Transplantation of olfactory mucosa as a scaffold for axonal regeneration following spinal cord contusion in rats

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Introduction

Traumatic spinal cord injury (SCI) is relatively common, and can result in severe nerve damage leading to partial or complete loss of motor and sensory function caudal to the level of injury. This occurs as a result of severing of descending and ascending fiber tracts. One of the most devastating permanent complications following SCI is paraplegia, management of which has been a constant challenge in clinical medicine. Facilitating restoration of nerve tract structure, and with it recovery of function, after SCI is of great interest to neuroscientists. The inability of the spinal cord to regenerate after SCI is due to the extremely limited regenerative capacity of most central nervous system (CNS) axons, along with the hostile environment of

the adult CNS, which does not support axonal growth. After an SCI, astroglial scarring occurs within lesioned areas [18]. It has been shown that axonal regeneration is in fact initiated in the injured spinal cord but that it is blocked by glial scar formation [10]. It seems that for successful axonal regeneration to take place, a supportive local environment is required from an early stage after the injury.

Recently [29], a team reported partial success in bridging the ends of the spinal cord after a complete resection using grafts of smooth muscle, peripheral nerve [11], fetal brain cells [21], semi-fluid collagen material [8], and embryonic spinal cord segments in the neonatal rat. These experiments suggest that regeneration of spinal nerve fibers across a spinal cord defect could be possible, under favorable conditions. To date, there have been very few studies regarding events that occur in the early stages of autografts transplantation.

We have previously reported that grafts of the olfactory mucosa are effective in restoring functional recovery in rats following spinal cord transaction, with histological evidence of neuronal regeneration [1,13,20]. In the present study, we examined histological features of olfactory mucosa autografts in rats subjected to a spinal cord contusion protocol. Respiratory mucosa was utilized as a control, as we have previously found that respiratory mucosa does not support neuronal generation.

Materials and Methods

Spinal cord injury model

Male Sprague-Dawley rats, weighing 250–300 g, were anesthetized using a pentobarbiturate sodium/atropine mixture (5/5 mg/kg, intraperitoneally). Rectal temperature was maintained at 37 ± 0.5 °C using a heating pad. A laminectomy was performed at the thoracic (Th) 8–9 vertebrae using a microsurgery bone rongeur to expose the spinal cord without touching it. The spinal cord, covered by the dura mater, was crush-injured by dropping a 10-g metal rod from a height of 7.5 cm using a New York University (NYU) impactor. Although crush injury is commonly simulated by dropping a

weight from a height of 2.5–5.0 cm [4], mild or moderate injuries tend to produce high rates of spontaneous locomotor recovery in controls. We dropped a rod from a height of 7.5 cm to cause severe crush injuries.

Dissection and preparation of olfactory and respiratory mucosa

Rats were deeply anesthetized using sodium pentobarbital (100 mg/kg) and sacrificed by decapitation. The nasal septum was freed by removing the lower jaw, upper teeth, and nasal turbinates. Both olfactory and respiratory mucasae were identified on the septum. The olfactory mucosa is located in the dorsocaudal portion and is easily identifiable by the yellowish appearance of its surface. The respiratory mucosa is located ventrorostral to the olfactory mucosa and identified by the grayish color of its surface. Each mucosa was carefully dissected to exclude the border region between the mucosae in order to avoid cross-contamination between the 2 types.

Transplantation of olfactory and respiratory mucosa

A couple of weeks after injury, the injury site was exposed, and the posterior sulcuses of the spinal cord were opened. Both olfactory and respiratory mucosae were divided into approximately 0.5–1.0-mm sections. Next, 2–3 sections of the olfactory and respiratory mucosae were gently inserted into the sulcuses respectively. The wound was sealed by suturing the muscle and the skin overlying the exposed spine.

Behavioral assessment

The BBB score is an operationally defined 21-point scale. It is designed to assess the degree of hind limb locomotor recovery following impact injury to the thoracic spinal cord in rats [3]. In the present study, the BBB score in each animal was determined by 2 independent observers, who were blinded to the purpose and other protocols of this study. The scores were averaged and compared between the 2 groups using the Student's *t* test (unpaired). Statistical significance was set at $p < 0.05$.

Preparation of tissue for histology and immunohistochemistry

For immunohistochemical examination, 3 rats from each of the transplantation groups were sacrificed 8 weeks after the transplantation. Rats were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (100 mg/kg), and perfused intracardially with 50 ml PBS, followed by 200 ml of a fixative containing 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Specimens were processed using a standard procedure for embedding in OCT compound, and cut horizontally into 7-μm-thick frozen sections with a cryostat (CM1510S; Leica). Frozen sections were mounted on coated glass slides.

