watanabe et al., 2004; Talbott et al., 2005). Thus, cells expressing these markers are heterogeneous, and neither of them appears to be specific for NPCs. Moreover, although the vast majority of GFP$^+$ cells were Olig2$^+$/Nkx2.2$^+$/NG2$^+$ *in vivo*, not all of these cells formed neurospheres *in vitro*. This could be because NPCs are only a fraction among cells expressing these markers, or alternatively, because currently available culture conditions do not support proliferation of all NPCs *in vitro*. More

studies are necessary to define the *in vivo* identity of NPCs in the adult spinal cord.

Overcoming environmental restriction by growth factors and genetic manipulations

Differentiation of NPCs into neurons and oligodendrocytes is tightly restricted by the environment in the injured spinal cord. Then, how do the manipulations described in this study overcome such restriction? First, it is unlikely that otherwise non-NPC cells transdifferentiated into NPC-like cells in response to exogenous manipulations. The molecular properties of the major fraction of GFP^+ cells early after infection were essentially identical between manipulated and unmanipulated tissues, and moreover, such phenotypes were preserved in GFP^+ cells-derived neurospheres. Thus, pre-existing, endogenous NPCs were likely responsible for generating new neurons and oligodendrocytes *in vivo*.

Previous studies reported various beneficial actions of GFs in spinal cord injury (Cheng et al., 1996; Lee et al., 1999; Teng et al., 1999; Rabchevsky et al., 2000; Kojima and Tator, 2002; Meijs et al., 2004). Their effects on neurogenesis by endogenous NPCs, however, have not yet been documented. We have demonstrated that direct administration of GFs into injured tissue can induce the production of new neurons in the otherwise non-neurogenic spinal cord. GFs increased the number of total GFP^+ cells *in situ*. GFs also increased the number of $GFP^+/TuJ1^+$ new neurons between DAI3 and DAI7. These results are consistent with the idea that GFs stimulated both proliferation and neuronal differentiation of endogenous NPCs. GFs might have enhanced survival of NPCs and newborn neurons as well. GFs act as mitogens for NPCs *in vitro* (Weiss et al., 1996; Kojima and Tator, 2002; Martens et al., 2002) and, thus, are generally thought to be inhibitory for their differentiation. Therefore, their neuron-inducing action *in vivo* is apparently puzzling. However, multiple extracellular molecules likely act simultaneously on NPCs *in vivo* so that the outcome of their combinatorial actions could be different from that observed *in vitro*. In fact, previous studies have shown that exogenous GFs can enhance neurogenesis after brain injury (Nakatomi et al., 2002; Teramoto et al., 2003). Our data, together with other previous studies, suggest that the induction of new neurons by GFs could be through interactions with multiple signaling pathways such as those for Notch, BMPs, and CNTF (Yamamoto et al., 2001b; Chojnacki et al., 2003; Mikami et al., 2004; Setoguchi et al., 2004). In this context, GFs could either directly act on NPCs, or indirectly modulate their activities through acting on other cell types such as inflammatory cells (Schwab, 2002; Hauben and Schwartz, 2003; Mikami et al., 2004; Yang et al., 2006). How GFs stimulate neurogenesis in the complex environment of injured tissue remains to be clarified.

Our data suggest that maturation is another limiting step in neuronal cell replacement in the injured spinal cord. Although a significant fraction of GFP^+ cells became HuC/D^+ cells in GF-treated animals, few cells were found to express NeuN that features a more mature phenotype of neurons. Although the mechanisms underlying this inhibition are currently unknown, we found that overexpression of Ngn2 can overcome this limiting step. Although Ngn2 alone strongly stimulated neurogenesis by NPCs *in vitro*, its effect on the production of HuC/D^+ immature neurons *in vivo* was rather weak in the absence of GFs. However, even without GFs, a small, but significant number of Ngn2-expressing cells became $NeuN^+$. Moreover, when combined with GFs, Ngn2 dramatically increased the number of $GFP^+/NeuN^+$ cells. Thus, the action of Ngn2 appeared to be distinct from that of GFs, and their combination was most effective in inducing neurogenesis *in vivo*.

In contrast, differentiation of GFP^+ cells into $GalC^+/GST\text{-}\pi^+$ immature oligodendrocytes was detectable even in GF-untreated animals. Yet, their maturation to MBP^+/PLP^+ myelin-forming cells did not occur at a detectable level. We showed that overexpression of Mash1 can enhance the production of $GalC^+/GST\text{-}\pi^+$ cells, and that at least some of these cells proceed to more mature PLP^+ oligodendrocytes. These results suggest that like neuronal cells, maturation and survival is a crucial step in replacement of oligodendrocytes in the injured spinal cord. This could be attributable to the absence of appropriate trophic support and/or the presence of cell death-inducing signals (Nakamura and Bregman, 2001; Velardo et al., 2004). Thus, a possible means to promote survival of new neurons and oligodendrocytes could be a sustained supply of neurotrophic factors and/or antagonists for cell death signals (McTigue et al., 1998; Lee et al., 1999; Liu et al., 1999; Namiki et al., 2000; Rabchevsky et al., 2000; Coumans et al., 2001; Meijs et al., 2004; Cao et al., 2005). Moreover, integration into the circuitry is probably important for their maturation and survival *in vivo* (Dobkin and Havton, 2004). Thus, strategies to enhance regeneration of these cells locally may need to be coordinated with those for reconstruction of long-range axonal tracts (Schwab, 2002; Silver and Miller, 2004).

