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Effects of continuous dexamethasone treatment on differentiation capabilities of bone marrow-derived mesenchymal cells

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Abstract

Human bone marrow-derived mesenchymal cells (hBMMCs) originate from cell populations in the bone marrow and are capable of differentiating along multiple mesenchymal lineages. To differentiate hBMMCs into osteoblasts, adipocytes and chondrocytes, dexamethasone has been used as a differentiation reagent. We hypothesized that dexamethasone would augment the responsiveness of BMMCs to other differentiation reagents and not define the lineage. This study investigated the effect of continuous treatment with 100 nM dexamethasone on the differentiation of BMMCs into three different lineages.

hBMMCs cultured with continuous dexamethasone treatment (100 nM) exhibited higher mRNA expression levels of osteogenic markers and higher positive rates of colony forming unit assays for osteogenesis compared to hBMMCs treated with dexamethasone only during the differentiation culture. Furthermore, continuous dexamethasone treatment augmented bone formation capability of monkey-derived BMMCs in a bone induction experimental model at an extra skeletal site. In addition, continuously dexamethasone-treated hBMMCs formed larger chondrogenic pellets and expressed SOX9 at higher level than the control BMMCs. Likewise, continuous dexamethasone treatment facilitated adipogenic differentiation based on mRNA level and colony forming unit analysis.

To investigate the mechanism of the augmentation of differentiation, further studies on apoptosis were conducted. The studies indicated that dexamethasone selectively induced apoptosis of some populations of hBMMCs which were thought to have poor differentiation capability.

Keywords: Dexamethasone; Bone marrow-derived mesenchymal cells; Osteogenesis; Multi-lineage potential; Apoptosis

Introduction

The most general clinical treatment for bone defect repair has been autologous bone graft in orthopaedic, oral and maxillofacial surgery. However, the availability of autograft bone is limited and furthermore the occurrence of complications including donor's morbidity, pain, infection and nerve and vascular injuries at the harvest site is relatively high [1–3]. Therefore, development of bone tissue regeneration techniques is necessary.

Human bone marrow mesenchymal cells (hBMMCs) are an attractive source for bone tissue engineering applications because they can be obtained from adult bone marrow aspirates and have excellent proliferation capability and versatility for pluripotent differentiation into various mesenchymal lineages, including the osteogenic lineage [4,5].

For clinical tissue engineered bone reconstructions, large amount of BMMCs are required and repeated cell multiplications are unavoidable. However, despite the multi-lineage potential of BMMCs, they lose their potential to differentiate

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into not only osteoblast but also adipocytes and chondrocytes after multiple doubling processes under standard culture conditions [6,7]. Therefore, it is necessary to determine culture protocols to establish optimum BMMCs able to retain their differentiation potential at an adequate level.

Previous studies reported that human BMMCs were induced to differentiate into osteoblasts by 100 nM dexamethasone, β-glycerophosphate and ascorbic acid phosphate [8,9]. Dexamethasone is also used to differentiate BMMCs into adipocytes [4] and chondrocytes [10–13]. Thus, we hypothesized that dexamethasone could augment the responsiveness of BMMCs to other differentiation reagents, and continuous dexamethasone treatment would establish BMMCs with higher differentiation potentials.

Materials and methods

Primary culture of hBMMCs

After obtaining informed consent, hBMMCs were cultured from bone marrow aspirates of 30 patients who received hip surgery at the Tokyo Medical and Dental University under a protocol approved by the institutional review board. These donors ranged in age from 30 to 87 years old.

Approximately 2 ml of bone marrow aspirate was obtained from the medullary cavity of the femoral shaft using a bone marrow biopsy needle (Cardinal Health, USA). The aspirate was added to 20 ml of standard medium (Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Co., St. Louis, USA)) containing 10% fetal bovine serum (Invitrogen Co., NY, USA) and 1% antibiotic–antimycotic (10,000 U/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B; Invitrogen)) containing 200 IU sodium heparin (Mochida Pharmaceutical Co. Ltd, Tokyo Japan) and then centrifuged to remove the fat layer.

Bone marrow cells were then resuspended in standard medium. Aliquots of cell suspensions were used to count nucleated cell number after hemolysis. Then, bone marrow cells including 1×10^8 nucleated cells were plated in two 75 cm^2 flasks (Becton, Dickinson and Company, USA).

After 3 h of plating, dexamethasone was added to one of the flasks (DEX group) at a final concentration of 100 nM, whereas the control group was maintained without dexamethasone. They were cultured in each medium at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. The medium was replaced every 3 days.

When culture flasks reached 80% confluency, cells were detached with 0.25% trypsin containing 1 mM EDTA (Invitrogen) and subsequently replated for each assay.

Analysis of cell surface makers

Flow cytometric analysis of representative cell surface makers for mesenchymal cells (CD44 and CD105), hematopoietic cells (CD4 and CD34) and endothelial cells (CD31) were conducted. BMMCs were cultured in each medium by primary culture and subsequent P1 culture and then used for the assay. Cells (1×10^6) were suspended in 200 μl PBS containing each FITC-conjugated antibody (20 μg) and incubated for 30 min at 4 °C. After the concentration of the cell suspension was adjusted to 1×10^6 ml, it was used for flow cytometry using a FACSCalibur (Becton, Dickinson and Company). All antibodies were purchased from Becton, Dickinson and Company.

Osteogenic differentiation

BMMCs obtained from each group were replated at 4×10^3 cells/cm^2 in a 6-well culture plate for the alkaline phosphatase (ALP) activity assay or a 100-mm culture dish for RNA isolation and maintained in each medium. When culture plates became 80% confluent, the culture media of both groups was changed to osteogenic medium containing 100 nM dexamethasone, 10 mM β-glycerophosphate (Sigma-Aldrich Co.) and 50 μg/ml ascorbic acid phosphate (Wako, Osaka, Japan) [9] and at 0, 5 and 10 days of osteogenic culture, they were used for each assay (Fig. 2A).

Chondrogenic differentiation

For chondrogenic induction, pellet culture was performed according to the method described by Indrawattana et al. [13]. Briefly, after culture medium was replaced by basal medium consisting of DMEM-high glucose with L-glutamine, sodium-pyruvate, pyridoxine hydrochloride (Invitrogen), 1% antibiotic–antimycotic (Invitrogen), 50 μg/ml ascorbic acid phosphate, 0.4 mM L-proline, 100 nM dexamethasone, 1% ITS$^+$1 (Sigma-Aldrich Co.) and 10 ng/ml recombinant human transforming growth factor-β3 (TGF-β3) (Sigma-Aldrich Co.), 2.5×10^5 BMMCs were recentrifuged to obtain aggregated cells and cultured at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. The medium was replaced every 2–3 days for 21 days.

Adipogenic differentiation

The BMMCs of each group were replated at 4×10^3 cells/cm^2 in a 100-mm culture dish and maintained until 80% confluence. Then, the culture medium was switched to adipogenic medium consisting of control medium supplemented with 100 nM dexamethasone, 500 μM isobutylmethylxanthine (Sigma-Aldrich Co.) and 100 μM indomethacin (Sigma-Aldrich Co.) [14] for an additional 14 days followed by total RNA isolation.

ALP activity assay

Cells were washed with PBS, lysed with 500 μl of 0.2% Triton X-100 (Sigma-Aldrich Co.) and sonicated to destroy cell membranes. The supernatant (10 μl) was added to 100 μl substrate buffer (10 mM disodium *p*-nitrophenylphosphate hexahydrate, 0.056 M 2-amino-2-methyl-1, 3-propanediol and 1 mM $MgCl_2$; Wako) in a 96-well plate. After incubation of the mixtures at 37 °C for 30 min, absorbance at 405 nm was measured. ALP activity was determined with a standard curve, employing the reaction of 10 μl of a *p*-nitrophenyl solution (Wako) and 100 μl of substrate buffer for 30 min. ALP activity is expressed as millimoles of *p*-nitrophenyl.

To normalize ALP activity, DNA content was measured using the Quant-iT PicoGreen dsDNA Assay kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Briefly, samples diluted with distilled water were mixed with the working solution and incubated for 3 min at room temperature. Fluorescence intensity was measured at emission and excitation wavelengths of 516 and 492 nm, respectively.

RNA isolation, real-time RT-PCR

Total RNA was isolated from culture dish or chondrogenic pellet cells disrupted by an electric crusher using RNeasy Mini Kits (Qiagen, GmbH, Germany), and first strand cDNA was prepared using the Superscript III First-strand synthesis system (Invitrogen) according to the manufacturer's instructions.

Quantification of gene expression was analyzed by real-time PCR using the Mx3000P® QPCR System (Stratagene). Primer sets were predesigned and purchased from Takara Bio Inc., Japan (Table 1). Real-time PCR was performed on 1 μl of the resulting cDNA in a total volume of 20 μl containing 10 μl of 2×SYBR® Premix Ex Taq™ (Takara Bio Inc.) and 0.4 μM of each primer. RT-PCR was performed using 40 cycles of 95 °C for 5 s and 62 °C for 20 s. β-Actin was used to normalize the amount of template presented in each sample.

CFU assay

For clonal analysis, colony forming unit (CFU) assays were performed. BMMCs were plated at 800 cells/100 mm dish and maintained in each medium for 14 days to form single cell-derived colonies. Then, the medium was changed to an osteogenic (CFU-ALP and CFU-OB) or adipogenic medium (CFU-adipocyte).

Table 1
Primers used for RT-PCR analysis and expressed sizes of PCR products

	Primer		Base pair
Osteocalcin		5′CCCAGGCGCTACCTGTATCAA3′	112
	Reverse	5′GGTCAGCCAACTCGTCACAGTC3′	
BMP-2	Forward	5′AACACTGTGCGCAGCTTCC 3′	167
	Reverse	5′CCTAAAGCATCTTGCATCTGTTCTC 3′	
BMP-6	Forward	5′AACCACGCGATTGTGCAGAC 3′	172
	Reverse	5′AGTGGCATCCACAAGCTCTTACAAC 3′	
SOX9	Forward	5′AACGCCGAGCTCAGCAAGA 3′	138
	Reverse	5′CCGCGGCTGGTACTTGTAATC 3′	
PPARγ	Forward	5′TGGAATTAGATGACAGCGACTTGG 3′	182
	Reverse	5′CTGGAGCAGCTTGGCAAACA 3′	
β-Actin	Forward	5′ATTGCCGACAGGATGCAGA 3′	89
	Reverse	5′GAGTACTTGCGCTCAGGAGGA 3′	

CFU-ALP

Dishes were stained with ALP stain at 7 days of osteogenic induction. Dishes fixed with 10% neutral buffered formalin were washed with PBS, then incubated with a filtered mixture of naphthol AS-MX phosphate (0.1 mg/ml, Sigma-Aldrich Co.), *N,N*-dimethylformamide (0.5%, Wako), $MgCl_2$ (2 mM) and Fast Blue BB salt (0.6 mg/ml, Sigma-Aldrich) in 0.1 M Tris-Cl (pH 8.5) for 30 min at room temperature.