For histological examination, horizontal sections were stained with hematoxylin and eosin (HE), and used for observing blood vessels, and measuring the volume of cavities in the spinal cord. For immunohistochemistry, sections were washed 3 times with PBS, and blocked with a 0.1% bovine serum albumin solution containing 0.1% Tween 20 in PBS for 30 min. Sections were then incubated overnight in a solution containing primary antibodies as follows: anti-p75NGFR (Chemicon, Cat. No. MAB365; 1:500 in 0.1 M PBS pH 7.4) for olfactory ensheathing cells, anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (1:300; Sigma) for astrocytes, and anti-neurofilament 200 kD rabbit polyclonal antibody (1:100; Chemicon) for axons. After washing, the sections were incubated overnight with secondary antibodies as follows: FITC- or Cy-3-labeled anti-mouse IgG antibody (1:1000; Amersham Biosciences) for astrocytes, or Cy-3-labeled anti-rabbit IgG antibody (1:1000; Amersham Biosciences) for axons. Sections were then mounted and examined by a fluorescence microscope (Axio Imager MI; Carl Zeiss).

All experimental procedures were approved by the Animal Ethics Committees of the Osaka University Medical School.

Results

The averaged BBB scores of the olfactory mucosa transplanted rats ($n = 5$) were 3.13 ± 1.12, 5.25 ± 1.21, 6.88 ± 1.34, and 10.83 ± 1.23, measured

1, 2, 4, and 8 weeks after transplantation, respectively. The averaged BBB scores of the respiratory mucosa transplanted rats (n = 5), measured over the same time frame, were 2.2 ± 0.84, 2.8 ± 1.15, 3.5 ± 1.02, and 4.0 ± 0.71. These data indicate that the recovery of hind limb movement in the olfactory mucosa transplanted rats improved significantly in comparison to the control, respiratory mucosa transplanted rats ($p < 0.05$, see fig. 1). In the histological assessment, expression of neurofilament was observed strongly at the injury site in the olfactory mucosa transplanted rats. The numerous fibers that were strongly stained with neurofilament were surrounding the GFP-positive cells and penetrating the transplanted olfactory mucosa. In contrast, there were no apparent neurofilament stained fibers at the marginal spinal cord of the respiratory mucosa transplanted rats.

Discussion

Injuries to the central nervous system (CNS) in humans are usually associated with a low degree of neurological recovery and, in the majority of cases, life-long debilitation. This lack of recovery, however, is not due to any intrinsic inability of CNS axons to regenerate; rather, the environment of the CNS is strongly inhibitory to axonal regeneration. Following SCI, astroglial scars form within lesioned areas of the spinal cord [18]. Although the majority of known inhibitors of neurite outgrowth are myelin membrane proteins, equally potent inhibitors have also been identified in astroglial scars, for example, chondroitin sulfate proteoglycans and semaphoring 3A. Manipulating the local environment in order to provide a favorable scaffold, supportive of axonal regeneration, is one of the more promising strategies for treatment of SCI.

Spinal cord reconstruction using implantation of cells from various sources has been gaining attention in recent years [15,22]. Neuronal stem cells have the potential to differentiate into both neuronal and glial cells, and are therefore prime candidates for cell replacement therapy following CNS injury. Neuronal stem cells constitutively secrete significant quantities of several neurotrophic factors that act to support host axonal regeneration after SCI [12]. Partial restoration of function after contusion of the spinal cord has been

accomplished by injecting neural/glial precursors (NSCs), differentiated *in vitro* from mouse embryonic stem cells (ESCs), into the lesion 9 days after injury [17]. However, implantation of NSCs alone did not produce any significant restorative effect because the majority of the NSCs grafted into the spinal cord differentiated with an astrocytic phenotype [6,12]. Although astrocytes can secrete neurotrophic factors and limit the extent of the inflammatory reaction, extensive astroglial scarring within the lesioned area blocks axon growth.

However, one of the major disadvantages associated with implantation or injection of cells alone is the limited proportion of viable cells surviving in the injury site after the procedure, as cells tend to migrate away from the injury site [23]. To achieve significant functional reconstruction of the spinal cord after spinal cord injuries, it is either necessary to populate lesion sites with tissue-specific, regeneration-competent cells that replace or rescue dying cells, or to activate endogenous neural progenitor cells that do likewise [27].

In this study, numerous neurofilaments were observed strongly in the transplanted olfactory mucosa. Unlike respiratory mucosa, it permits axonal regeneration after SCI and therefore may be an appropriate scaffold on which to reconstruct axons.