Cell replacement strategies for spinal cord injury

In this study, we detected ~9400 $NeuN^+$ new neurons in Ngn2 virus/GF-treated animals. This level of neuronal cell replacement by endogenous NPCs is comparable with those reported for other parts of the CNS (Arvidsson et al., 2002; Nakatomi et al., 2002; Teramoto et al., 2003; Chmielnicki et al., 2004), and also to those achieved by grafting exogenous cells (Chow et al., 2000; Q. L. Cao et al., 2001, 2002; Hofstetter et al., 2005). Considering that our retrovirus-mediated method labeled only a small fraction of NPCs within tissue, the maximum neurogenic capacity of endogenous NPCs is likely larger than this level. However, poor long-term survival of new neurons is still the major issue common to the strategies using endogenous and exogenous NPCs. Thus, in terms of functional recovery, significance of supplying new neurons at this level of quantity remains to be explored. In case of transplantation of exogenous NPCs, many cell types other than neurons are supplied to lesions, which, as a whole, exert beneficial effects (Lu et al., 2003; Hofstetter et al., 2005). Under certain circumstances, grafted cells appear to exert detrimental effects as well (Enzmann et al., 2005; Hofstetter et al., 2005). Similar situations may also need to be considered in case of mobilizing endogenous NPCs by growth factor treatment and genetic manipulations. Additional improvement of such strategies may lead to development of novel cell replacement therapy for spinal cord injury.

References

Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A (2003) Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 425:968–973.

Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O (2002) Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 8:963–970.

Belachew S, Chittajallu R, Aguirre AA, Yuan X, Kirby M, Anderson S, Gallo V (2003) Postnatal NG2 proteoglycan-expressing progenitor cells are intrinsically multipotent and generate functional neurons. J Cell Biol 161:169–186.

Cao Q, Benton RL, Whittemore SR (2002) Stem cell repair of central nervous system injury. J Neurosci Res 68:501–510.

Cao Q, Xu XM, DeVries WH, Enzmann GU, Ping P, Tsoulfas P, Wood PM, Bunge MB, Whittemore SR (2005) Functional recovery in traumatic spinal cord injury after transplantation of multineurotrophin-expressing glial-restricted precursor cells. J Neurosci 25:6947–6957.

Cao QL, Zhang YP, Howard RM, Walters WM, Tsoulfas P, Whittemore SR (2001) Pluripotent stem cells engrafted into the normal or lesioned adult rat spinal cord are restricted to a glial lineage. Exp Neurol 167:48–58.

Cao QL, Howard RM, Dennison JB, Whittemore SR (2002) Differentiation of engrafted neuronal-restricted precursor cells is inhibited in the traumatically injured spinal cord. Exp Neurol 177:349–359.

Chen J, Leong SY, Schachner M (2005) Differential expression of cell fate determinants in neurons and glial cells of adult mouse spinal cord after compression injury. Eur J Neurosci 22:1895–1906.

Cheng H, Cao Y, Olson L (1996) Spinal cord repair in adult paraplegic rats: partial restoration of hind limb function. Science 273:510–513.

Chmielnicki E, Benraiss A, Economides AN, Goldman SA (2004) Adenovirally expressed noggin and brain-derived neurotrophic factor cooperate to induce new medium spiny neurons from resident progenitor cells in the adult striatal ventricular zone. J Neurosci 24:2133–2142.

Chojnacki A, Shimazaki T, Gregg C, Weinmaster G, Samuel Weiss S (2003) Glycoprotein 130 signaling regulates notch1 expression and activation in the self-renewal of mammalian forebrain neural stem cells. J Neurosci 23:1730–1741.

Chow SY, Moul J, Tobias CA, Himes BT, Liu Y, Obrocka M, Hodge L, Tessler A, Fischer I (2000) Characterization and intraspinal grafting of EGF/bFGF-dependent neurospheres derived from embryonic rat spinal cord. Brain Res 874:87–106.

Coumans JV, Lin TT, Dai HN, MacArthur L, McAtee M, Nash C, Bregman BS (2001) Axonal regeneration and functional recovery after complete spinal cord transection in rats by delayed treatment with transplants and neurotrophins. J Neurosci 21:9334–9344.

Dawson MR, Polito A, Levine JM, Reynolds R (2003) NG2-expressing glial progenitor cells: an abundant and widespread population of cycling cells in the adult rat CNS. Mol Cell Neurosci 24:476–488.

Dobkin BH, Havton LA (2004) Basic advances and new avenues in therapy of spinal cord injury. Annu Rev Med 55:255–282.

Enzmann GU, Benton RL, Woock JP, Howard RM, Tsoulfas P, Whittemore SR (2005) Consequences of noggin expression by neural stem, glial, and neuronal precursor cells engrafted into the injured spinal cord. Exp Neurol 195:293–304.

Goldman SA (2004) Directed mobilization of endogenous neural progenitor cells: the intersection of stem cell biology and gene therapy. Curr Opin Mol Ther 6:466–472.

Han SS, Kang DY, Mujtaba T, Rao MS, Fischer I (2002) Grafted lineage-restricted precursors differentiate exclusively into neurons in the adult spinal cord. Exp Neurol 177:360–375.

