

**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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells (green, indicated by arrows). *F*, Stimulation of oligodendrocyte differentiation by Mash1. The percentages of GFP<sup>+</sup> cells expressing oligodendroglial markers in spinal cords infected with control (open bars) and Mash1 (filled bars) viruses were quantified at DAI7 or DAI28. GalC<sup>+</sup> cells were examined using dissociated single cells, whereas GST-π<sup>+</sup> and PLP<sup>+</sup> 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<sup>+</sup> cells at DAI7 and DAI28, respectively, in animals treated with Mash1 viruses and GFs. The estimated number of GFP<sup>+</sup>/

GST-π<sup>+</sup> cells at DAI7 was, thus,  $1.87 \times 10^4$  cells per spinal cord. Despite this relatively large number of immature cells detected early, only 2.7% of them appeared to advance to PLP<sup>+</sup> cells at DAI28 (510 GFP<sup>+</sup>/PLP<sup>+</sup> cells per spinal cord). Moreover, GFP<sup>+</sup>/PLP<sup>+</sup> and GFP<sup>+</sup>/GST-π<sup>+</sup> cells were barely detectable at DAI56 and later time points (data not shown). Instead, the majority ( $50.8 \pm 6.3\%$ ; *n* = 3 animals) of Mash1-expressing cells remained NG2<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells that formed neurospheres were Olig2<sup>+</sup> and Nkx2.2<sup>+</sup>, and ~30% of them were also NG2<sup>+</sup>. Cells expressing these markers were also the predominant cell type among the whole neurosphere-forming cells derived from the injured spinal cord.

NG2<sup>+</sup> 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<sup>+</sup> cells in the adult forebrain possesses the ability to produce neurons (Belachew et al., 2003; Nunes et al., 2003). NG2<sup>+</sup> 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<sup>+</sup> cells are the predominant cell type that divides early after injury. Other studies have shown that Olig2<sup>+</sup> and Nkx2.2<sup>+</sup> 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<sup>+</sup>/Nkx2.2<sup>+</sup>/NG2<sup>+</sup> 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<sup>+</sup> cells coexpressed Olig2 and Nkx2.2 in injured tissue. Likewise, Olig2<sup>+</sup> and Nkx2.2<sup>+</sup> cells contain both NG2<sup>+</sup> and NG2<sup>-</sup> 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<sup>+</sup> cells were Olig2<sup>+</sup>/Nkx2.2<sup>+</sup>/NG2<sup>+</sup> *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<sup>+</sup> cells early after infection were essentially identical between manipulated and unmanipulated tissues, and moreover, such phenotypes were preserved in GFP<sup>+</sup> 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<sup>+</sup> cells *in situ*. GFs also increased the number of GFP<sup>+</sup>/TuJ1<sup>+</sup> 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<sup>+</sup> cells became HuC/D<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup>. Moreover, when combined with GFs, Ngn2 dramatically increased the number of GFP<sup>+</sup>/NeuN<sup>+</sup> 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<sup>+</sup> cells into GalC<sup>+</sup>/GST- $\pi$ <sup>+</sup> immature oligodendrocytes was detectable even in GF-untreated animals. Yet, their maturation to MBP<sup>+</sup>/PLP<sup>+</sup> myelin-forming cells did not occur at a detectable level. We showed that overexpression of Mash1 can enhance the production of GalC<sup>+</sup>/GST- $\pi$ <sup>+</sup> cells, and that at least some of these cells proceed to more mature PLP<sup>+</sup> 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<sup>+</sup> 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.

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# 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

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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。それは脊髄の可塑性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ところによる。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。

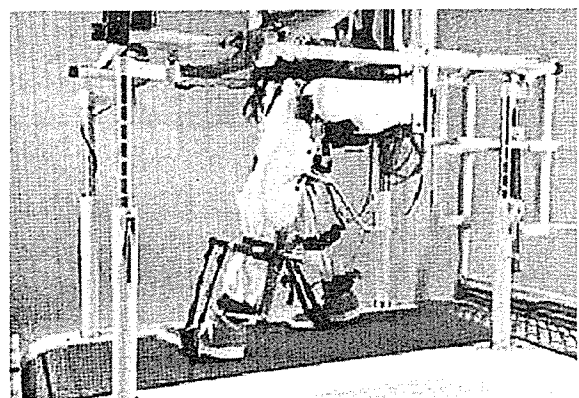
筆者ら国立身体障害者リハビリテーションセンターと東京農工大、芝浦工大の研究グループは近年、動力付き歩行装具と免荷装置から成る移動型の歩行訓練装置を開発した。この装置は、現在のところ自立歩行が困難な後期高齢者や股関節術後患者を主たる対象としているが、近い将来、対麻痺者や片麻痺者の歩行リハビリテーションへとその用途を広げる予定である。以下ではこの装置の基本コンセプトの理論的背景、開発要件ならびに装置の概要について紹介する。

### 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プさせる(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増加される。ステップにとま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脳神経系を賦活する。それが繰り返されることで中枢神経系の再組織化が促進される。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増加することで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への喚起、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