CFU-OB

Mineralized colonies were identified by Von Kossa stain and designated as colony-forming unit-osteoblasts (CFU-OB). At 14 days of osteogenic induction, cells were washed twice with Gey's balanced salt solution, fixed with 10% formalin, rinsed with 0.1 mol/L cacodylic buffer and covered with 1.0 ml of 5% silver nitrate (Wako). Cells were then exposed to UV light for 1 h. Finally, the dishes were rinsed with distilled water and air-dried.

CFU-adipocyte

At 14 days of adipogenic culture, dishes were fixed in 4% paraformaldehyde and washed with deionized water. Oil Red O stock solution was prepared by adding 0.5 g Oil Red O (Sigma-Aldrich Co.) to 100 ml isopropyl alcohol. Prior to staining, 30 ml of the stock solution and 20 ml of deionized water was mixed and filtered to use as a working solution. Cells were stained with the working solution for 30 min and washed with deionized water.

After counting positive colonies in each assay, dishes were stained with crystal violet to recognize all colonies presented on the dishes, and the total number of colonies was determined. Colonies <2 mm in diameter and faintly stained colonies were ignored.

Bone formation study

All animal experiments were conducted according to the guidelines of the Tokyo Medical and Dental University for the care and use of laboratory animals. To obtain monkey BMMCs, bone marrow was aspirated from femurs of Japanese macaque (female, 5 years old, 4–5 kg body weight, $n=5$) under general anesthesia by intramuscular injection of medetomidine hydrochloride (0.1 mg/kg) and ketamine hydrochloride (10 mg/kg). Monkey bone marrow-derived mesenchymal cells (mBMMCs) were cultured in culture media with or without dexamethasone as described above. When primary cultures became nearly confluent, cells were passaged at a density of 5×10^3 cells/cm^2 and cultured with each medium respectively. When the culture flasks became subconfluent, culture media was supplemented with the osteogenic reagents described above maintained for 4 days. Cells were then used for subsequent bone formation analysis.

Preparation of the implants

Implants for bone formation assay were prepared according to previous reports [15,16]. Briefly, to combine cells and porous β-tricalcium phosphate (β-TCP) blocks (5 mm×5 mm×5 mm, porosity: 75%, pore size: 100–200 μm, Olympus Co., Tokyo Japan) as scaffolds for bone formation, autologous blood plasma containing citrate phosphate dextrose for anticoagulation at a ratio of 1/10 by volume was prepared. Detached cells were suspended in plasma (1×10^6 cells/ml), and the cell suspension (2 ml) was mixed with 300 μl of 2% calcium chloride and immediately introduced into porous β-TCP blocks under vacuum at 50 mM Hg. When calcium chloride was added, fibrinogen presented in the cell suspension started to transform to fibrin within a few minutes and form fibrin networks in the porous scaffolds. The blocks were incubated at 37 °C until gelation was completed at 30 min and were then used as implants.

Implantation

General anesthesia was induced with intramuscular administration of ketamine (5 mg/kg) and maintained with inhalational anesthesia of isoflurane (1.0–2.0%). After intubation, the monkeys were placed in a prone position and shaved. Subsequently, they were draped in the usual sterile manner. Three incisions were made on each side of the monkeys' back, and three muscle pouches were created at each side. On the left, three β-TCP blocks with control BMMCs were implanted into each pouch, whereas DEX blocks were placed on the right. At 4 weeks after transplantation, implants were harvested under sterile condition for histological analysis using inhalational anesthesia of isoflurane, and then the splittings of the muscle and skin were sutured.

Histological analysis

The harvested composites were fixed in 10% neutral buffered formalin and decalcified with K-CX solution (Falma, Tokyo, Japan). For each implant, 5 μm paraffin sections at 0.5, 1.5, 2.5, 3.5 and 4.5 mm positions from the surface were prepared. Each section was stained with hematoxylin and eosin. Bone formation areas were measured using Adobe Photoshop 7.0 software.

Apoptotic assays

Caspase-3 activity assay

Caspase-3 activity was assayed using the ApoAlvert Caspse-3 Colorimetric Assay Kit (Clontech Laboratories, Inc., USA). Cells in 100 mm culture dishes were treated with 100 nM dexamethasone for 5 days. Cells were detached by trypsinization and lysed in 50 μl of cell lysis buffer. After centrifugation, the supernatant was added to 50 μl of 2× reaction buffer/DDT Mix and 5 μl of 1 mM Caspase-3 Substrate. After incubation of the mixtures at 37 °C for 1 h, absorbance at 405 nm was measured.

Apoptotic cell staining

Detection of early apoptotic cells was performed with the APOPercentage Apoptosis assay kit (Biocolor CO., Belfast, Northern Ireland). Briefly, cells were seeded on 100 mm culture dishes at cloning density and cultured for 14 days to form single cell-derived colonies, which were then treated with 100 nM dexamethasone for 0, 3, 6, 24, 72 and 120 h s. The dishes were then incubated with 1 ml of dye provided in the kit for 1 h to detect apoptotic cells.

Flow cytometry analysis

BMMCs, cultured on a 100-mm culture dish, were harvested by trypsinization after dexamethasone treatment (100 nM, 72 h). To identify ALP-positive cells, the collected cells were incubated for 30 min at 4 °C with mouse monoclonal anti-human ALP antibody (R&D systems, Inc., Minneapolis, MN, USA), washed and incubated with FITC-conjugated secondary antibody (rat anti-mouse IgG1 monoclonal antibody; Becton, Dickinson and Company) for 30 min at 4 °C. To detect cells at early stages of apoptosis, the cells were incubated with PE conjugated annexin-V (Becton, Dickinson and Company), which binds phosphatidylserine (PS). Flow cytometric analyses were performed using a FACSCalibur.

Statistical analysis

Average values were expressed as the arithmetic mean ± standard deviation (SD) and analyzed using the paired Student's *t*-test. Differences were considered statistically significant when the p value was <0.05.

Results

Surface marker profiles

BMMCs of both the DEX and control group were negative for all hematopoietic and endothelial markers. For CD44 and CD105 profiles, there were no obvious differences between the two groups (Fig. 1).

ALP activity

At the beginning of the osteogenic culture, ALP activity of the dexamethasone-treated group was significantly higher than that of the control group, and ALP activity of the DEX group gradually decreased as osteogenic induction progressed, although it still remained higher than the control group (Fig. 2B).

Expression of osteoblastic markers

At 5 and 10 days of osteogenic differentiation, BMP-2 and BMP-6 mRNA expression levels were statistically higher in the DEX than control group (Figs. 2C and D). The most remarkable difference between the groups was mRNA expression of BMP-6 at day 0. BMP-6 expression in the DEX group had already been intensely stimulated by continuous dexamethasone treatment prior to osteogenic induction culture. mRNA expression of osteocalcin at 5 and 10 days was higher in the DEX group than the control group, whereas it was statistically lower at day 0 (Fig. 2E).

CFU assay

Bone marrow is composed of a heterogeneous mixture of cells including matured cells, lineage restricted progenitors and a small proportion of stem cells. Therefore, MSCs cultured without any purification process including the protocol used in this study consist of heterogeneous cell-derived populations. Therefore, there were conjectures that the dexamethasone treatment facilitated the osteogenic capability of each cell or selectively increased the populations of BMMCs with osteogenic potential.

To verify the compositional alteration of the populations, a CFU assay was performed. The ratios of CFU-ALP and CFU-OB to total colonies are shown in Figs. 2G and I. The positive ratios of the DEX group in the both assays were higher than those of the control group.

Analysis of bone formation capability in vivo

To confirm the bone formation capability of BMMCs with continuous dexamethasone treatment, an ectopic bone formation model of autograft BMMCs in non-human primates was applied. CFU-ALP and CFU-OB were also examined to ensure correspondence between hBMMCs and mBMMCs. The positive ratios of the DEX group were higher than those of the control group in both CFU-ALP and CFU-OB, and these results agreed with the results of human BMMCs analyses (Figs. 3A and B).

Bone formation analysis demonstrated significantly increased bone formation in the DEX group compared to controls. Although the implants of the control group also showed bone formation in all cases, the amount of bone formation was smaller than that of the DEX group in each animal and the average bone formation amount was about half that of the DEX group (Figs. 3C and D).

Effect of dexamethasone on chondrogenic differentiation of BMMCs

The macro finding on the left in Figs. 4A and B is the control pellet and the right is the DEX group pellet. The diameter of the DEX group pellet was significantly larger than that of the control (Fig. 4C). SOX9 mRNA expression levels in the DEX group at day 0 was lower than that of the control group, but at 21 days was significantly higher than that of control group (Fig. 4D).

Effect of dexamethasone on adipogenesis

The ratio of CFU-Adipocyte to total colonies is shown in Fig. 4F. The ratio of the DEX group was significantly higher than that of the control group.

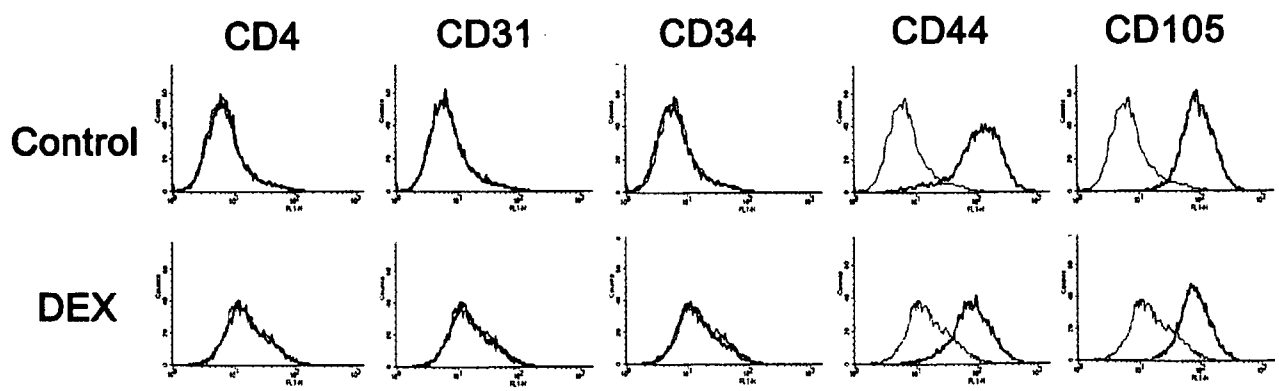

Fig. 1. Surface marker profiles. Cell surface markers were analyzed using flow cytometry. The top line is the control group and the bottom is the DEX group. Both BMMCs were negative for hematopoietic and endothelial markers and positive for mesenchymal markers.