Han SS, Liu Y, Tyler-Polsz C, Rao MS, Fischer I (2004) Transplantation of glial-restricted precursor cells into the adult spinal cord: survival, glial-specific differentiation, and preferential migration in white matter. Glia 45:1–16.

Hauben E, Schwartz M (2003) Therapeutic vaccination for spinal cord injury: helping the body to cure itself. Trends Pharmacol Sci 24:7–12.

Hill CE, Proschel C, Noble M, Mayer-Proschel M, Gensel JC, Beattie MS, Bresnahan JC (2004) Acute transplantation of glial-restricted precursor cells into spinal cord contusion injuries: survival, differentiation, and effects on lesion environment and axonal regeneration. Exp Neurol 190:289–310.

Hofstetter CP, Holmstrom NA, Lilja JA, Schweinhardt P, Hao J, Spenger C, Wiesenfeld-Hallin Z, Kurpad SN, Frisen J, Olson L (2005) Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. Nat Neurosci 8:346–353.

Horky LL, Galimi F, Gage FH, Horner PJ (2006) Fate of endogenous stem/progenitor cells following spinal cord injury. J Comp Neurol 498:525–538.

Horner PJ, Gage FH (2000) Regenerating the damaged central nervous system. Nature 407:963–970.

Horner PJ, Power AE, Kempermann G, Kuhn HG, Palmer TD, Winkler J, Thal LJ, Gage FH (2000) Proliferation and differentiation of progenitor cells throughout the intact adult rat spinal cord. J Neurosci 20:2218–2228.

Ishii K, Toda M, Nakai Y, Asou H, Watanabe M, Nakamura M, Yato Y, Fujimura Y, Kawakami Y, Toyama Y, Uyemura K (2001) Increase of oligodendrocyte progenitor cells after spinal cord injury. J Neurosci Res 65:500–507.

Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisen J (1999) Identification of a neural stem cell in the adult mammalian central nervous system. Cell 96:25–34.

Kamakura S, Oishi K, Yoshimatsu T, Nakafuku M, Masuyama N, Gotoh Y (2004) Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. Nat Cell Biol 6:547–554.

Kitada M, Rowitch DH (2006) Transcription factor co-expression patterns indicate heterogeneity of oligodendroglial subpopulations in the adult spinal cord. Glia 54:35–46.

Kojima A, Tator CH (2002) Intrathecal administration of epidermal growth factor and fibroblast growth factor 2 promotes ependymal proliferation and functional recovery after spinal cord injury in adult rats. J Neurotrauma 19:223–238.

Leber SM, Sanes JR (1991) Lineage analysis with a recombinant retrovirus: application to chick spinal motor neurons. Adv Neurol 56:27–36.

Lee TT, Green BA, Dietrich WD, Yezierski RP (1999) Neuroprotective effects of basic fibroblast growth factor following spinal cord contusion injury in the rat. J Neurotrauma 16:347–356.

Lim DA, Tramontin AD, Trevejo JM, Herrera DG, Garcia-Verdugo JM, Alvarez-Buylla A (2000) Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. Neuron 28:713–726.

Liu Y, Kim D, Himes BT, Chow SY, Schallert T, Murray M, Tessler A, Fischer I (1999) Transplants of fibroblasts genetically modified to express BDNF promote regeneration of adult rat rubrospinal axons and recovery of forelimb function. J Neurosci 19:4370–4387.

Lu P, Jones LL, Snyder EY, Tuszynski MH (2003) Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Exp Neurol 181:115–129.

Martens DJ, Seaberg RM, van der Kooy D (2002) In vivo infusions of exogenous growth factors into the fourth ventricle of the adult mouse brain increase the proliferation of neural progenitors around the fourth ventricle and the central canal of the spinal cord. Eur J Neurosci 16:1045–1057.

McTigue DM, Horner PJ, Stokes BT, Gage FH (1998) Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. J Neurosci 18:5354–5365.

McTigue DM, Wei P, Stokes BT (2001) Proliferation of NG2-positive cells and altered oligodendrocyte numbers in the contused rat spinal cord. J Neurosci 21:3392–3400.

Meijs MF, Timmers L, Pearse DD, Tresco PA, Bates ML, Joosten EA, Bunge MB, Oudega M (2004) Basic fibroblast growth factor promotes neuronal survival but not behavioral recovery in the transected and Schwann cell implanted rat thoracic spinal cord. J Neurotrauma 21:1415–1430.

Mikami Y, Okano H, Sakaguchi M, Nakamura M, Shimazaki T, Okano HJ, Kawakami Y, Toyama Y, Toda M (2004) Implantation of dendritic cells in injured adult spinal cord results in activation of endogenous neural stem/progenitor cells leading to de novo neurogenesis and functional recovery. J Neurosci Res 76:453–465.

Mocchetti I, Rabin SJ, Colangelo AM, Whittemore SR, Wrathall JR (1996) Increased basic fibroblast growth factor expression following contusive spinal cord injury. Exp Neurol 141:154–164.

Morita S, Kojima T, Kitamura T (2000) Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther 7:1063–1066.

Nakamura M, Bregman BS (2001) Differences in neurotrophic factor gene expression profiles between neonate and adult rat spinal cord after injury. Exp Neurol 169:407–415.