Indeed, the olfactory mucosa is an excellent autologous source of adult neuronal precursor cells. The neurons and the sustentacular cells there renew themselves constantly throughout life by proliferation of basal global stem cells [5,7,25]. Furthermore, the mucosa contains olfactory ensheathing cells, which have previously been the subject of much attention for their potential in the repair of spinal cord injuries [2,26,16,24]. Recent studies of spinal cord axon regeneration have reported good long-term results using various types of tissue scaffolds [14,19,28]. Olfactory tissue would allow autologous transplantation, is easily accessible, and can be obtained by a simple biopsy that is performed through the external nares 9. These considerations, combined with the results of the present study, make nasal mucosa an attractive potential scaffold for axonal regeneration.

Conclusions

As we have previously reported, olfactory mucosa transplantation following spinal cord injury can support at least partial hind limb motor recovery. In this study, we identified numerous axons surrounding the transplanted cells, and penetrating the mucosa at the transplant site without marginal spinal white matter. Olfactory mucosa might therefore be a more suitable scaffold for axonal regeneration than white matter, which contains inhibiting factors for axonal regeneration in the spinal cord.

Figure Legends

Fig.1 A significantly greater degree of functional recovery as measured by hindlimb usage was observed in the olfactory mucosa transplanted rats (OM) compared with the respiratory mucosa transplanted rats (RM) 4 weeks after the transplantation (□). BBB: Basso, Beattie, and Bresnahan Locomotor Rating Scale.

Fig.2 Histology (HE). A transplanted mucosa (indicated by an arrow) is recognizable in the contused spinal cord.

Fig.3 Immunohistological study. Numerous fibers (arrow), strongly stained with neurofilament, are seen penetrating the transplanted olfactory mucosa (a). The fibers surround the GFP-positive cells (b).

Fig.4 Immunohistological study. No apparent fibers stained with neurofilament are found in the respiratory mucosa (GFP positive) transplanted spinal cord.

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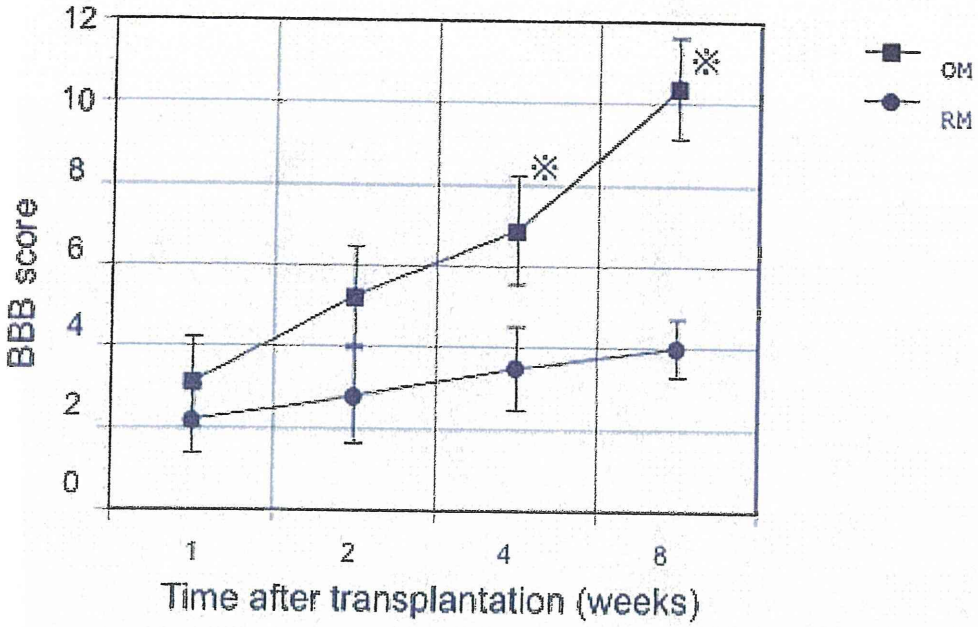
12
10
8
6
4
2
0
BBB score
※
※
1
2
4
8
Time after transplantation (weeks)
CM
RM