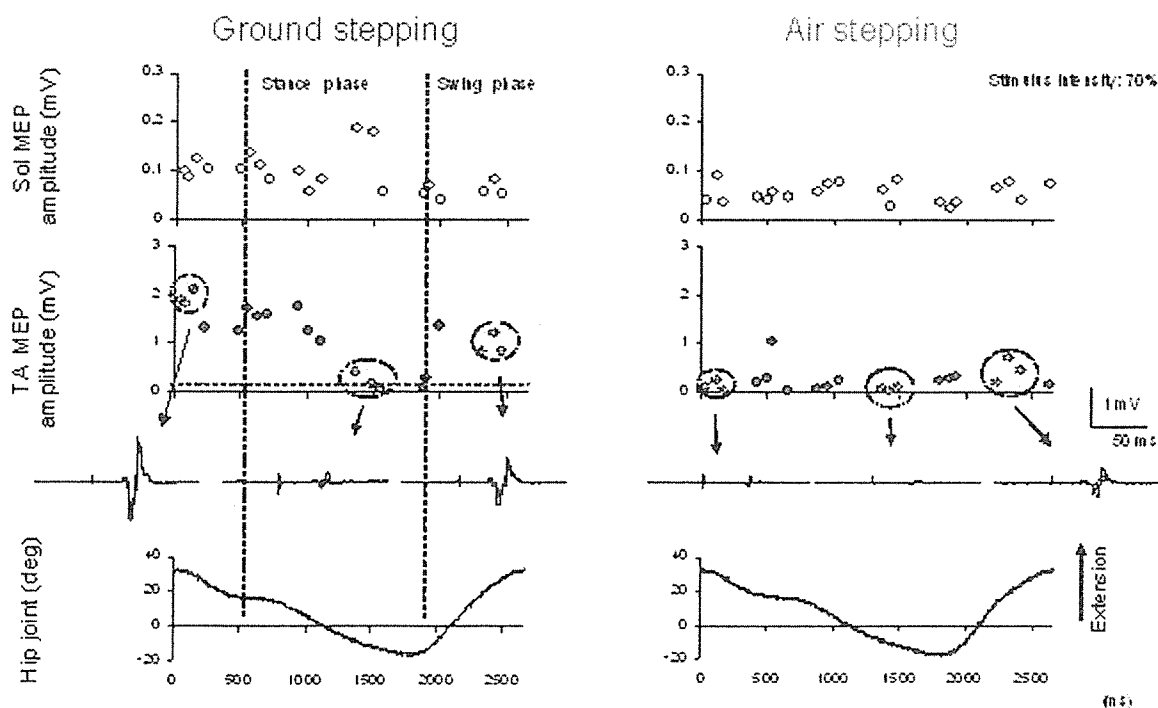
対麻痺患者であっても下肢を受動的に動かしステップングを行うと下肢の各筋群にステップングと同調した筋活動が誘発される。これは下肢のステップングに伴う感覚入力から脊髄の歩行パターンジェネレーターおよびその他の歩行に関連する神経回路を介し反射性出力を誘発した結果生じると考えられている。受動歩行トレーニングを続けると下肢の麻痺筋群に誘発されるそれらの筋放電が増大するとともに筋間の放電パターンも改善することが知られている。筆者らはさらにロボット型歩行訓練機を用いた実験によって、受動的ステップングを行うと足関節屈筋である前脛骨筋の皮質脊髄路興奮性が増大することを見出した(図2)。この筋はそもそも皮質との結合が強く、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<sup>2) 3)</sup>。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プ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プング動作を実現する機構を組み込むこととした。