Nakashima K, Takizawa T, Ochiai W, Yanagisawa M, Hisatsune T, Nakafuku M, Miyazono K, Kishimoto T, Kageyama R, Taga T (2001) BMP2-mediated alteration in the developmental pathway of fetal mouse brain cells from neurogenesis to astrocytogenesis. Proc Natl Acad Sci USA 98:5868–5873.

Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M (2002) Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell 110:429–441.

Namiki J, Kojima A, Tator CH (2000) Effect of brain-derived neurotrophic factor, nerve growth factor, and neurotrophin-3 on functional recovery and regeneration after spinal cord injury in adult rats. J Neurotrauma 17:1219–1231.

Nunes MC, Roy NS, Keyoung HM, Goodman RR, McKhann G II, Jiang L,

Kang J, Nedergaard M, Goldman SA (2003) Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. Nat Med 9:439–447.

Parras CM, Galli R, Britz O, Soares S, Galichet C, Battiste J, Johson JE, Nakafuku M, Vescovi A, Guillemot F (2004) Mash1 specifies neurons and oligodendrocytes in the postnatal brain. EMBO J 23:4495–4505.

Rabchevsky AG, Fugaccia I, Turner AF, Blades DA, Mattson MP, Scheff SW (2000) Basic fibroblast growth factor (bFGF) enhances functional recovery following severe spinal cord injury to the rat. Exp Neurol 164:280–291.

Schwab ME (2002) Repairing the injured spinal cord. Science 295:1029–1031.

Setoguchi T, Yone K, Matsuoka E, Takenouchi H, Nakashima K, Sakou T, Komiya S, Izumo S (2001) Traumatic injury-induced BMP7 expression in the adult rat spinal cord. Brain Res 921:219–225.

Setoguchi T, Nakashima K, Takizawa T, Yanagisawa M, Ochiai W, Okabe M, Yone K, Komiya S, Taga T (2004) Treatment of spinal cord injury by transplantation of fetal neural precursor cells engineered to express BMP inhibitor. Exp Neurol 189:33–44.

Shihabuddin LS, Horner PJ, Ray J, Gage FH (2000) Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. J Neurosci 20:8727–8735.

Silver J, Miller JH (2004) Regeneration beyond the glial scar. Nat Rev Neurosci 5:146–156.

Sun Y, Nadal-Vicens M, Misono S, Lin MZ, Zubiaga A, Hua X, Fan G, Greenberg ME (2001) Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell 104:365–376.

Talbott JF, Loy DN, Liu Y, Qiu MS, Bunge MB, Rao MS, Whittemore SR (2005) Endogenous Nkx2.2+/Olig2+ oligodendrocyte precursor cells fail to remyelinate the demyelinated adult rat spinal cord in the absence of astrocytes. Exp Neurol 192:11–24.

Teng YD, Mocchetti I, Taveira-DaSilva AM, Gillis RA, Wrathall JR (1999) Basic fibroblast growth factor increases long-term survival of spinal motor neurons and improves respiratory function after experimental spinal cord injury. J Neurosci 19:7037–7047.

Teramoto T, Qiu J, Plumier JC, Moskowitz MA (2003) EGF amplifies the replacement of parvalbumin-expressing striatal interneurons after ischemia. J Clin Invest 111:1125–1132.

Torii M, Matsuzaki F, Osumi N, Kaibuchi K, Nakamura S, Casarosa S, Guillemot F, Nakafuku M (1999) Transcription factors Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system. Development 126:443–456.

Velardo MJ, Burger C, Williams PR, Baker HV, Lopez MC, Mareci TH, White TE, Muzyczka N, Reier PJ (2004) Patterns of gene expression reveal a temporally orchestrated wound healing response in the injured spinal cord. J Neurosci 24:8562–8576.

Vroemen M, Aigner L, Winkler J, Weidner N (2003) Adult neural progenitor cell grafts survive after acute spinal cord injury and integrate along axonal pathways. Eur J Neurosci 18:743–751.

Watanabe M, Toyama Y, Nishiyama A (2002) Differentiation of proliferated NG2-positive glial progenitor cells in a remyelinating lesion. J Neurosci Res 69:826–836.

Watanabe M, Hadzic T, Nishiyama A (2004) Transient upregulation of Nkx2.2 expression in oligodendrocyte lineage cells during remyelination. Glia 46:311–322.

Weiss S, Dunne C, Hewson J, Wohl C, Wheatley M, Peterson AC, Reynolds BA (1996) Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. J Neurosci 16:7599–7609.

Yamamoto S, Yamamoto N, Kitamura T, Nakamura K, Nakafuku M (2001a) Proliferation of parenchymal neural progenitors in response to injury in the adult rat spinal cord. Exp Neurol 172:115–127.

Yamamoto S, Nagao M, Sugimori M, Kosako H, Nakatomi H, Yamamoto N, Takebayashi H, Nabeshima Y, Kitamura T, Weinmaster G, Nakamura K, Nakafuku M (2001b) Transcription factor expression and Notch-dependent regulation of neural progenitors in the adult rat spinal cord. J Neurosci 21:9814–9823.

Yang H, Lu P, McKay HM, Bernot T, Keirstead H, Steward O, Gage FH, Edgerton VR, Tuszynski MH (2006) Endogenous neurogenesis replaces oligodendrocytes and astrocytes after primate spinal cord injury. J Neurosci 26:2157–2166.

Zai LJ, Wrathall JR (2005) Cell proliferation and replacement following contusive spinal cord injury. Glia 50:247–257.