Fig 1

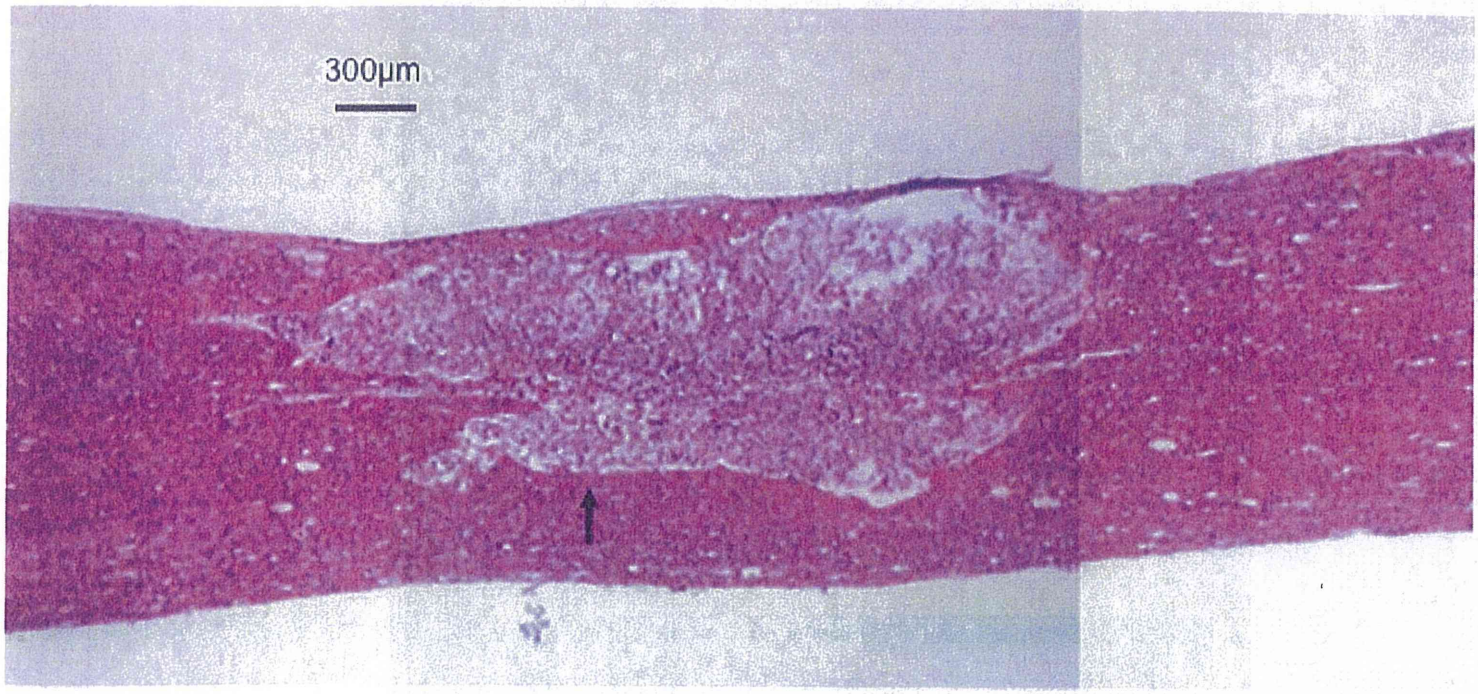
300μm

Fig.2

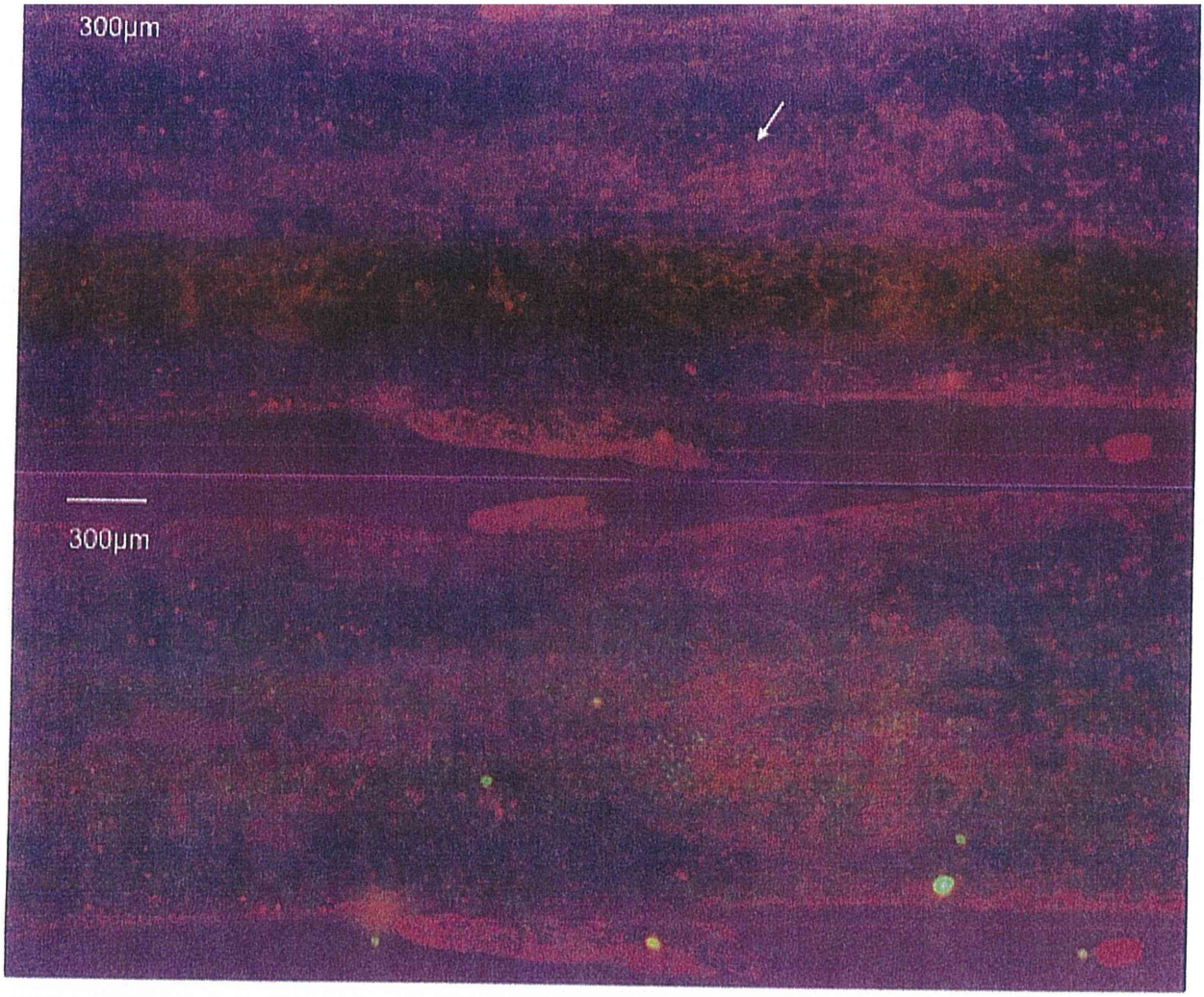

Fig 3

