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지도 교수 강 성 범

이 논문을 의학석사 학위논문으로 제출함
2016년 8월

서울대학교 대학원
의학과
손 일 태

손 일 태의 의학석사 학위논문을 인준함
2016년 8월

위 원 장 김 태 우 (인)
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Isolation of internal and external sphincter progenitor cells for fecal incontinence: first study using human anal sphincter

by

Il Tae Son

A thesis submitted to the Department of Medicine, Graduate School in partial fulfillment of the requirements for the Degree of Master of Science in Clinical Medical Sciences at Seoul National University College of Medicine

August 2016

Approved by Thesis Committee:

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Professor Kang Sung-Bum Vice chairman

Professor Duck-Woo Kim

ABSTRACT

Isolation of internal and external sphincter progenitor cells for fecal incontinence: first study using human anal sphincter

Son Il Tae

Department of Medicine

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Seoul National University

Introduction: We investigated internal and external sphincter progenitor cells as potential tools for tailored cell therapies for fecal incontinence.

Methods: Sphincter progenitor cells were isolated with the preplate technique from normal internal and external anal sphincters from 10 patients with rectal cancer who underwent abdominoperineal resection. The isolated cells and differentiated muscle fibers were identified with an immunofluorescence assay, western blotting, and RT–PCR. The proliferation of the progenitor cells with or without radiotherapy was compared with quantitative clonogenic and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.

Results: Immunofluorescence assay before differentiation confirmed that the internal anal sphincter progenitor cells expressed CD34 and neural-glial antigen 2 (NG2), whereas the external anal sphincter progenitor cells expressed CD34, PAX7, and NG2. After differentiation, the internal anal sphincter progenitor cells expressed α-SMA, calponin, and desmin, whereas the external anal sphincter progenitor cells expressed MYC, MYOG, and desmin. Differential expression profiles were observed for the two cell types

with western blotting and RT–PCR, which were identical to those observed with immunofluorescence. In MTT assays of the internal anal sphincter progenitor cells, cell viability was lower in the radiotherapy group than in the nonradiotherapy group at 24 h (14·1% vs 11·5%, respectively, p = 0·033), 48 h (14·7% vs 11·9%, respectively, p = 0·022), 72 h (15·3% vs 12·5%, respectively, p = 0·012), 96 h (16·2% vs 13·1%, respectively, p = 0·012), and 120 h (14·8% vs 13·6%, respectively, p = 0·536), as confirmed in the external anal sphincter progenitor cells and with clonogenic assays.

Conclusions: This is the first study to differentially harvest internal and external sphincter muscle progenitor cells from humans. We suggest that internal- or external-anal-sphincter-muscle-derived cells can be used for tailored cell therapies for fecal incontinence.

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Funding: This trial was supported by a grant from the National Research Foundation of Korea (grant number: 2013R1A1A2061651).

Keywords: fecal incontinence, anal sphincter progenitor cell, tailored cell therapy

Student Number: 2014-25035

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INTRODUCTION

Fecal incontinence (FI) is still placed within the uncertain domain treatment adversely affecting all aspects of an individual's life, although it is considered a benign condition without life-threatening sequelae.[1,2] The commonest etiology of FI is damage to the anal sphincter or nerve complex resulting from trauma, including obstetric injuries and anorectal interventions. Although an insufficiency of skeletal muscle cells in the external anal sphincter (EAS) contributes to FI,[3] the critical factor for FI is the functional loss of the fatigue-resistant smooth muscle in the internal anal sphincter (IAS), which play a crucial role in the maintenance of normal anal canal tone.[4–9] The symptoms of FI can develop differently according to its pathophysiology, and require management that is targeted to the etiology in these patients. The restoration of anal function with various autologous-cell-based therapies and tissue engineering has recently been reported,[2–12] although some limitations have hampered the recovery of incontinent sphincters.