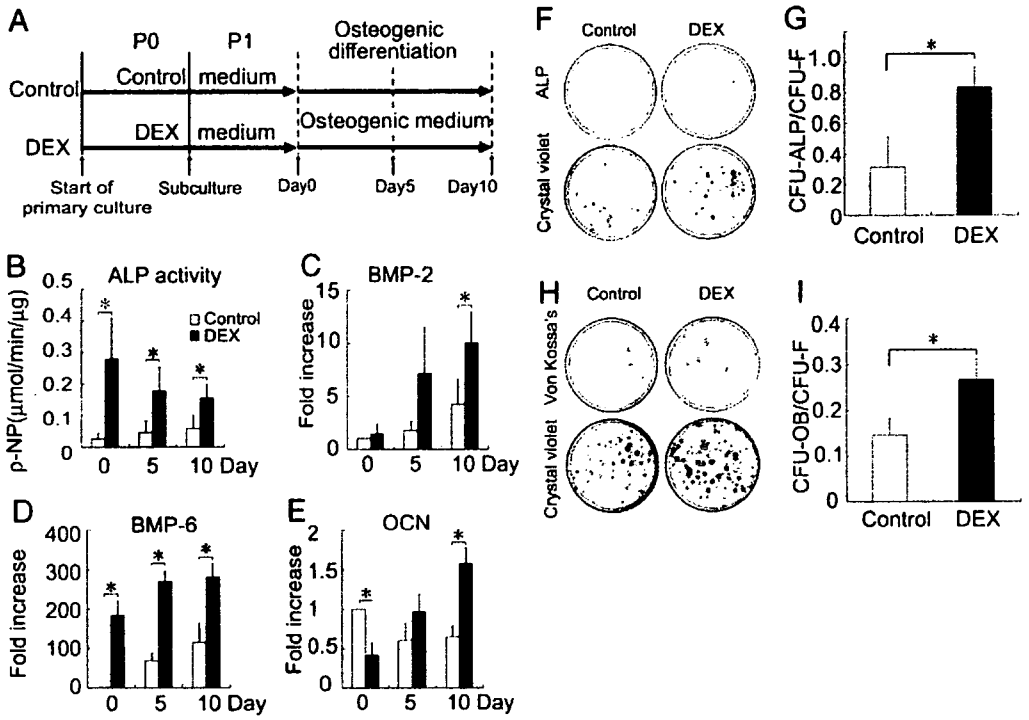

Fig. 2. Osteogenic differentiation. (A) Schematic representation of the cell culture protocol. (B) ALP activity ($n=4$). (C–E) Quantitative analyses of mRNA expression of osteogenic markers: (C) BMP-2 and (D) BMP-6. (E) Osteocalcin. The fold change of gene expression was normalized to cell culture at control day 0 ($n=4$). (F–I) CFU assays. (F) Images of dishes after ALP staining (upper) and subsequent crystal violet staining (lower). (G) CFU-ALP-positive rate ($n=4$). (H) Images after von Kossa's staining (upper) and subsequent crystal violet staining. (I) CFU-OB-positive rate ($n=5$). Bars show the mean and SD. $*p<0.05$.

The PPARγ mRNA expression levels in the DEX group at days 0 and 14 were higher than those of the control group (Fig. 4G).

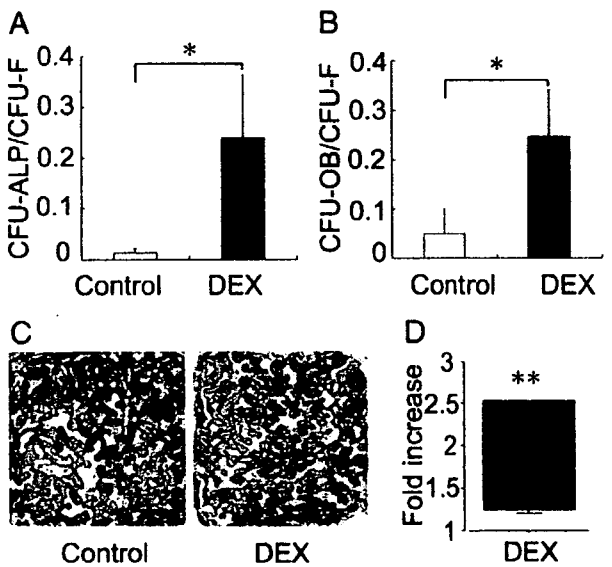

Fig. 3. Bone formation analyses. (A) CFU-ALP-positive rate and (B) CFU-OB-positive rate of monkey-derived BMMCs ($n=5$). (C) *In vivo* bone formation analysis at an extra skeletal site. Histological specimens were stained with hematoxylin and eosin. Scale bars indicate 1 mm. (D) Ratio of bone formation area of the DEX group to that of the control group at 4 weeks ($n=5$). Bars show the mean and SD. $*p<0.05$, $**p<0.01$.

From these results, we confirmed that continuous dexamethasone treatment through the proliferation stage of BMMCs augments differentiation potentials at least into the three lineages. Furthermore, from the results of CFU assays, we speculate that the compositional alteration of BMMCs may have played a role in this augmentation.

Colony formation rate analysis

BMMCs cultured with the control medium during primary culture (P0) were plated at clonal density and cultured in the same medium with or without dexamethasone through the colony formation period for 14 days. Each dish was then incubated in osteogenic medium for an additional 7 days (Fig. 5A). Colonies were stained with ALP stain, and ALP-positive colonies were counted.

Though there was no significant difference between the numbers of ALP-positive colonies in both groups, the total colony number of the dexamethasone-treated dishes was smaller than that of the control group (Figs. 5B and C).

Caspase-3 assay

To test whether apoptosis was involved in dexamethasone-induced inhibition of colony formation, dexamethasone-induced caspase-3 activity was assayed. Fig. 6A shows a schematic representation of the experimental protocol. BMMCs cultured with the control medium were passaged and, after the

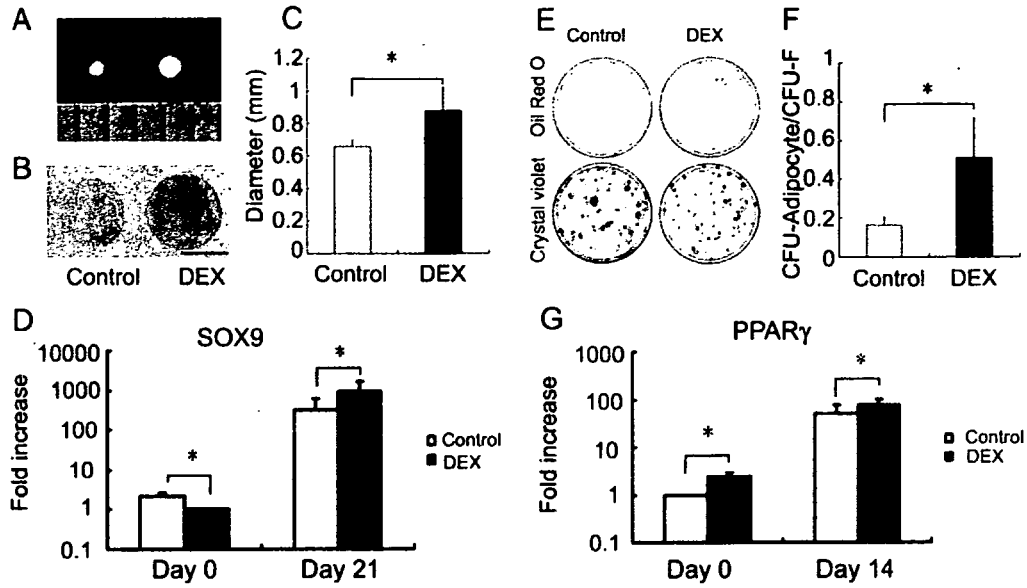

Fig. 4. Chondrogenic and adipogenic differentiation. (A–D) Chondrogenic differentiation. (A) Chondrogenic pellets of the control and DEX groups after 21 days of chondrogenic culture. Pellets were placed next to a 1-mm scale ruler. (B) Toluidine blue staining of pellets. The scale bar indicates 0.5 mm. (C) The diameters of the pellets ($n=4$). (D) mRNA expression of sox9 at day 0 and 21 of the chondrogenic culture. The fold change of gene expression was normalized to the control at each time point ($n=6$). (E–G) Adipogenic differentiation. (E) CFU-adipocyte assay. Dishes were stained with Oil Red O (upper) and subsequently stained with crystal violet staining (lower). (F) Positive ratio of Oil Red O-positive colonies (CFU-Adipocyte) ($n=4$). (G) mRNA expression of PPARγ relative mRNA expression level at day 0 and 14 ($n=4$). Bars show the mean and SD. $^*p<0.05$.

culture dishes became subconfluent, $DEX^{-/+}$ group cells were cultured in the presence of dexamethasone for 5 days. Subsequently, caspase-3 activity was measured. Caspase-3 activity was significantly increased in the $DEX^{-/+}$ group (Fig. 6B), indicating dexamethasone induced apoptosis of BMMCs.

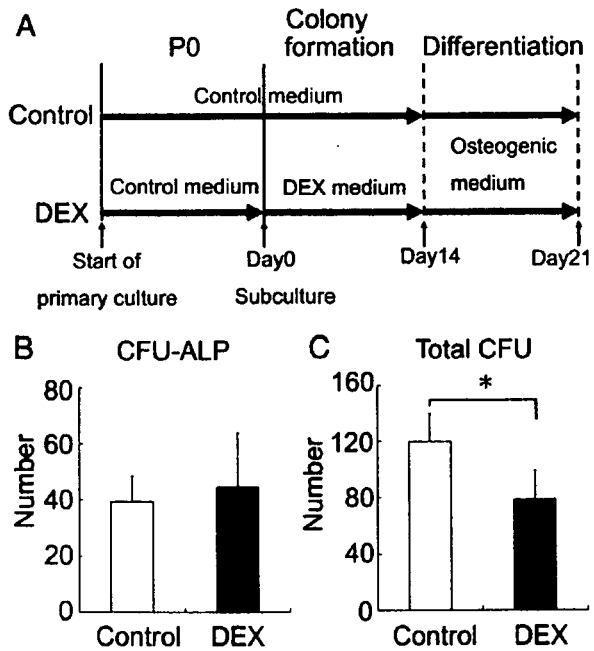

Fig. 5. (A) Scheme of CFU culture protocol. (B, C) The numbers of ALP-positive colonies and total colony number. Bars show the mean and SD. $n=5$, $^*p<0.05$.