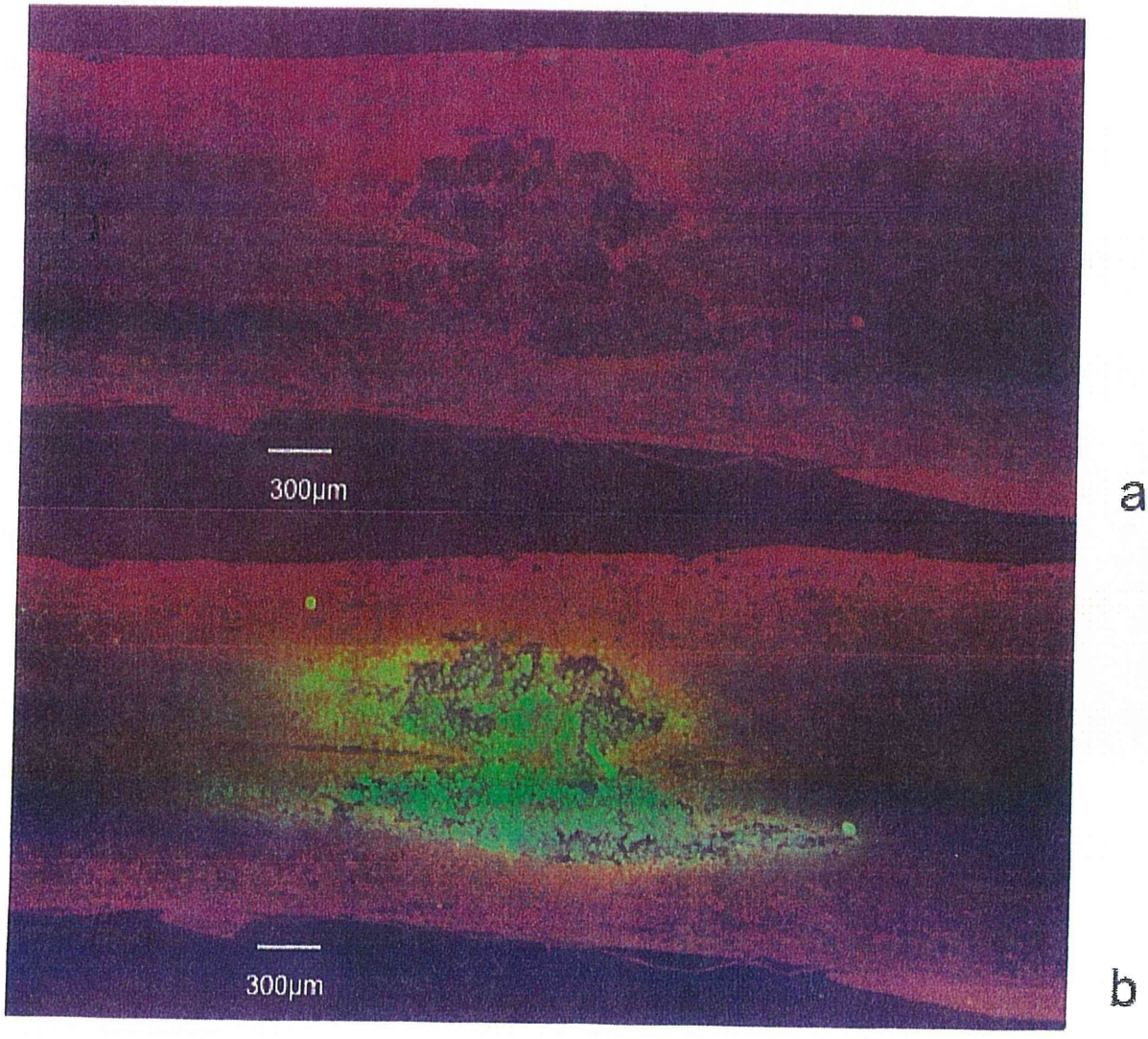

Fig 4

Basic Procedures of

脊椎脊髄外科
サージカル・テクニック

編集

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