The first functional in vitro model of the IAS has been reported,[4] which showed the feasibility of a bioengineered, intrinsically innervated human IAS construct, with viable myogenic and neuronal components, in an animal model.[5–9] In terms of cell therapies and animal models related to EAS, several studies have used autologous-muscle-derived cells or progenitor cells.[2,3,10–12] However, we consider that the issues of tissue denervation, the functional connection of a graft to the host nervous system, and the in-growth of new blood vessels have not yet been adequately addressed. Furthermore, these

outcomes have been based on previous studies that targeted the smooth muscle cells of the IAS[5–9] or the skeletal muscle cells of the EAS.[2,3,10–12] However, there has been no study for the differential isolation and culture of smooth and skeletal muscle cells from the human anal sphincter. We hypothesize that tailored cell therapies might provide the appropriate tissue regeneration to alleviate dysfunctional IAS- or EAS-based incontinence, because there are embryological, anatomical, and functional differences between the IAS and EAS.[13] Therefore, in this study, we aimed to identify IAS and EAS progenitor cells as potential tools for tailored cell therapies for FI.

METHODS

Tissue harvest from human IAS and EAS

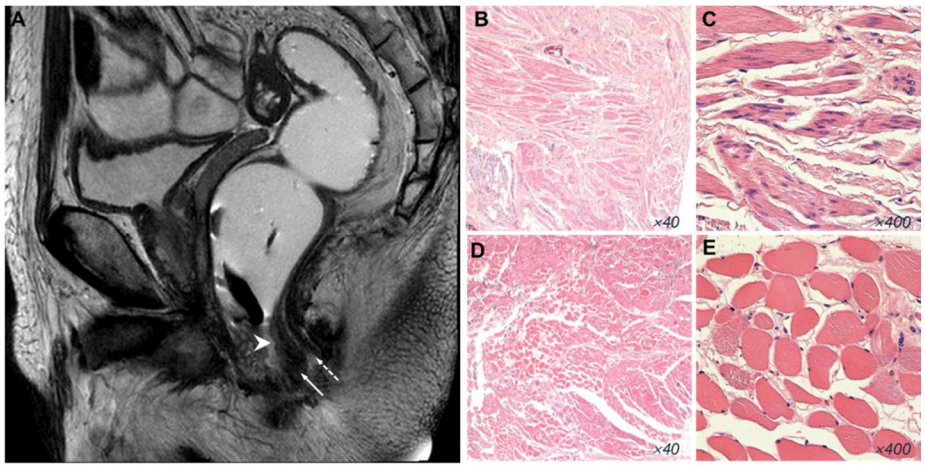
Normal IAS and EAS muscles were obtained from 10 patients who had undergone abdominoperineal resection for rectal cancer ***(Table 1)***. Of these 10 patients, six received preoperative long-course concurrent chemoradiotherapy, which was given at a dose of 50·4 Gy, 45 Gy of which was administered in 25 fractions to the pelvis, and a 5·4 Gy boost was administered in three fractions to the primary tumor ***(Figure 1).***[14] The study protocol was approved by the Ethics Committee of Seoul National University Bundang Hospital, and all the patients gave their informed consent to the procedure.

Table 1 Patient demographics

Patient	Sex	Age, years	Radiation therapy*	Differentiation type	Tumor location† (cm)	Laparoscopic surgery	Pathologic stage‡
1	M	70	Yes	MD	1.5	Yes	ypT3N0M0
2	M	76	Yes	MD	3.5	Yes	ypT3N1bM0
3	M	49	Yes	MD	3.0	No	ypT2N0M0
4	M	80	No	WD	0	Yes	rpT3N1bM0
5	M	76	No	MD	3.0	Yes	T2N0bM1
6	M	59	Yes	MD	2.5	Yes	ypT2N0M0
7	F	69	No	MD	2.0	Yes	rpT3N0M0
8	M	73	Yes	MD	5.0	No	ypT0N0M1
9	M	70	Yes	MD	3.0	Yes	ypT1N0M0
10	M	55	No	MD	0	Yes	pT2N2M1