歩行困難者への工学的支援

Assistive technology for locomotor disabilities

キーワード：脊髄損傷、歩行、二次障害、歩行訓練機
Keywords ：Spinal cord injury, locomotion, secondary disorder, gait trainer

中澤 公孝[1)]
Kimitaka Nakazawa

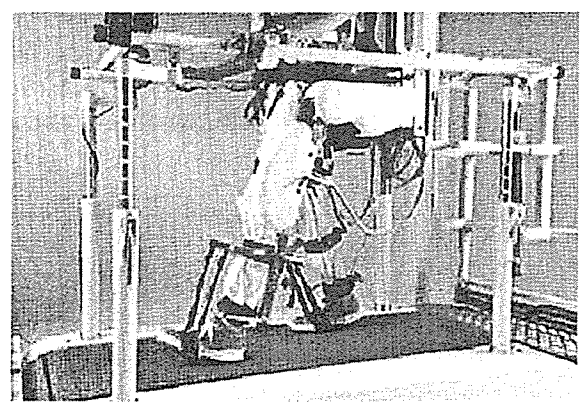
歩行困難者、中でも脊髄損傷者の歩行リハビリテーション理論は過去10年余りで大きく変貌した。それは脊髄の可塑的性質の発見に代表されるように、周辺の神経生理学的研究が急速に発展したことによるところが大きい。リハビリテーションの理論的枠組みの転換は、関連する機器開発にも反映され、従来とは異なったさまざまな装置が開発されるようになった。本稿では、近年筆者らのグループが開発した歩行訓練装置の基本コンセプトとそれが構築されるにいたった理論的背景を紹介しながら、この分野の新しい動向を解説したい。

筆者ら国立身体障害者リハビリテーションセンターと東京農工大、芝浦工大の研究グループは近年、動力付き歩行装具と免荷装置から成る移動型の歩行訓練装置を開発した。この装置は、現在のところ自立歩行が困難な後期高齢者や股関節術後患者を主たる対象としているが、近い将来、対麻痺者や片麻痺者の歩行リハビリテーションへとその用途を広げる予定である。以下ではこの装置の基本コンセプトの理論的背景、開発要件ならびに装置の概要について紹介する。

理論的背景

神経生理学的視点

本装置の理論的背景の第一は、1990年代中盤以降、主に脊髄損傷のニューロリハビリテーションとして考案された受動歩行トレーニング理論にある。受動歩行トレーニングでは、トレッドミル上で訓練者の下肢を理学療法士あるいは最近ではロボットがベルトスピードに合わせてステッピングさせる（図1）。それによって下肢の感覚受容器が刺激され、脊髄への感覚入力が喚起される。ステッピングにともなってパターン化した感覚入力が脊髄の歩行パターンジェネレーターおよび脊髄より上位の中枢神経を賦活する。それが繰り返し行われることで中枢神経系の再組織化が促進される[1)]。この時、不全脊髄損傷者のように上位中枢から脊髄運動ニューロンへの入力がわずかでも残っていると脊髄での伝達効率が改善され、自立歩行回復の可能性が高くなる。これが受動歩行トレーニングの理論である。



ロボット型歩行訓練機Lokomat

図1

1）国立身体障害者リハビリテーションセンター　Research Institute National Rehabilitation Center for Persons with Disabilities

我々は、受動歩行トレーニングのポイントを次の２点に集約し、新たな歩行訓練装置の要件とした。それは、①受動歩行あるいは半受動歩行による末梢感覚入力の喚起、および②脳からの下行性司令の確保、である。それぞれについて以下で詳しく説明する。