CFU-apoptosis assay

The mechanism of dexamethasone-induced apoptosis in the compositional alteration of BMMCs was analyzed by the CFU-apoptosis assay. BMMCs (P0) cultured in control medium were seeded at the density used for CFU assays and cultured for 14 days. After colonies formed, dexamethasone was added to the medium and dishes were sequentially stained to detect apoptotic and ALP-positive colonies.

Most of the colonies that stained positive for apoptosis or ALP were stained homogeneously across the whole colony, demonstrating that apoptosis was induced selectively at specific single cell-derived populations (Fig. 6C). The distribution of apoptotic colonies and ALP-positive colonies is shown in Fig. 6D. Most of the apoptotic colonies were negative for ALP.

Flow cytometry analysis

To make sure that dexamethasone induces apoptosis in ALP-negative cells and increases ALP-positive cells, we also analyzed the control and dexamethasone-treated cells by flow cytometry. There was an increase in annexin-V-positive/ALP-negative cells from 1.52% to 3.55% and annexin-V-negative/ALP-positive cells from 10.07% to 21.06% after incubation with DEX for 3 days. (Fig. 6E) Annexin-V-positive cells were also confirmed to be 7-AAD negative, which indicates cell necrosis.

Discussion

Recently, many studies [4,5,17–19] and clinical trials [20–27] focused on the multi-potency of human BMMCs have been

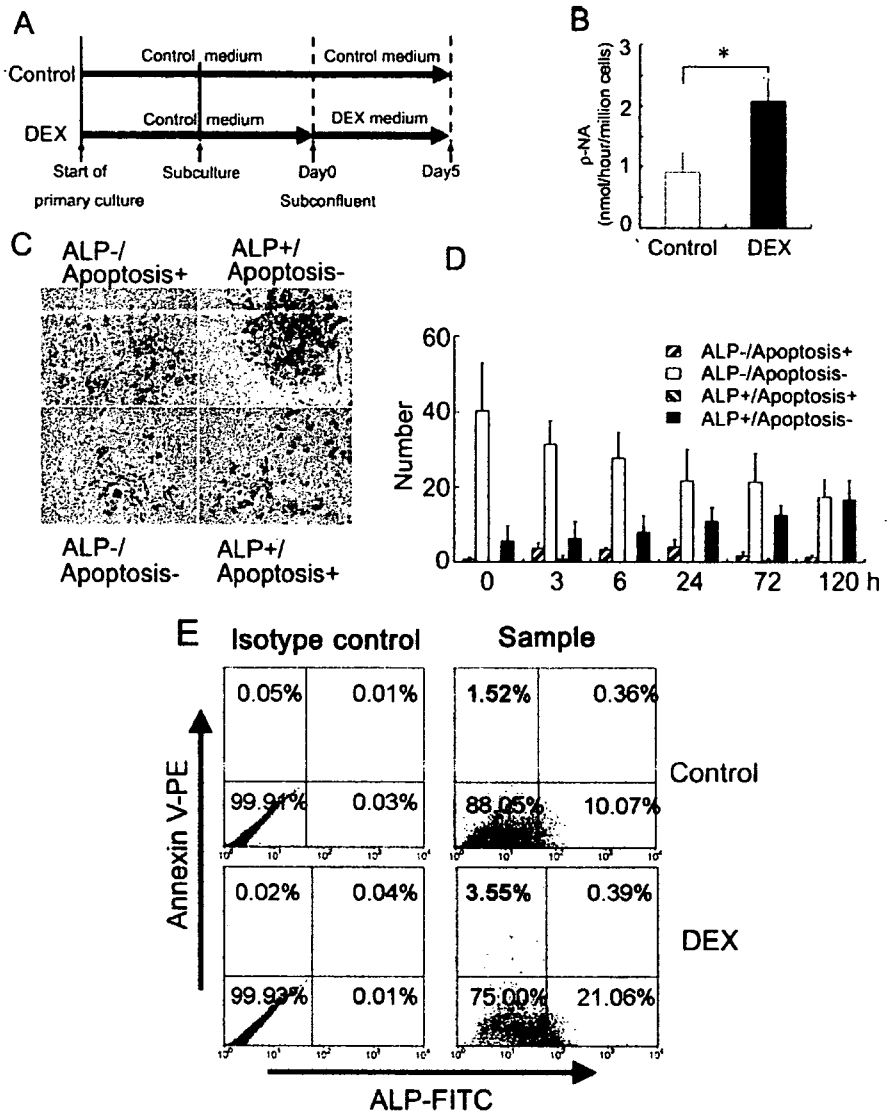

Fig. 6. Apoptotic assays. (A, B) Effects of dexamethasone on caspase-3 activity. (A) Schematic representation of the cell culture protocol. (B) Caspase-3 activity of each group ($n=4$). (C, D) Apoptotic stain for detection of early apoptotic cells. (C) Apoptotic cells were stained red, and ALP-positive cells blue. (D) Colony number of ALP (+ or −)/apoptosis (+ or −) ($n=3$). Bars show the mean and SD. *$p<0.05$. (E) Flow cytometric analysis for annexin V and ALP after 3 days of dexamethasone treatment. As a control, hBMMCs cultured without dexamethasone were analyzed. The left panels indicate the isotype control of each group.

reported. Whereas the BMMCs in most of these reports were expanded with standard protocols using combinations of culture media and bovine serum, it is well known that the differentiation capability of the cells diminishes with cell proliferation. We focused on dexamethasone, which has been used for differentiation of BMMCs into osteogenic, chondrogenic and adipogenic lineages. Based on these facts, we hypothesized that dexamethasone would facilitate the responsiveness of BMMCs to other differentiation reagents, which are used in combination with dexamethasone, and that continuous dexamethasone treatment throughout the culture period would facilitate BMMC responsiveness.

First, the differentiation capabilities of continuous dexamethasone-treated BMMCs were compared with those of BMMCs cultured in standard medium. The results of osteogenic differentiation studies indicated that the dexamethasone-treated cells showed higher osteogenic capability and bone formation capability than the control group. In particular, ALP activity and mRNA expression of BMP-6 were prominently higher in the DEX group. mRNA levels of osteocalcin a bone specific marker of later stages of osteogenic differentiation were two-fold higher in the DEX group than the control group at 10 days of differentiation, contrary to the comparison at the beginning of osteogenic induction. From these results, we presumed that continuous treatment retained the differentiation stage of BMMCs at premature osteoblast or less differentiated stages and raised their responsiveness to osteogenic induction. Consequently, the differentiation capability of BMMCs into an osteogenic lineage

was augmented. Furthermore, results of the bone formation study, in which the BMSCs were combined with osteoconductive β-TCP scaffolds providing an osteogenic environment, support our presumption. Our results are consistent with previously published reports about human BMMCs. Cheng et al. [28] reported that dexamethasone treatment of BMMCs increased ALP activity and suppressed secretion of osteocalcin. However, when BMMCs were cultured in dexamethasone free medium after 1 week of dexamethasone pretreatment, osteocalcin production stimulated by 1, 25-$(OH)_2D_3$, was increased compared to BMMCs without dexamethasone pretreatment [28]. Diefenderfer et al. [29] indicated that human BMMCs pretreated with dexamethasone were highly responsive to osteogenic induction by BMP-2 while BMMCs without dexamethasone treatment responded poorly [29]. Continuous dexamethasone treatment strongly enhanced BMP-6 gene expression and was consistent with previous reports [30], although the BMP-6 protein concentration secreted in the culture medium was not measured in our experiment. Friedman et al. [31] reported BMP-6 as the only BMP expressed prior to differentiation and as a key regulator of BMMC osteogenic differentiation, demonstrating the synergistic effects of the combination of dexamethasone/BMP-6 and dexamethasone/BMP-7 as well as BMP-6/BMP-7. Boden et al. [32] also confirmed using fetal rat calvarial cells (RC cells), which consist of a heterogeneous mixture of mesenchymal progenitors, that dexamethasone treatment up-regulates BMP-6 secretion and that dexamethasone pretreated cells exhibit more abundant bone nodules and osteocalcin production by osteoinduction by BMP-2 in comparison to no pretreatment. In addition, even when the pretreatment was substituted with BMP-6 protein, the increments in osteogenic differentiation were almost the same level as those in the dexamethasone pretreated cells [32]. Augmentation of the differentiation capability yielded by continuous dexamethasone treatment in our study would involve the enhancement of the BMP signaling pathway.

The effects of continuous dexamethasone treatment on chondrogenic and adipogenic differentiation were also tested, and the differentiation capability of dexamethasone-treated BMMCs into both lineages was also promoted. The mechanism of dexamethasone's role in chondrogenesis and adipogenesis is elusive. However, similar mechanisms to that of osteogenic differentiation are inferred from our results. There are some reports demonstrating enhancement of chondrogenic differentiation of human BMMCs by BMPs [10,11] and involvement of BMPs in adipogenic differentiation, although not of human BMMCs [33]. Another factor, FGF-2, was reported as a critical factor to maintain multi-potency of human adipose-derived stem cells [34]. In this study, cellular differentiation capabilities were diminished accompanied by decreased FGF2 secretion after multiple doublings (more than 70–80), while expression levels of FGF receptors were unaltered. In addition, decline of the differentiation capabilities of adipogenesis, osteogenesis and chondrogenesis were rescued by addition of FGF-2. In our study, although dexamethasone treatment showed ununiform regulation of FGF-2 mRNA expression in BMMCs from 4 donors, mRNA expression of FGFR-1 was upregulated 2–4 times in 3 of 4 BMMCs (data not shown). From these, enhancement of BMPs and/or FGFs signals might also facilitate multi-lineage differentiation potentials although this remains to be clarified.