*Preoperative long course concurrent chemoradiotherapy; †distance from anal verge; ‡ American Joint Committee on Cancer, 7th edition; MD = moderated differentiated; WD = well differentiated

Figure 1 Magnetic resonance (MR) image and histological sections with hematoxylin-and-eosin staining of an 80-year-old man with pathologically proven rectal cancer



(A) T2-weighted sagittal MR image obtained from patient No. 4 shows well-separated internal anal sphincter (IAS) (indicated by arrow), external anal sphincter (EAS) (indicated by dotted arrow), and distal rectal cancer (arrowhead); **(B–E)** hematoxylin-and-eosin staining of normal human IAS and EAS; **(B and C)** IAS consists of smooth muscle fibers; **(D and E)** EAS consists of striated muscle fibers.

After the subcutaneous fat and connective tissue were removed from the surgical specimens with sterile scissors, without electrocauterization, the muscle samples were divided into the IAS and EAS muscles. Muscle tissues from the IAS were plunged into modified Krebs–Henseleit buffer (Sigma, Japan) containing 2% penicillin/streptomycin, and tissues from the EAS were plunged into chilled Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 1% penicillin/streptomycin. The tissues were immediately transferred to the laboratory for the isolation and culture of the progenitor cells.

Cell isolation and differentiation

The modified preplate technique was used for the purification of the IAS and EAS progenitor cells.[15,16] After the harvested IAS muscle tissues were centrifuged for 10 min at 25 °C (1200 rpm), the cells were enzymatically dissociated with the addition of collgenase/dispase solution (0·01% soybean trypsin inhibitor plus 0·1% collagenase type I) in modified Krebs–Henseleit buffer for 1 h at 37 °C, and the cells obtained from the last preplate were used. SmBm2 (Lonza, Switzerland) was used as the culture medium, and the differentiation medium was 75% DMEM (Gibco, USA) plus 20% media199 (Gibco, USA) supplemented with 7% horse serum and 1% penicillin/streptomycin. The external sphincter muscle tissues were centrifuged for 10 min at 25 °C (1200 rpm), dissociated in collgenase/dispase solution plus 0·1% collagenase type I with DMEM for 2 h at 37 °C. After 2 h, the supernatant was cultured with SkGM-2 medium (Lonza) for 5 days. The progenitor cells were maintained in the proliferative state until they had

reached 80% confluence without splitting. The growth medium was then replaced with differentiation medium (PromoCell, Germany) at 37 °C in a CO2 incubator to allow the cells to differentiate.

The differentiation of the progenitor cells was observed for 1, 3, and 5 days after the addition of differentiation medium and assessed after differentiation.

Histopathology and immunofluorescent staining

A histological examination with hematoxylin-and-eosin (H&E) staining was performed of all frozen sections to confirm the IAS and EAS. Monoclonal antibodies, including those directed against PAX7, CD34, neural-glial antigen 2 (NG2), myosin heavy chain (MYC), myogenic factor 4 (MYOG), alpha-smooth muscle actin (α-SMA), calponin, and desmin, were used to detect specific biomolecular markers before and after differentiation ***(Table 2)***. An immunofluorescent (IF) assay, western blotting, and reverse transcription–PCR (RT–PCR) were performed before and after cell differentiation.

For the IF assay, the cells were fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7·4), then washed, and incubated for 90 min at 37 °C in PBS plus 0·3% of Triton X-100 (Sigma, Japan). All the tissue sections were then deparaffinized, rehydrated, and processed to block any endogenous peroxidases. The fixed cells were diluted with 1 ml of PBS supplemented with Triton X-100 and then with normal goat serum or normal horse serum. These cells were incubated in culture medium containing monoclonal antibodies at –4 °C for 24 h. After the cells were tagged with a secondary antibody (Alexa-Fluor-488-conjugated goat anti-mouse IgG or Alexa-Fluor-594-conjugated goat anti-rabbit IgG antibodies), the nuclei were

conterstained with 4 ,6-diamidino-2-phenylindole (DAPI). Negative control staining was performed for each marker without the primary antibody. The expressed markers were examined with fluorescence microscopy (Zeiss, AxioVision 4, Germany).