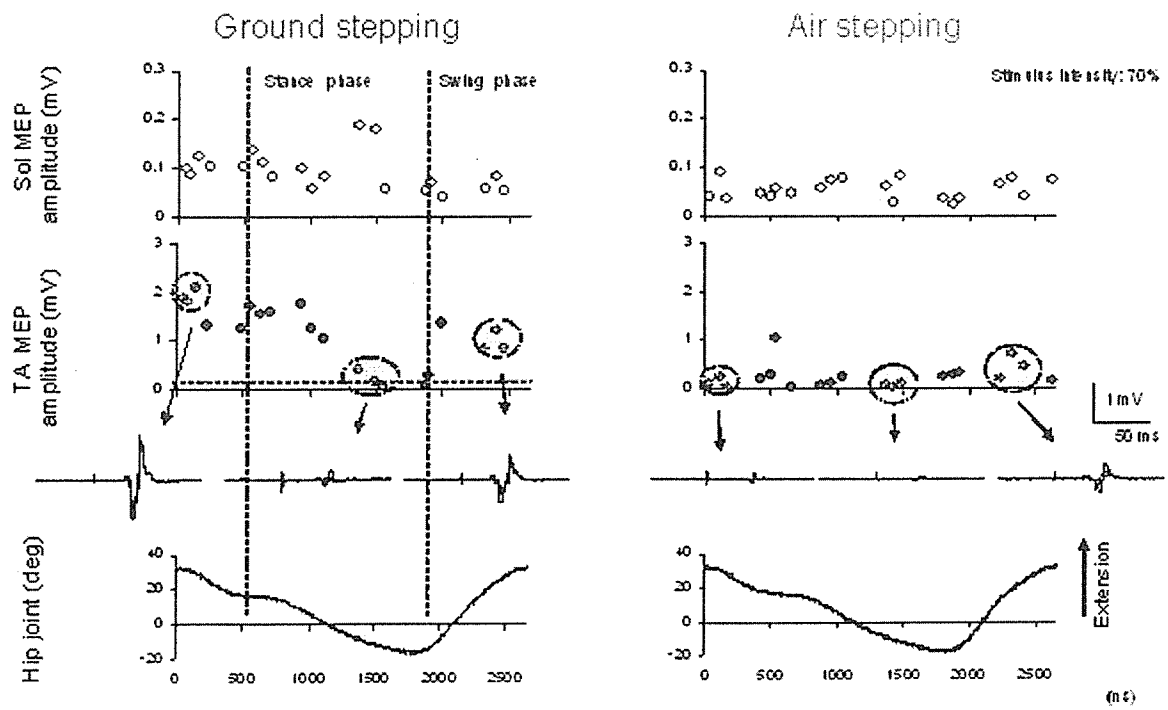
①　受動歩行の効果

対麻痺患者であっても下肢を受動的に動かしステッピングを行うと下肢の各筋群にステッピングと同調した筋活動が誘発される。これは下肢のステッピングに伴う感覚入力が脊髄の歩行パターンジェネレーターおよびその他の歩行に関連する神経回路を介し反射性出力を誘発した結果生じると考えられている。受動歩行トレーニングを続けると下肢の麻痺筋群に誘発されるそれらの筋放電が増大するとともに筋間の放電パターンも改善することが知られている。筆者らはさらにロボット型歩行訓練機を用いた実験によって、受動的ステッピングを行うと足関節屈筋である前脛骨筋の皮質脊髄路興奮性が増大することを見出した（図２）。この筋はそもそも皮質との結合が強く、足関節伸筋のヒラメ筋に比べて歩行中の皮質脊髄路興奮性が高いことが知られている[2) 3)]。今回の筆者らの結果は、随意歩行に似た下肢の動きを他動的に与えるだけで、皮質脊髄路の興奮性が増強することを示している。それはさらに他動的ステッピングを長期的に行うこと、あるいは下行性司令が加わることによってこの経路の再組織化が起こることも示唆する結果といえる。