Although the differentiation studies revealed that continuous dexamethasone treatment enhanced the multi-lineage differentiation potential, the mechanism responsible could not involve only BMP-signaling because results of CFU assays of osteogenic and adipogenic differentiation showed compositional alteration of BMMCs' subpopulations. BMMCs are a population originating from heterogeneous cells. Thus, the response of each cell subpopulation to dexamethasone must be varied. We postulated that dexamethasone-induced apoptosis would play a role in the alteration of subpopulations. The CFU-ALP assay demonstrated that dexamethasone selectively inhibited colony formation from the cells which would not turn ALP positive. The caspase-3 assay proved that dexamethasone induced apoptosis of BMMCs. Furthermore, the CFU-ALP assay, apoptosis assay and flow cytometric analysis demonstrated definitively that dexamethasone selectively induced apoptosis of ALP-negative BMMCs. We could not find a report of dexamethasone-induced apoptosis of BMMCs though there were a few reports of primary cultured osteoblasts [35], osteocytes [36] and osteogenic cell lines [37]. In these reports, dexamethasone was reported to induce apoptosis of relatively mature osteogenic cells, which is suggestive of and consistent with our results. Our studies revealed that dexamethasone induced apoptosis of BMMCs and has a role in BMMCs' differentiation. However, we assume that the effect of dexamethasone treatment on the selective proliferation of BMMCs can not be explained by apoptosis alone. Studies using the nodule formation assay with RC cells have revealed that dexamethasone treatment redistributed cell populations, resulting in increased populations that were capable of osteogenic [38], chondrogenic [39] and adipogenic differentiation [40] because the effects of dexamethasone were most potent at the log phase of cell proliferation. In these studies, the ability of dexamethasone to stimulate proliferation capability of cells with differentiation potentials was suggested to be a mechanism for the observed redistribution. Based on the fact that FGFR-1 mRNA expression of BMMCs in our studies were upregulated by dexamethasone, as mentioned above, dexamethasone might promote proliferation of some populations with high differentiation capability.

In conclusion, continuous dexamethasone treatment augments the multi-lineage differentiation capability of BMMCs. In addition, dexamethasone alters BMMC populations, and apoptosis is one of the mechanisms responsible for redistribution.

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Retrospective cohort study between selective and standard C3-7 laminoplasty. Minimum 2-year follow-up study

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Abstract A total of 64 patients with cervical spondylotic myelopathy (CSM) were assessed in this study. Forty-two patients underwent selective expansive open-door laminoplasty (ELAP). Twenty-two patients who underwent conventional C3-7 ELAP served as controls. There were no significant differences in recovery rate of JOA scores, C2–C7 angle or cervical range of motion between two groups. Incidence of axial symptoms and segmental motor paralysis in selective ELAP was significantly lower than those in the C3-7 ELAP. Size of anterior compression mass, postoperative spinal cord positions and decompression conditions were evaluated using preoperative or postoperative MRI in 50 of 64 patients. There was a positive correlation between number of expanded laminae and maximum anterior spaces of spinal cord. Incomplete decompression was developed in three of 37 patients in selective ELAP and in two of 13 patients in C3-7ELAP. Mean size of anterior compression mass at incomplete decompression levels was significantly greater than that at complete decompression levels. Since, there was less posterior movement of the spinal cord in selective ELAP than that in C3-7ELAP, minute concerns about size of anterior compression mass is necessary to decide the number of expanded laminae. Overall, selective ELAP was less invasive and useful in reducing axial symptoms and segmental motor paralysis. This new surgical strategy was effective in improving the surgical outcomes of CSM, and short-term results were satisfactory.

Keywords Selective cervical laminoplasty · Cervical spondylotic myelopathy · Axial symptoms · Segmental motor paralysis · Posterior shift of spinal cord

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Introduction

In most patients with cervical spondylotic myelopathy (CSM), cervical laminoplasty has been performed for posterior cervical surgery. There are many variations in surgical procedure and good clinical results have been reported for many of them [11]. However, there are no clear criteria for the number of the laminae that require treatment and C3–C7 laminoplasty has been performed in most cases [3, 7, 13].

Although the long-term outcomes of the laminoplasty are stable [7,8], postoperative problems such as cervical malalignment [7], restriction of range of motion (ROM) [9], persistent axial symptoms [4] and segmental motor paralysis [1] have been reported. To prevent these problems, recent studies have attempted less invasive surgical procedures [14].

Since 2001, we have indicated selective expansive open-door laminoplasty (selective ELAP) to reduce the damage to cervical posterior elements such as muscle and facet joints. English literature dealing with selective cervical laminoplasty is scant. The purpose of this study was to evaluate the efficacy and short-term results of selective ELAP for CSM.

Materials and methods

A total of consecutive 64 patients with CSM were assessed in this study. Patients with kyphotic deformity treated with decompression in conjunction with correction were excluded.

In selective ELAP, open-door laminoplasty was performed only at stenosis levels, and partial laminectomy of the upper half of lamina at the most inferior stenosis level was combined with laminoplasty. To determine the canal stenosis, magnetic resonance imaging (MRI) of cervical spine was used. The stenosis levels were defined by the disappearance of the subarachnoid space at the T2-wighted sagittal images (Fig. 1a). Forty-two patients underwent selective ELAP since 2001 and the mean follow-up period was 33 months. The mean age at the time of surgery was 62 years. The mean pre-operative Japanese Orthopaedic Association (JOA) score was 9.2 points. The mean preoperative C2–C7 angle was 19.9°. The mean cervical range of motion (ROM), which was measured as the angle between C2–C7 on plain radiographs of lateral flexion and extension, was 41.2°. The mean level of canal stenosis was 2.8 levels. Assuming that partial laminectomy was calculated at 0.5 laminae, the mean number of treated laminae was 3.2 (range 1.5–4.5).

Twenty-two patients with CSM who underwent conventional C3-7 ELAP in our hospital before 2001 served as controls and the mean follow-up period was 72 months. The mean age at the time of surgery was 64 years. The mean pre-operative JOA score was 10.4 points. The mean preoperative C2–C7 angle was 21.6°. The mean cervical ROM was 40.6°. The mean level of canal stenosis was 2.5 levels. There was no significant difference of pre-operative parameters between the two groups (Table 1).

Table 1 Preoperative patient demographics

	Selective ELAP	C3-7ELAP
Gender	Man: 28, woman: 14	Man: 17, woman: 5
Age	62 (39–85)	64 (39–79)
JOA score	9.2 (4–14.5)	10.4 (2.5–14.5)
C2–C7 angle	19.9 ± 1.9°	21.6 ± 4.7°
Range of motion	41.2 ± 1.8°	40.6 ± 2.9°
Stenosis level	2.8 (1–4)	2.5 (1–4)
Number of expanded laminae	3.2 (1.5–4.5)	5

Values are indicated as mean ± standard error and range is given in parenthesis

Surgical indication and technique for selective ELAP

The criteria of the surgical method used in the current study was as follows: anterior spinal fusion was indicated for patients with CSM in whom the spinal canal stenosis caused by anterior compression mass is one or two levels and the spinal canal is 13 mm and over. On the other hand, ELAP was indicated for patients with multilevel compression myelopathy, developmental spinal canal stenosis (less than 13 mm), or spinal canal stenosis caused by posterior elements such as the ligamentum flavum.

To determine the decompression area, MRI and plain radiographs of cervical spine were used for selective ELAP. The laminae to expand were determined as follows: from one level above the most cranial stenosis level to upper half partial laminectomy of the most caudal stenosis level. The method described below is based on three-level stenosis case from C4–C5 to C6–C7 (Fig. 1a). After longitudinally dividing the nuchal fascia in line with the mid line incision, the laminae from C4 to upper half lamina of C7 were exposed. The C3–C4 and C6–C7 interlaminar spaces were exposed and the ligamentum flavum at those levels were removed. In accordance with the so-called Hirabayashi's open-door method, [3] open-side and hinge-side gutters were made using a high-speed drill at the C4, C5 and C6 laminae. Before the 'open-door' technique, the upper half laminectomy of C7 was performed leaving untouched the attachment of the semispinalis cervicis and

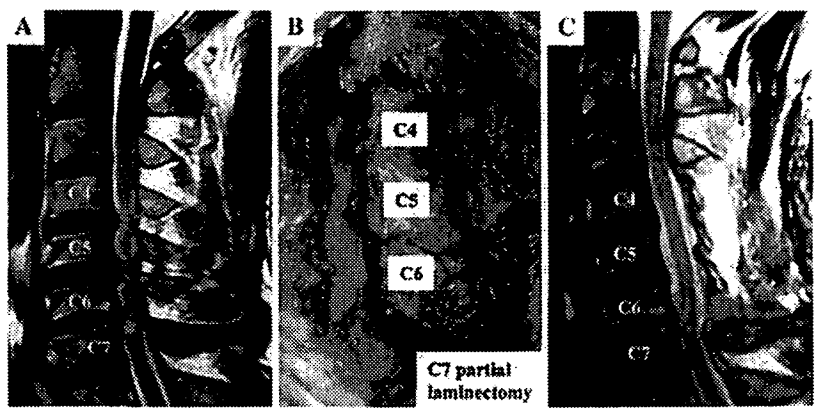

Fig. 1 Case presentation of selective ELAP. **a** Preoperative MRI shows three-level stenosis from C4/5–C6/7. The stenosis levels were defined by the disappearance of the subarachnoid space. **b** Intraoperative photograph. In this three levels stenosis case, the laminae from C4 to C6 laminae were opened in conjunction with upper half laminectomy of C7. **c** Postoperative MRI

multifidus muscle of C7. The laminae were elevated and secured to the facet joints by using sutures (Fig. 1b, c).

Surgical technique for conventional C3-7 ELAP

For conventional C3-7 ELAP, surgical procedures were performed according to Hirabayashi's method [3].

Postoperative care

The patients of both groups were allowed to sit up or walk between 2 and 5 days after surgery with a cervical brace for 3 weeks. Isometric and isotonic muscle contraction of the posterior neck was recommended after surgery as soon as possible.

Evaluation

Clinical and radiological results were evaluated at 1 year and 2 years after surgery. Clinical recovery rates were evaluated using Japanese Orthopaedic Association (JOA) scores. Local symptoms (pain and stiffness), or so-called axial symptoms, were evaluated using an axial symptom scoring system that was developed at our institution. In this scoring system, there were four parameters (i.e., posterior neck pain, posterior neck stiffness, shoulder pain, shoulder stiffness) and each parameter was classified into four grades (i.e., none: 3, occasional mild: 2, continuous mild or occasionally severe: 1, continuously severe: 0). The analgesics utilized after surgery were also evaluated. The incidence of postoperative segmental motor paralysis, or so-called C5 palsy, was examined in both groups during the follow-up period.