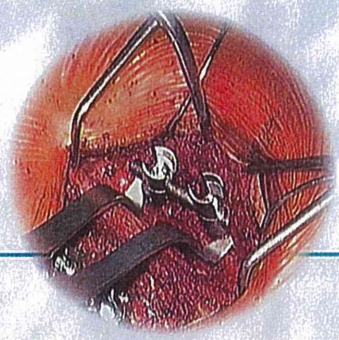
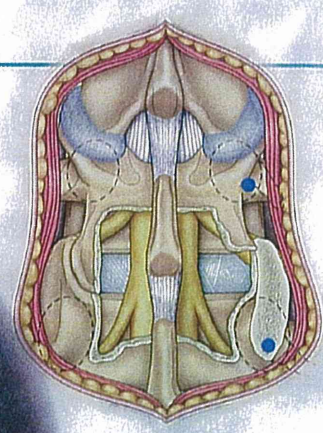
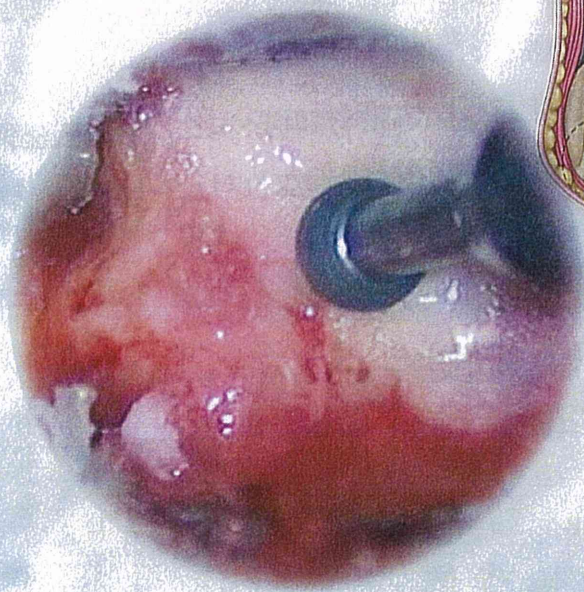