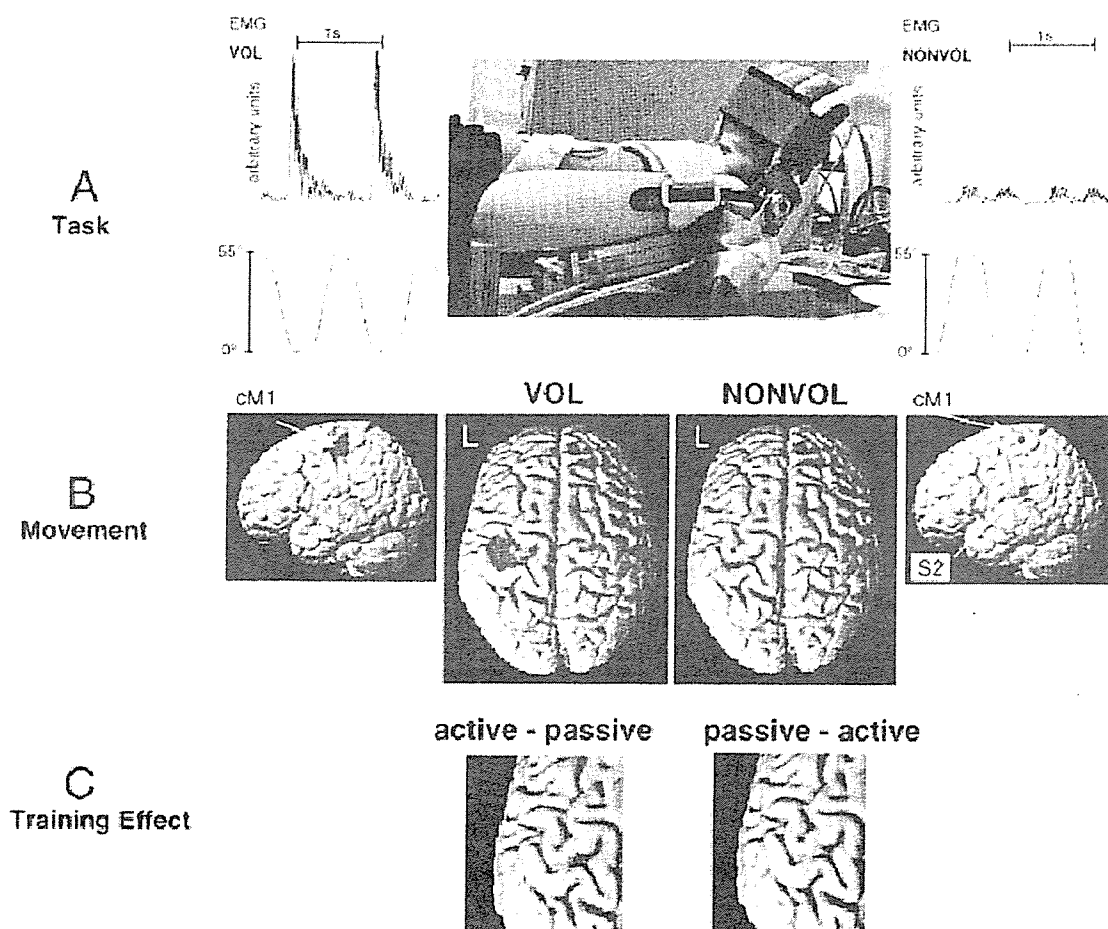
### ② 下行性司令の効果について

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)<sup>4)</sup>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)<sup>5)</sup>は受動運動トレーニングと随意命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プング; Air stepping; 空中に吊られた状態でのステップング。

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<sup>6)</sup>、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

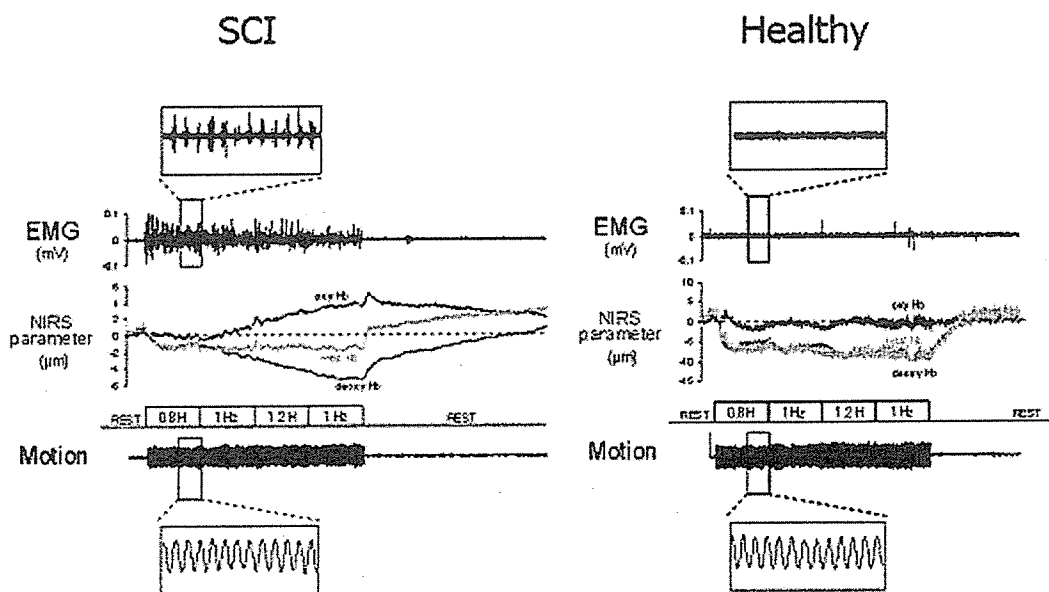
取量を過小評価していることを意味する。歩行装具を用いた立位歩行は体幹の筋など残余機能を十分に賦活させ、結果として心臓循環系、呼吸系へのトレーニングに適した負荷を与えることが容易になると考えられる。筆者らはまた、装具歩行や受動的ステップングにおいて下肢の麻痺筋群に運動周期に一致した筋活動が誘発されることに着目し、それらの筋活動によって筋の末梢循環や代謝レベルが変化するのかどうかを調べた<sup>7)</sup>。実験には立位歩行を模した受動的な下肢の左右交互性スウィング運動を用いた。この運動においても下肢麻痺領域に筋活動が誘発されることを確認した。実験では近赤外分光法を用い下腿三頭筋の酸素化レベルを定量化した。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. 下肢のステップ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プング運動を実現し、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伸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プングを行う。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

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