Preoperative and postoperative cervical alignment was evaluated using C2–C7 angle. Cervical ROM was measured as the angle between C2–C7 on plain radiographs of lateral flexion and extension. The % ROM was calculated using the following formula: (post operative ROM/preoperative ROM) × 100.

The size of anterior compression mass, postoperative spinal cord positions and decompression conditions were evaluated using preoperative or postoperative MRI in 50 of 64 patients (selective ELAP: 37, C3-7ELAP: 13), in which a quality of MRI was enough to investigate. Postoperative MRI was obtained 1–6 months after surgery. MRI of T2-weighted sagittal image was analyzed using Scion Image Beta 4.02 (Scion Co., Maryland, USA). A baseline was drawn to link the middle point of posterior vertebral body margin, and the distances from the baseline to the posterior edge of anterior compression mass (i.e., size of anterior compression mass) or to the anterior edge of spinal cord (i.e., anterior space of spinal cord) at the disc level were measured at the stenosis levels in the selective ELAP group and at all four levels in the C3-7 ELAP group (Fig. 2).

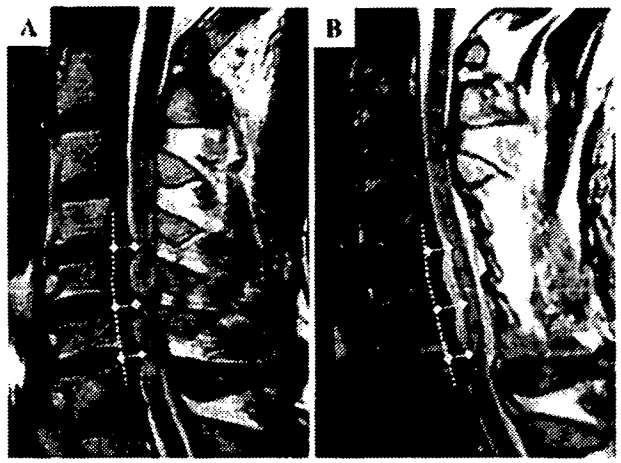

Fig. 2 Size of anterior compression mass and anterior space of spinal cord. Figure shows C4–C6 expansive open-door laminoplasty in conjunction with C7 upper half laminectomy of three-level stenosis case. A baseline (*dotted line*) was drawn to link the middle point of posterior vertebral body margin and the anterior compression mass (**a**) and anterior space of the spinal cord (**b**) were measured at the disc levels

Statistical analysis

Data were expressed as the mean ± standard error. Comparisons between the selective and conventional C3-7 ELAP groups were made by a *t* test, a Mann–Whitney's *U* test or Fisher's exact probability test. Correlations were evaluated using Spearman's correlation coefficient by rank test. Differences with *P* values of less than 0.05 were considered significant.

Results

Clinical and radiological results were shown in Table 2. There was no significant difference of the recovery rate of JOA score, C2–C7 angle and %ROM between the two groups.

The mean axial symptoms score of 11.5 (1-year) and 11.4 (2-years) in selective ELAP group was significantly higher than those of 9.5 (1-year) and 10.1 (2-years) in C3-7 ELAP group. The analgesics utilized in the selective ELAP group were significantly less than those in C3-7 ELAP group at 1 year after surgery, but not significantly different at 2 years after surgery.

Although none of the patients in the selective ELAP group exhibited postoperative segmental motor paralysis,

Table 2 Clinical and radiological results

	Selective ELAP	C3-7 ELAP	Statistical analysis
Recovery rate of JOA score (1-year)	58.7 ± 3.6%	52.7 ± 5.8%	NS
Recovery rate of JOA score(2-year)	57.7 ± 3.6%	53.4 ± 5.0%	NS
Axial symptoms (1-year)	11.5 ± 0.1	9.5 ± 0.5	$P < 0.001$
Axial symptoms (2-year)	11.4 ± 0.1	10.1 ± 0.5	$P = 0.026$
Analgesics (1-year)	3/42	8/22	$P = 0.006$
Analgesics (2-year)	4/42	4/22	NS
C5 palsy	0/42	3/22	$P = 0.037$
C2–C7 angle (1-year)	21.2 ± 2.0° (107%)	21.4 ± 4.0° (99%)	NS
C2–C7 angle (2-year)	20.9 ± 1.9° (105%)	22.4 ± 3.8° (99%)	NS
%ROM (1-year)	70.4 ± 5.2%	72.3 ± 9.9%	NS
%ROM (2-year)	70.6 ± 7.8%	65.4 ± 8.9%	NS

Post/pre-operation % are given in parenthesis

the paralysis developed in three of 22 patients in the C3-7 ELAP group. The incidence of segmental motor paralysis in selective ELAP group was significantly lower than that in C3-C7 ELAP group. C5, C6 and C7 palsy was developed at two-day, four-day and 1.5-month after surgery, respectively in C3-7 ELAP groups. Spontaneous recovery was observed in two cases at 5 and 6 months after surgery.

There was no significant difference of the mean size of anterior compression mass between the two groups (Table 3). The postoperative anterior space of the spinal cord was shown in Fig. 3. The anterior space of the spinal cord ranged from 3.0 to 8.7 mm, and the maximum spaces were obtained at the middle of laminoplasty. The mean value of maximum anterior space of the spinal cord was 5.5 mm in the selective ELAP group and was 5.9 mm in C3-C7 ELAP group (Table 3). The more the number of expanded laminae increased, the more the maximum anterior space of the spinal cord enlarged. There was a positive correlation between the number of expanded laminae and the maximum anterior spaces of the spinal cord (Fig. 4).

Incomplete decompression was defined as residual concave deformity of spinal cord caused by compression mass using postoperative MRI. Incomplete decompression was developed in 3 of 37 patients in the selective ELAP and in 2 of 13 patients in the C3-7ELAP group (Table 3). All of compression mass at incomplete decompression levels were 6 mm and over. The mean size of anterior compression mass at incomplete decompression level was significantly greater than that at complete decompression level (Fig. 5). However, the recovery rates of JOA scores were 64.2 ± 5.5% in incomplete decompression cases and 57.9 ± 3.0% in complete decompression cases.

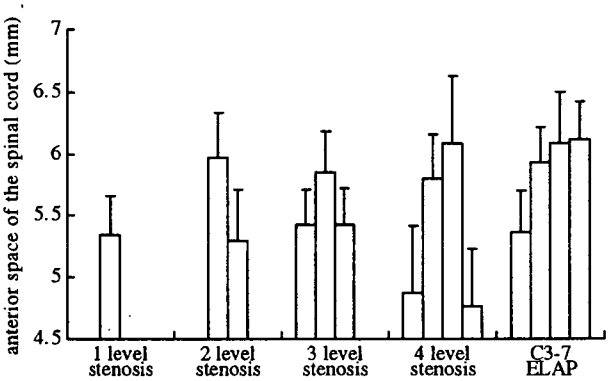

Fig. 3 Anterior space of spinal cord. The anterior space of spinal cord increased in the middle position of the laminoplasty. The maximum anterior space of spinal cord showed a tendency to increase in proportion to the number of expanded laminae

Discussion

The optimal surgical treatment for multilevel cervical spinal cord compression caused by cervical spondylosis remains controversial. Anterior decompression and fusion [2, 18], laminectomy with or without fusion [5], and expansive laminoplasty [3] have all been performed to treat multilevel CSM. In Japan, laminoplasty is the most common procedure out of concern for complications [2, 18]

Table 3 Preoperative and postoperative MRI findings

	Selective ELAP	C3-7 ELAP	Statistical analysis
Anterior compression mass	3.9 ± 0.1 mm	3.8 ± 0.2 mm	NS
Maximum anterior space of spinal cord	5.5 ± 0.1 mm	5.9 ± 0.1 mm	$P = 0.015$
Incomplete decompression	3/37	2/13	NS

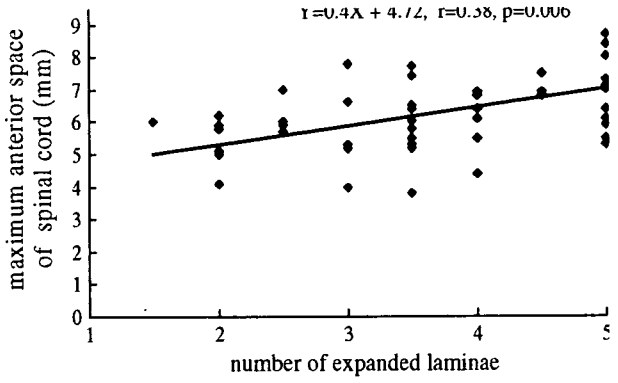

Fig. 4 Maximum anterior space of spinal cord associated with number of expanded laminae. There was a significant correlation between the maximum anterior space of spinal cord and the number of expanded laminae ($r = 0.38$, $P = 0.006$)

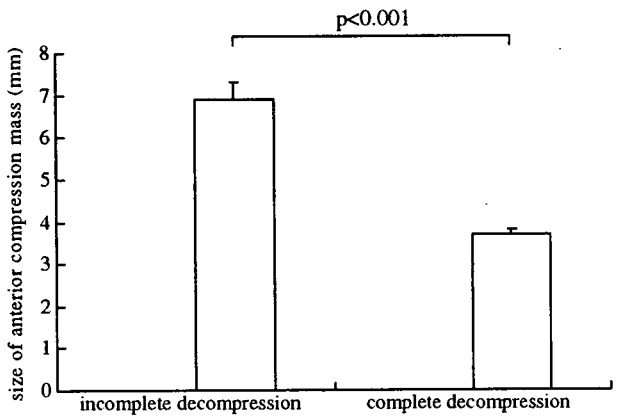

Fig. 5 Size of anterior compression mass associated with postoperative decompression condition. The mean size of anterior compression mass was 6.4 ± 0.2 mm at incomplete decompression levels and 3.7 ± 0.1 mm at complete decompression levels ($P < 0.001$)

such as dislodgement of graft bone, pseudoarthrosis, adjacent level's stenosis, kyhotic deformity and instability.

Although satisfactory results of the laminoplasty are maintained for more than 10 years after surgery [7], several postoperative problems including neurological deterioration caused by malalignment [8], restriction of ROM [18], axial symptoms [4] and postoperative C5 palsy [1, 12] have been reported.