以上を踏まえ、今回開発する機器には受動的ステッピング動作を実現する機構を組み込むこととした。

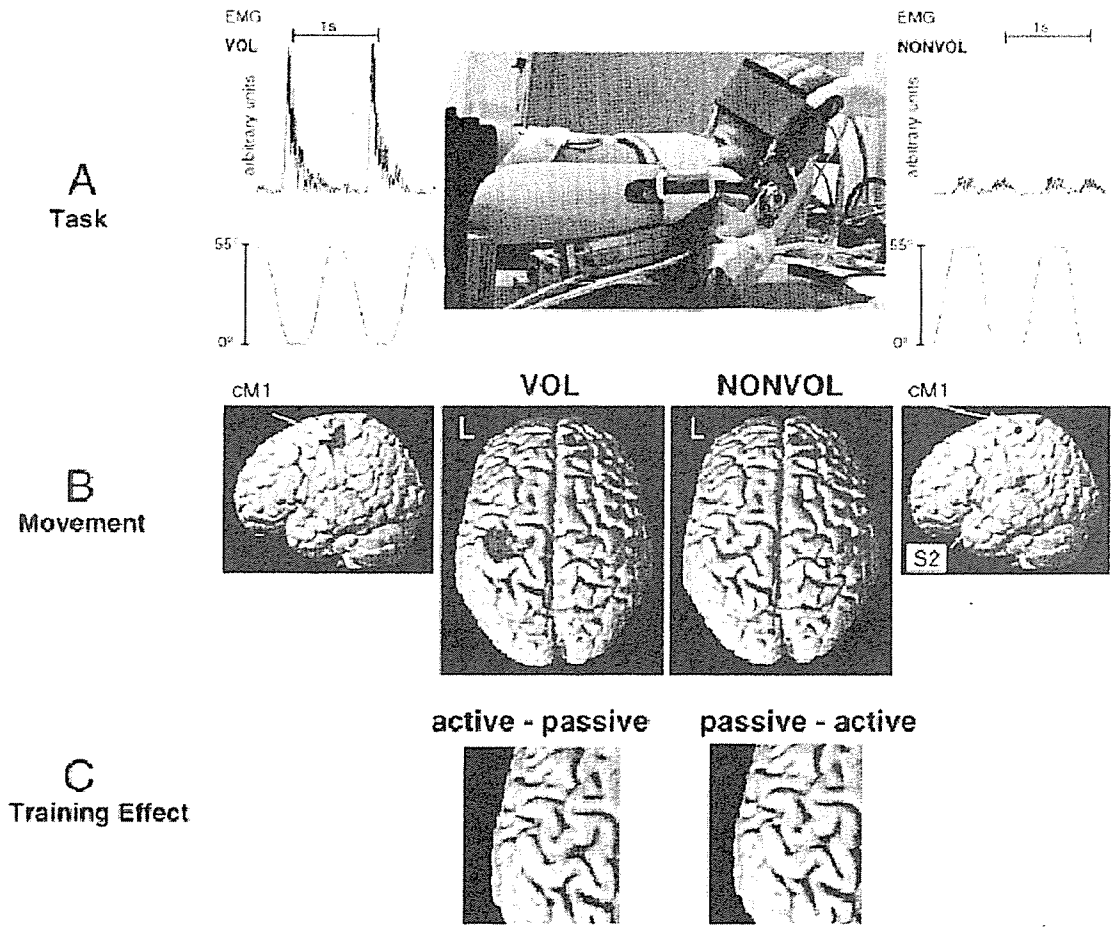
②　下行性司令の効果について

前述したように免荷式受動歩行トレーニングは不全脊髄損傷者の歩行能力回復に効果がある。逆に言えば脳と脊髄との結合が完全に遮断された完全脊髄損傷者では効果が見込めない。自立歩行の回復にはわずかであっても脳から脊髄への下行性入力の存在が必要なのである。最近のThomas and Gorasini（2005）[4)]の報告によれば不全脊髄損傷者の皮質脊髄路機能は歩行トレーニングで改善する。またLotze ら（2003）[5)]は受動運動トレーニングと随意司令を要する随意運動トレーニングを比較し、後者の方が大脳一次運動野の再組織化が著明であることを示した（図３）。この結果は、随意性の低い麻痺肢であっても、運動を起こそうとする意志が運動機能の回復



受動歩行中に経頭蓋磁気刺激によって誘発したヒラメ筋（SOL）と前脛骨筋（TA）の誘発電位（MEP）の変化。Ground stepping；トレッドミルベルト上でのステッピング、Air stepping；空中に吊られた状態でのステッピング。

図２



手関節の受動トレーニング（NONVOL）と能動トレーニング（VOL）による脳の再組織化の比較。A:動作課題特性の比較。VOLでは筋活動電位が発生するのに対し、NONVOLではほとんど生じていない。B:それぞれの運動課題で活性化する脳の領域の違い。C:トレーニング後に生じる活性領域の違い。文献5より引用。

図3

には重要であることを示唆している。すなわち、不全脊髄損傷者の受動歩行トレーニングにおいて、ただ他動的に下肢を動かされているのに比べて、自分の意志の下に動かそうとする随意司令がきわめて重要であることが示唆される。随意司令を引き出すためには、強い動機付けが必要である。この動機付けのため、今回の歩行訓練機は自分の意志の下で移動できることを重要な要件の一つとした。

体力医学的視点

上記したポイントは歩行回復にかかわる神経生理学的視点であった。もう一方の視点は体力医学的視点である。

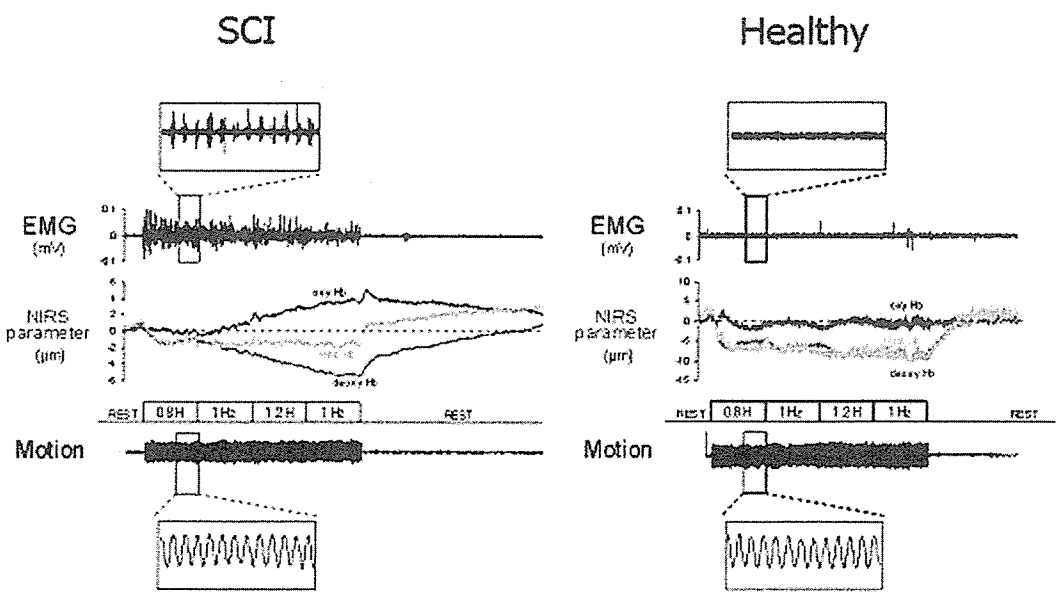
前述したように、上位中枢と脊髄との連絡が完全に遮断された完全損傷者では現状で理論的には自立歩行の改善は期待できない。そのため、完全損傷者にとって歩行機能回復を目指した受動歩行トレーニングを行うことの臨床的な意義は無い。しかし、立位での受動運動は麻痺領域の血液循環を促進するなど、神経系以外の運動に関連する諸器官の刺激効果にも優れる。筆者らは、歩行装具を用いた装具歩行トレーニング実験の結果から対麻痺者が立位で運動を行うことのいくつかの利点を知るに至った。例えば、装具歩行中の心拍数は150拍程度、酸素摂取量は16ml/kg/min程度となり[6]、これらの数値は座位で腕エルゴメーターをオールアウトまで行ったときの最大値で相対化するといずれも80%以上の数値となる。これは上肢の運動では心臓循環系を十分に働かせるに至っていないこと、すなわち最大心拍数と最大酸素摂