Spinal Surgery

MEDICAL VIEW

目　次

Ⅳ 胸腰椎後方手術

頸椎前方固定術
プレート

手術適応

頸椎前方プレートの登場

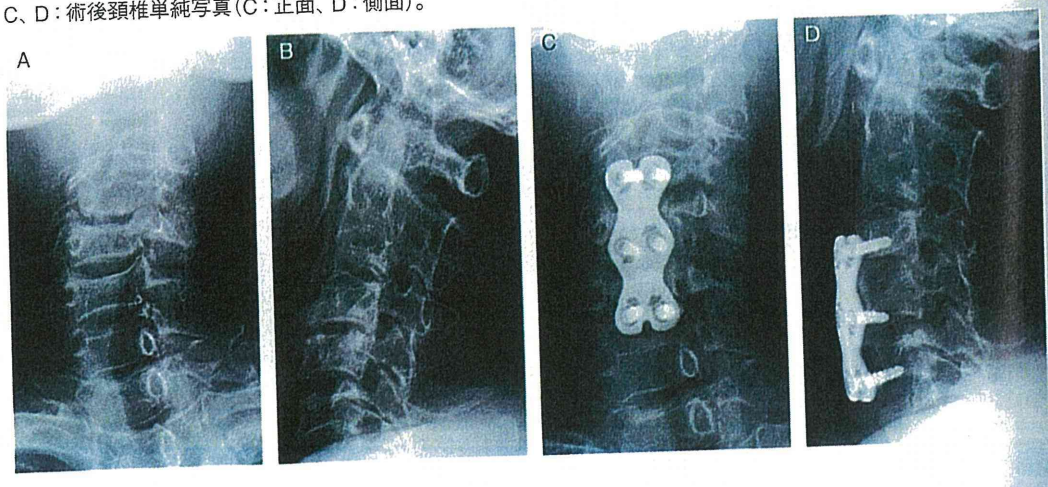
頸椎前方固定術、なかでも多椎間固定を必要とする症例においては、術後の偽関節や移植骨の圧潰または転位により企図した前彎矯正を失い、術後後彎変形をきたすことがある。これに対してより確実な骨癒合とアラインメントの維持を目指して頸椎前方プレートが併用されるようになった。1990年代にプレート、スクリュー間のロック機構が開発され、これにより椎体後方皮質を貫通させずにプレートを椎体前面に固定できるようになり、広く普及することとなった。当初プレートは大きく、嚥下障害や呼吸困難を引き起こすこともあったが、現在ではlow profileとなり、自家骨移植を用いた多椎間固定術に頸椎前方プレートの併用が一つの標準術式となってきている[3,4,7]【図1】。

プレート使用の利点

プレートを使用することにより、固定術後の偽関節、移植骨の転位、後彎、移植骨の沈み込み等を防ぐことが期待される。これにより術後の外固定期

【図1】症例写真

A、B：術前頸椎単純写真（A：正面、B：側面）。
C、D：術後頸椎単純写真（C：正面、D：側面）。



間の短縮と早期離床が促進される。プレート併用の適応については、変性疾患における多椎間固定、外傷による不安定性、関節リウマチや破壊性脊椎関節症等の合併等とされ、変性疾患に対する単椎間固定には適応があまりないとされている[3,6]。

プレート併用の効果については、頚椎アライメントの維持や早期離床があげられる。

問題点、合併症

問題点として、スクリューの椎間板内誤刺入、設置中の食道等の隣接臓器損傷、術後隣接椎間へのdynamic stressの増加や、術後のプレート、スクリューの移動といったinstrument failureがあげられる。適切なプレートサイズの選択、正確なプレートbending、隣接椎間に干渉しないプレート位置の決定等が重要である。Bone quality不良な場合は、決してプレートを過信することなく、慎重な術後管理が望まれる。スクリューやプレートのloosening による二次的な食道への侵食は重大な合併症となるため、術後綿密な経過観察が必要である。

プレート、スクリューの種類

現在市販されているプレートは、ほぼプレートとスクリュー間のロッキングシステムを有するものである。スクリューの刺入角度が固定されているもの(fix screw)と、ある程度角度が自由に刺入できるもの(variable screw)とがある。さらにプレート自体は変化しないconstrained plate、プレートに伸縮機能のついたdynamic plateがある。プレート、スクリューともに自由度がないものをrigid type、スクリューの角度が変化しうるものをsemi-rigid type、スクリュー自体が平行移動もしくはプレートそのものに伸縮機能があるものをdynamic typeとし分類すると**表1**のようになる。プレートには移植固定後loadがかかるとともに、移植骨の萎縮によりさらに長期間にわたってloadがかかることになる。それによって椎体骨の圧潰や偽関節につながる。Variable screwもdynamic plateも、このloadによりプレートにかかるdynamic stressの緩和を目指したものであるが、このようなflexibilityを有するプレートが、主流となっている[1]。主にvariable screwは回旋運動を許容し、dynamic plateはプレート内のスクリューの平行移動を許容する[8]**【図2】**。後者のほうがより均等な荷重分散を達成することが報告されている。

多椎間固定

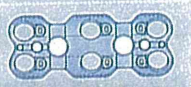
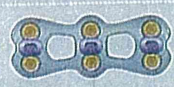
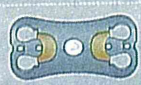
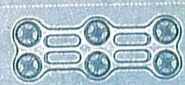
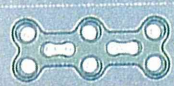
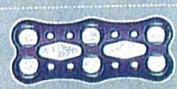
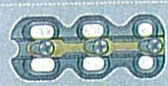
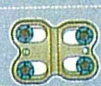
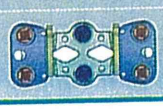
多椎間のプレート固定が術後早期のgraftの転位を防ぎ、癒合率を向上さ

せる一方、多椎間では graft dislodgementの報告もある[2]。術後の頚椎前彎が過度であることがその原因と考えられており、特にlong fusionの場合は、矯正前彎が過度にならないよう注意が必要である。またスプレッダーの使用による無理な移植骨の打ち込みが、椎体骨の圧潰そしてプレートへの過荷重につながることも報告されている[5]。