The common purpose of various laminoplasty procedures is to obtain the posterior movement of the spinal cord. Generally, five laminae (i.e., C3–C7) are indicated for expansion in most institutions. Before 2001, C3-7 ELAP was indicated for all patients in our institute for the purpose of obtaining sufficient posterior movement of the spinal cord. However, in a postoperative MRI of C3-7 ELAP, excessive posterior movement of the spinal cord is not unusual. Therefore, it is doubtful whether C3-7 laminoplasty, which expands five laminae, is necessary for all patients with CSM. Since 2001, we have indicated selective ELAP to reduce postoperative problems.

Although the present study has some limitations of research methodology (i.e., irregular operator or appraiser and the difference of therapeutic period between the two groups), the pre-operative parameters of both groups were almost equal between the two groups. Therefore, the study design of this study is a retrospective cohort study indicating different surgical strategies for two groups.

The results of the present study demonstrated that there was no significant difference in neurological recovery rates between the two groups. Because the mean number of the laminae managed with surgery was 3.2 in selective ELAP, this procedure was less invasive for facet joints, ligaments and muscle than conventional C3-7 ELAP.

The high incidence of a change from a preoperative lordotic alignment to a postoperative straightened or kyphotic alignment after laminoplasty has been reported [7,8]. Since there is a relationship between severe local kyphosis and poor surgical outcomes [15], maintaining the cervical alignment is one of the keys to satisfactory neurological recovery. In our results, the cervical alignments evaluated using the C2–C7 angle were maintained in both groups, in contrast to previous reports [7, 13, 18]. In the present study, cervical alignments may have been maintained in both groups because we encouraged the patients to do early active cervical exercises after surgery and the cervical brace was worn only for 3 weeks.

There are some confusing aspects about cervical ROM in the literature on laminoplasty. Some authors have asserted that limitation of ROM leads to a reduction of dynamic factors and is associated with satisfactory neurological improvement [10]. In contrast, other authors have noted the importance of preserving cervical ROM to reduce axial symptoms [6] or to maintain cervical alignment [9]. The decrease of cervical ROM was not significantly different between the two groups in the present study. That is, reducing the surgical invasion did not contribute to maintenance of cervical ROM. However, other authors of long-term follow-up studies have demonstrated a clear trend toward chronological loss of cervical ROM after laminoplasty [7, 13], so significant differences in the decrease of ROM between the selective ELAP and C3-7 ELAP may emerge at long-term follow-up.

The incidence of axial symptoms (pain or stiffness in the neck or shoulder) varies markedly [4, 17] largely because of differences in the definition of what constitutes axial symptoms or when axial symptoms are recorded. Although several studies have recently reported the importance of axial symptoms [4, 6, 14] their underlying mechanisms are not fully understood. Potential sources include the cervical disc, musculature, facet joints, spinal cord, and nerve roots [4,17]. Axial symptoms were evaluated using semi-

quantitative scoring system in this study. The present study demonstrated that selective ELAP, which was less invasive for the posterior elements, could reduce the axial symptoms not only at 1 year but also at 2 years after surgery. This suggests that axial symptoms may be caused in part by musculature or facet joints.

Segmental motor paralysis, or so-called C5 palsy, is seen occasionally in patients treated with laminoplasty, and in studies in which C5 palsy is reported, the mean incidence is about 5–8% [11, 12]. It is interesting that C5 palsy was not observed after selective ELAP in the present study. Although the mechanisms underlying C5 palsy remain unclear, some possible causes (i.e., nerve root and spinal cord) have been reported [1, 12]. One of the causes of C5 palsy has been attributed to a nerve root tethering effect [16] caused by excessive posterior movement of the spinal cord after decompression. The present study demonstrated that the number of expanded laminae is correlated with the maximum anterior space of the spinal cord. Therefore, the mechanism of less incidence of C5 palsy in selective ELAP may be elucidated by less posterior movement of the spinal cord in selective ELAP than after C3-7 ELAP. All these results suggest that selective ELAP has the potential to reduce the incidence of C5 palsy. However, high incidence of C5 palsy in C3-7 ELAP may affect the statistical significance in the current study; further, a large study is necessary to evaluate the evidence.

Incomplete decompression caused by anterior compression mass developed in five patients. The size of anterior compression mass at incomplete decompression level was significantly greater than that of complete decompression level, and was 6 mm and over at all incomplete decompression levels. The results of the current study demonstrated that one of the risk factors for incomplete decompression is the size of anterior compression mass. In the selective ELAP, there was less posterior movement of the spinal cord than that in C3-7ELAP, minute concerns about the size of anterior compression mass is necessary to decide the number of expanded laminae. Therefore, in the case of anterior compression mass over 6 mm at most inferior or superior level, one more lamina is expanded in our new strategy.

Overall, selective ELAP was useful in reducing surgical invasions and postoperative problems compared with conventional C3-7 ELAP. However, further study and long-term follow-up investigations will be necessary to certify the firm evidence.

Conclusions

Selective ELAP was less invasive and useful in reducing axial symptoms and segmental motor paralysis. This new surgical strategy was effective in improving the surgical outcomes of CSM, and short-term results were satisfactory.

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特集

脊髄損傷再生医学の最前線

脊髄損傷再生の研究動向—各施設から

ラットを用いた胸髄レベルでの脊椎・脊髄短縮モデル*

脊髄損傷後の慢性期における完全麻痺の治療を想定して

片岡　秀雄**　吉田佑一郎　鈴木　秀典
屋良　貴宏　田口　敏彦

はじめに

本邦では，年間約5,000人の脊髄損傷患者が発生すると言われている[9]．脊髄損傷により，運動・知覚麻痺，膀胱直腸障害などが起こり，ステロイド大量投与や手術による除圧，固定が行われるが麻痺の改善が不十分な場合も多い．

脊髄損傷の動物実験モデルにおいて，薬物療法[8]や細胞移植療法[7]が行われている．われわれは，細胞移植療法として骨髄間質細胞移植を行い，その有効性を報告している[4]．薬物療法や細胞移植療法は，脊髄不全麻痺に対してはある程度の効果があるが，完全麻痺に対する十分に効果的な療法は，いまだに報告されていない．

脊髄損傷により，損傷部で上行，下行伝導路が遮断され，運動，知覚，膀胱直腸障害が起きる．われわれは，脊髄損傷慢性期の患者の特に胸髄レベル損傷による完全麻痺の治療としての脊髄短縮術を考案した．脊髄短縮術では，損傷部の脊髄を切除した後に脊髄を短縮し，断端部を吻合させる(図1)．われわれのモデルは現在，開発の途中の段階ではあるが，これまでの成果を報告する．

Key words
脊髄損傷 (spinal cord injury)
完全麻痺 (complete paralysis)
脊髄短縮 (spinal cord shortening)

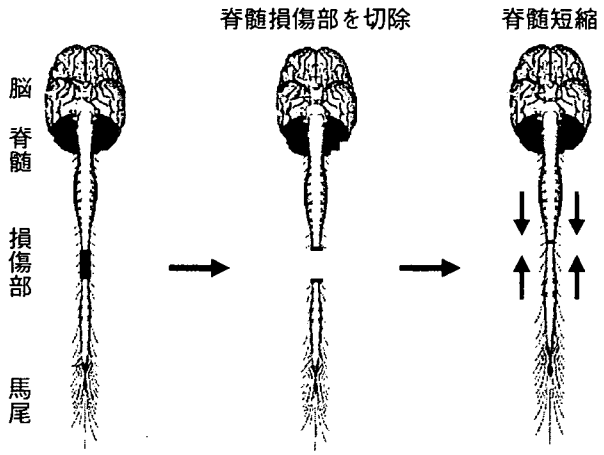

図1　胸髄レベルにおける脊髄短縮術

対象と方法

生後13～16週の雌のWistarラットを実験に使用した．手術時の麻酔はケタミンとキシラジンの筋肉内注射にて行った．ラットの背側に皮切を行い，傍脊柱筋を剝離し，第6胸椎から第10胸椎までの展開を行った．次に，第7胸椎から第9胸椎までの椎弓切除を行った．第6胸椎，第10胸椎に直径1.0 mm，長さ4.0 mmのステンレス製の

* A New Rat Model of Vertebra Body and Spinal Cord Shortening at Thoracic Level—For Future Application to Treat Patients in Chronic Period with Complete Paralysis after Spinal Cord Injury

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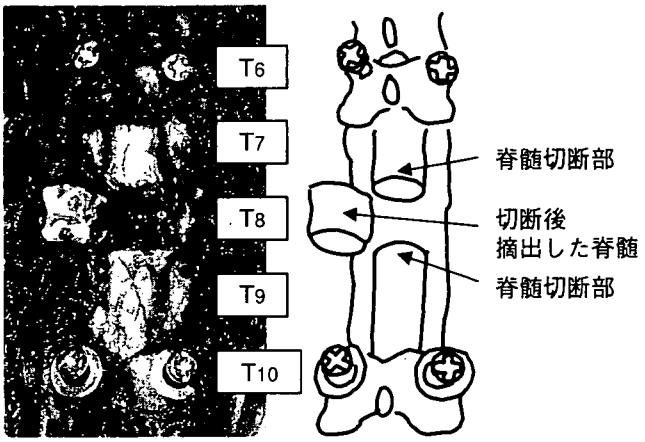

図 2　ラット胸髄レベルの脊髄短縮術（1）
脊髄切断後.

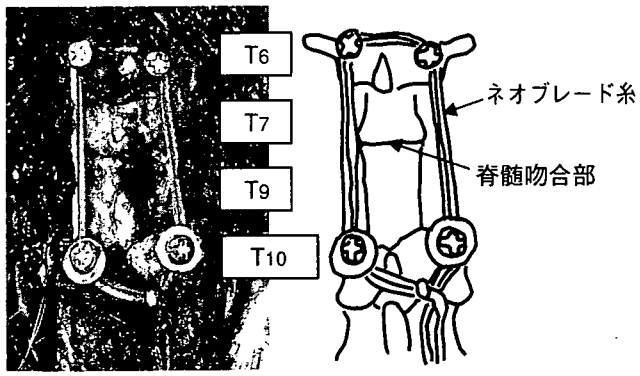

図 3　ラット胸髄レベルの脊髄短縮術（2）
脊髄短縮後.