取量を過小評価していることを意味する。歩行装具を用いた立位歩行は体幹の筋など残余機能を十分に賦活させ、結果として心臓循環系、呼吸系へのトレーニングに適した負荷を与えることが容易になると考えられる。筆者らはまた、装具歩行や受動的ステッピングにおいて下肢の麻痺筋群に運動周期に一致した筋活動が誘発されることに着目し、それらの筋活動によって筋の末梢循環や代謝レベルが変化するのかどうかを調べた[7]。実験には立位歩行を模した受動的な下肢の左右交互性スウィング運動を用いた。この運動においても下肢麻痺領域に筋活動が誘発されることを確認した。実験では近赤外分光法を用い下腿三頭筋の酸素化レベルを定量化した。その結果、健常者の場合と異なり、対麻痺者では受動運動の結果、当該筋の酸素化レベルが大きく変化し、末梢循環が変調したことが示唆された（図４）。麻痺領域の末梢循環は普段の生活ではほとんど変化しないことが予想されることから、たとえ受動的運動であってもこれを促進することの意味は大きいと思われる。

脊髄損傷者では麻痺領域の不使用に伴う諸種の二次的障害が深刻な問題となる。その意味で、立位での受動運動はその予防に有効であるといえる。この点は完全損傷、不全損傷を問わない。したがって、脊髄完全損傷者にとっても立位運動を行うことは十分な臨床的意義があるといえる。本装置の理論的背景の第二のポイントがここにある。

以上の神経生理学的、体力医学的知見を踏まえ、本装置の要件を以下のように設定した。

1．下肢のステッピング運動を装置によって実現可能とする。対麻痺者など自力での歩行が困難な人に対して受動的ステッピング運動を実現し、歩行の再学習、神経の再組織化を促す。
2．移動型免荷装置によって訓練者の意志に基づく移動を可能にする。それによって訓練者の動機付け、トレーニング意欲を高める。上位中枢からの下行性指令を可能な限り高めることで脊髄への伝達効率を改善する。同時に残余機能を最大限動員することで呼吸循環系をはじめとする全身諸機関への刺激効果を高める。



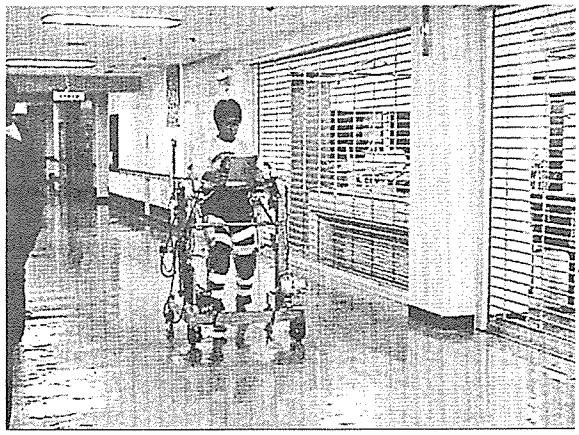
立位での受動的股関節屈曲伸展動作中の下腿三頭筋酸素化レベル。脊髄損傷者（SCI）と健常者（NORMAL）でのデータの比較。SCIでは動作中（Motion）に筋放電（EMG）が誘発され、近赤外分光法で計測した酸素化ヘモグロビンなどの変量（NIRS parameters）が変化していることがわかる。文献７より引用

図４

歩行訓練装置の概要

装置の基本構成はモーターで駆動される歩行装具部と身体を支え、あわせて免荷もおこなう免荷機構部から成る（図５）。歩行装具部は訓練者の状態、下肢のサイズに応じたステッピングを行う。免荷機構部は最大で約50kgの免荷をおこなうとともに、26 m/mの速度での移動を可能とする。

既に屋外での動作確認を行い、上記要件を満たす装置の試作機が完成した。今後さらに細部を改良し、臨床試験を行う予定である。



移動型歩行訓練装置を用いた試験歩行の様子

図５

参考文献

1）Raineteau O, Schwab ME. Plasticity of motor systems after incomplete spinal cord injury. Nature Rev 2: 263-273, 2001
2）Capaday C, Lavoie BA, Barbeau H, Schneider C, Bonnard M (1999) : Studies on the corticospinal control of human walking. I. Responses to focal transcranial magnetic stimulation of the motor cortex. J Neurophysiol 81: 129-139
3）Christensen LO, Andersen JB, Sinkiaer T, Nielsen J (2001) : Transcranical magnetic stimulation and stretch reflexes in the tibialis anterior muscle during human walking. J Physiol 531: 545-557
4）Thomas S and M A Gorassini: Increases in corticospinal tract function by treadmill training. J Neurophysiol 94:2844-2855, 2005
after incomplete spinal cord injury
5）Lotze M, Braun C, Birbaumer N, Anders S, Cohen LG: Motor learning elicited by voluntary drive. Brain 126: 866-872, 2003
6）Kawashima N, Sone Y, Nakazawa K, Akai M, Yano H: Energy expenditure during walking with weight-bearing control (WBC) orthosis in thoracic level of paraplegic patients. Spinal Cord 41, 506-510, 2003
7）Kawashima K, Nakazawa K, Akai M. Muscle oxygenation of the paralyzed lower limb in spinal cord injured persons. Med Sci Sport Exerc 37, 915-921, 2005.