強固な固定

強固な固定が必要となる腫瘍、外傷および一部変性疾患の治療においては、fix screwのみによってプレートとスクリューの間に可動性のない固

【表1】市販されている頚椎前方プレート各種

		製品名	メーカー
Rigid type		CSLP 青龍	Synthes
Semi-rigid type		TRESTLE	Alphatec
		Slimlock	Depuy Spine
		Atlantis	Medtronic
		Venture	Medtronic
		Zephire	Medtronic
		VueLock	Biomet
		CSLP VA	Synthes
		Vectra	Synthes
Dynamic type		Premier	Medtronic
		ABC	Aesculap
		Vectra-T	Synthes

るだけ上位終板に近づける。頭尾側のスクリューホールから移植骨が見えてはならない。

② プレートの形状

ほとんどの製品であらかじめ前彎がつけられており、術中新たに前彎を追加することはほとんどないが、追加する場合は専用のプレートベンダーで、プレートの強度を損ねないよう、特に一カ所に過負荷がかからぬよう注意する。変性が強く前方骨棘があり、プレートと椎体前面とのコンタクトが不十分な場合は、骨棘の切除も必要である。

③ プレート位置の確認

プレートの上下のスクリューホールが、椎体のほぼ中央にくるように置く。椎間板や椎体を切削する前に、上下の椎体中央をマーキングしておくと思わぬmalplacementを防ぐことができる。側面X線透視等によって、プレートの位置、サイズの確認を行う【図3】。

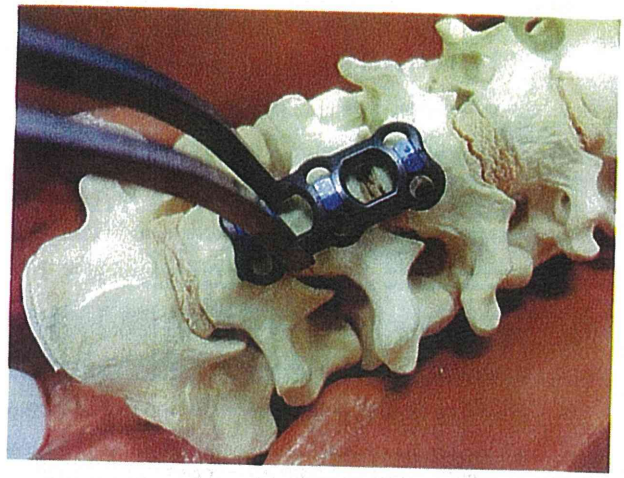
④ スクリュー刺入

プレートホルダーでプレートを保持し、透視下に下穴を作成しタッピングを行う。筆者は椎体の80％程度長さのスクリューを好んでいる。1本目のスクリューが問題なく刺入されれば、その対角線上に2本目を刺入し、問題なければ残り2本を刺入する。Long fusionの場合は、移植骨にもスクリューを刺入し、転位を防止する【図4】。

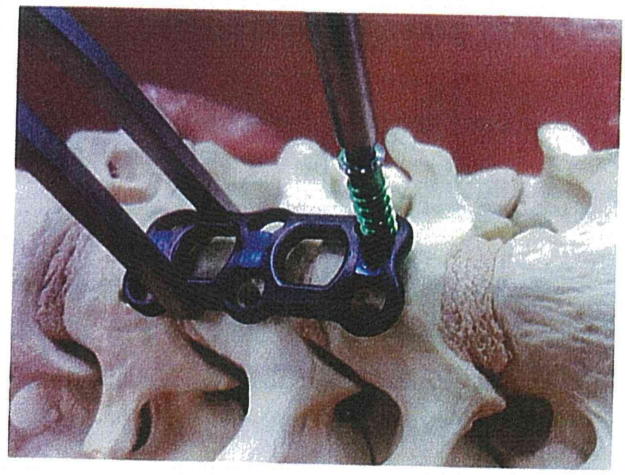
プレートの抜去について

プレートを用いた強固な椎間固定は、隣接椎間の変性を加速させる可能性がある。プレート等によるインストゥルメンテーションの合併症としては、金属過敏症、CTやMRI等におけるイメージの阻害、プレートのボリュームによる嚥下障害、遅発性感染等があるが、ノンプレートと比較して、その合併症率は高くないとする報告もある[9]。しかし合併症率3％、インプラントフェイラーによる再手術率3～45％、感染率5～10％とも報告されており[10]、骨癒合が得られた後は、プレート抜去が望ましい。

【図3】プレート位置、サイズの確認



【図4】スクリューの刺入



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（岩月幸一）