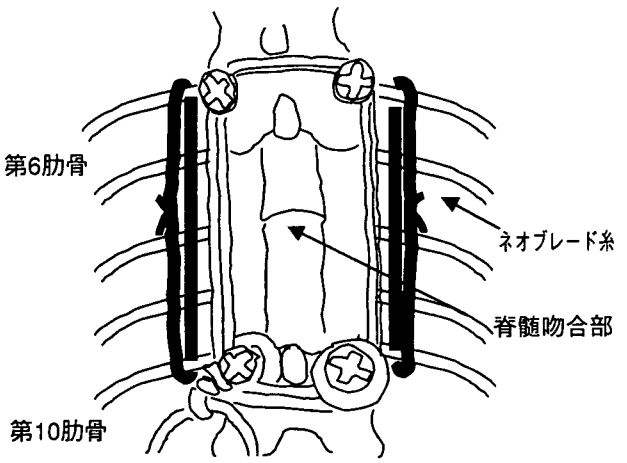

図 4　ラット胸髄レベルの脊髄短縮術（3）
第 6 肋骨と第 10 肋骨を糸を用い両側で固定する.

screw を pedicle screw として両側に挿入した. 第 10 胸椎には内径 1.5 mm のステンレス製のワッシャーも使用した.

脊髄を第 8 胸椎上縁，下縁レベルの 2 カ所（幅 4 mm）において Feather 社製の No. 11 のメス刃を用いて水平断の方向に完全切断した（図 2）.

切断した脊髄を摘出した後に，第 8 胸椎を電動ドリルにて削り，尖刃を用いて $T_{7/8}$, $T_{8/9}$ の椎間板を切除した. 第 6 胸椎，第 10 胸椎に挿入した screw，ワッシャーに 1-0 ネオブレード糸(アルフレッサ・ファーマ社製）をかけた. 第 7 胸椎椎体と第 9 胸椎椎体が接し，脊髄切断部の中枢端と末梢端が接合するように引き寄せ固定した（図 3）.

脊髄吻合部の転位が起こるため，両側の第 6 肋骨と第 10 肋骨のネオブレード糸による固定を追加することにした（図 4）.

術後 2 週間程度で自律性反射により膀胱が収縮して排尿が起こるまで，腹部上から膀胱を手指で圧迫して，排尿を 1 日 2 回行った. 以上の実験は山口大学医学部動物使用委員会の審査を受けた後に許可を得て行った.

結果

当初は術後のラットの長期生存が困難であったが，手術時の出血量の減少や，術後管理の工夫により術後 2 カ月以上の生存がほぼ可能となった.

実験ラット 1：生後 16 週，体重 252 g のラットを使用. 術後 19 日目から右下肢の筋肉部の周期的な収縮が時々みられるようになった. 術後 25 日目から両上肢を使っての前方移動時に，体の動きに合わせてそれを補助する形で，右下肢の股，膝，足関節がリズミックに動くのがみられた（図 5）. 右下肢の BBB スコアは足・膝関節 extensive，股関節 slight で 5 であった.

術後 62 日目に還流固定し，組織を摘出した. 右下肢の動きにより，脊髄の組織的吻合を予測していた. しかし，脊髄は短縮部より頭側部が背側に脱臼し，脊髄断端どうしの連続性はなく，非常に薄い瘢痕組織のみにより連続性が保たれていた.

実験ラット 2：生後 13 週，体重 240 g のラットを使用. 脊髄吻合部の転位を防ぐため T_6 と T_{10} 椎体間の screw＋ネオブレード糸固定に加えて，両側の第 6 肋骨と第 10 肋骨のネオブレード糸に

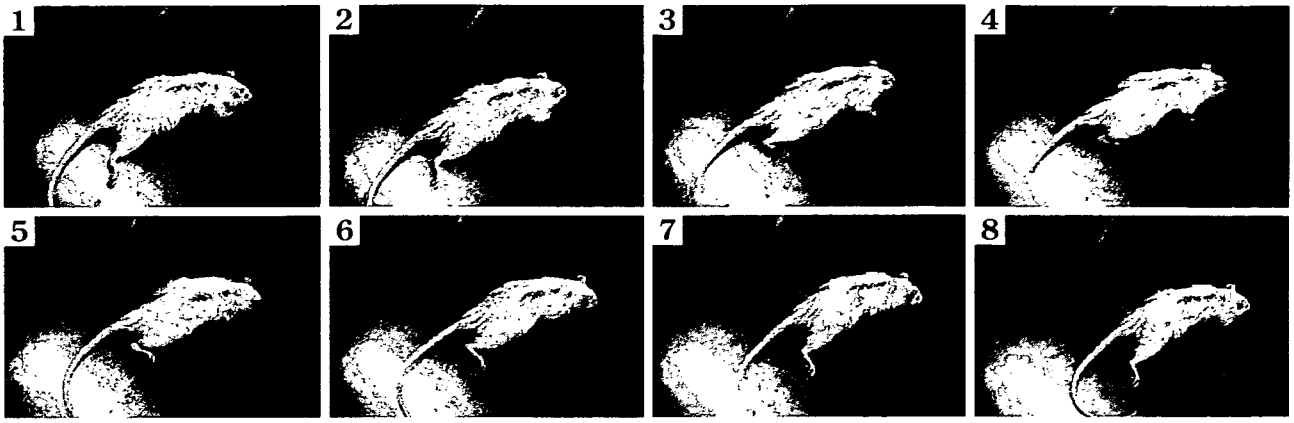

図 5　実験ラット 1：術後 25 日目
前進時に右下肢の動きがみられる．

よる固定を追加した（図 4）．

術後 5 週目に組織をみると椎体固定部は吻合部において転位がなく，脊髄断端の吻合部の転位もなかった．しかし，吻合部の間は瘢痕が入り込んでおり，脊髄神経組織の連続性はなかった（図 6）．

考察

脊髄腰膨大部には，central pattern generator (CPG) があると言われている．CPG は上位脊髄や末梢からの刺激入力がなくても，歩行のための足を動かす信号（step like efferent pattern）を発することができるとされている[2]．臨床のリハビリテーションの場においては，コンピューター制御されたトレッドミル上の免荷歩行を行うことにより脊髄不全麻痺に効果があることが，すでに欧米より数多く報告されている[10]．この効果は，末梢からの刺激が CPG に伝わり，CPG が活性化することによるとされている．われわれは CPG 存在の概念に基づき，脊髄損傷後の治療法確立に向けてラットを用いた機能的な神経筋の電気刺激法の基礎実験も行い，報告してきた[3]．

われわれの実験では，図 4 のように脊髄短縮術後にラットの前方移動時に上肢の動きと協調した右下肢の動きがみられた．脊髄の切断端どうしの組織の連続部は瘢痕しかなく，切断部を越えた神経軸索の連続性はみられなかった．右下肢の上肢の動きに同調したリズミックな動きは，体幹移動時に腹部が擦過されるなどによりなんらかの刺激

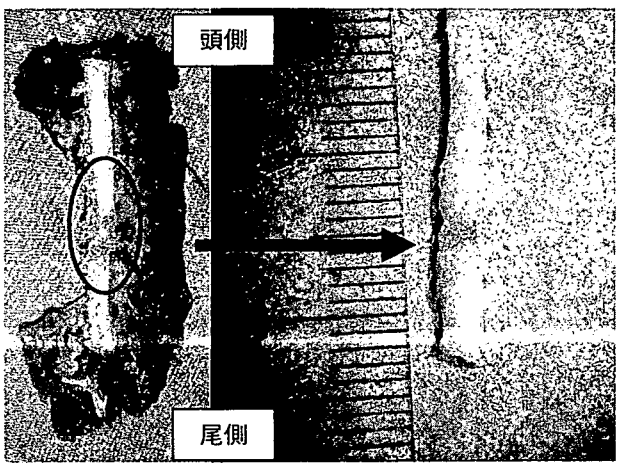

図 6　実験ラット 2：術後 5 週で脊髄を摘出
脊髄吻合部の転位（−），間に瘢痕組織（+）．

が腰膨大部の CPG に伝わり，引き起こされたと考えられた．

脊髄完全麻痺の場合，脳からの信号が損傷部を超えられず，腰膨大部の CPG まで神経電気信号が到達していないと考えられる．脊髄不全麻痺の場合，Swiss の Balgrist 大学の Dietz らは，10〜15%の軸索が損傷部を超えて，再接合すれば，脳からの信号が腰膨大部の CPG に伝わり，ヒトが歩行可能になると推察している[6]．われわれはその概念に基づき，ラットにおいて脊髄短縮術を行い，脳からの信号が損傷部を超えて，弱いながらも腰膨大部の CPG まで伝導すれば歩行可能になると考えている（図 7）．

20 世紀初頭に Cajal は，中枢神経はいったん損傷を受けると修復が困難であると提唱し[1]，以後

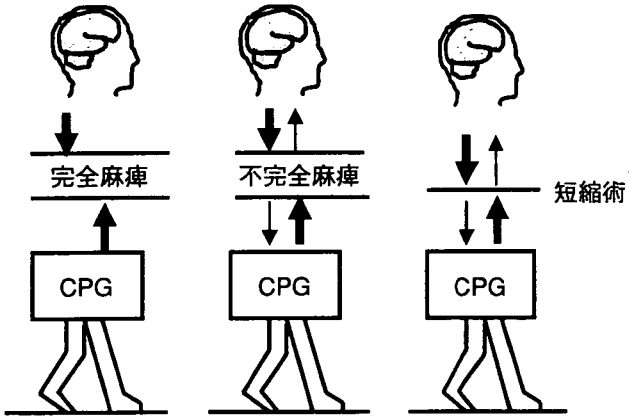

図 7　脳から CPG，CPG から脳への信号伝達の模式図[2)]

それは広く信じられてきた．しかし，最近になって脊髄切断後の中枢神経の軸索の良好な伸展の報告が多くみられるようになってきた[5)]．

われわれの実験の現在の課題の1つとして脊髄切断，除去後の吻合部に瘢痕が形成されており，吻合部を越えた神経軸索の伸長は起こっていないことが挙げられる．脊髄を外科手術用のメス刃を用いて切断しているが，刃が厚いため脊髄切断部に組織の比較的強い挫滅を起こしている．そのため瘢痕形成が起こり，神経軸索の伸展が阻害される一因となっていると思われる．現在は，より薄い工業用の剃刀を用い，脊髄を可及的に鋭利で atraumatic になるように切断している．

また脊髄吻合部の固定性を強めるために 10-0 ナイロン糸などで吻合部の縫合も必要である可能性がある．

将来的にはモデルに改良を加え，脊髄の吻合部を越えた神経軸索の接合が起きれば，脊椎・脊髄短縮術は脊髄損傷後の完全麻痺に対して臨床応用できる可能性があると考えられる．

まとめ

開発の途中の段階ではあるが，ラットを用いた脊椎・脊髄短縮術のこれまでの成果について報告した．

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