Western blotting and RT–PCR

To confirm the expression of protein markers during the proliferation of the progenitor cells, we used western blotting to detect a number of markers. The protein concentrations were assessed with the bicinchoninic acid method (Bradford assay) with a Protein Assay Kit (Bio-Rad, USA). After 10–15 μg of protein was loaded into each lane of a precast 12·5% Tris-HCl gel (Ready Gel, Bio-Rad), the proteins were separated with SDS-PAGE and transformed to membranes. The membranes were blocked with 5·0% skim milk (Bio-Rad, USA) and Tris-buffered saline containing Tween 20 (TBST; BioPlus, Korea) for 1 h 30 min. They were then incubated with primary antibodies at 4 °C for 24 h. After incubation, the membranes were rinsed to remove any unbound primary antibody and then incubated with secondary antibody (Jackson ImmunoResearch, USA) in TBST for 1 h 30 min at room temperature. The band densities were determined with densitometry after scanning, and were evaluated with the ImageJ software (Kodak, M35, USA).

Primer of RT–PCR to assess cell differentiation was performed to extract total RNA. Cells were homogenized in TRIzol Reagent (iNtRON, Korea). Specific primers were designed for the markers of skeletal and smooth muscle cells (Bioneer, Korea). cDNA was synthesized before and after cell differentiation with random priming and the SuperScript III One-Step RT–PCR System

(Invitrogen, USA), and amplified (Solg™ 2X Taq PCR Smart mix 2, Solgent, Korea).

Viability of progenitor cells

The proliferation of progenitor cells was assessed according to the sphincter muscle type and the exposure to radiotherapy. A clonogenic assay and an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Amresco, USA) were used to assay cell viability. The control group was unirradiated cells, which were compared with radiated cells in the IAS and EAS. In the clonogenic assay, the radiated and nonradiated progenitor cells were seeded in six-well plates and cultured for 5 days. The culture medium was replaced with fresh medium every day. The colonies were fixed and stained with 1·25% crystal violet. In the MTT assay, progenitor cells seeded in six-well plates and incubated at 37 °C were washed twice with PBS, and 5 mg/mL MTT in PBS was added to each well for 4 h. After the MTT solution was removed, solubilization solution (dimethyl sulfoxide) was added to each well to dissolve the formazan crystals. The absorbance at 550 nm was measured with a paradigm microplate reader (Spectramax Plus 384, USA). After 24, 48, 72, 96, and 120 h, the cells were diluted with a working solution of dimethyl sulfoxide and counted to construct a growth curve. A quantitative analysis was performed to compare the cell viability in the radiated and nonradiated groups using Student's t test and a gross examination. All experiments were performed in triplicate.

Role of the funding source

This trial was supported by a grant from the National Research Foundation of

Korea (grant number 2013R1A1A2061651). The funding source had no role in the design or performance of the study; in the data collection, analysis, or interpretation; or in writing the report. The corresponding author had full access to all the data in the study and accepts the final responsibility for the decision to submit for publication.

Table 2 Antibodies used for the immunofluorescence assay and western blotting and, primers used for RT–PCR

Marker	Dilution*	Primer sequence (5′ - 3′)†	Size†	Type of muscle cell‡	Time of expression
Desmin	1:150	F: CTGTCCCTCCCACCTCTGT R: AGCCCCTGCTTTCTAAGTCC	250 bp	Smooth and skeletal	Before differentiation
CD 34	1:200	F: TGAAAAAGCTGGGGATCCTAGA R: TCCCAGGTCCTGAGCTATAGCC	5110 bp	Smooth and skeletal	Before differentiation
MHC	1:400	F: CTCAGGCTTCAAGATTTGGTGG R: TTGTGCCTCTCTTCGGTCATTC	265 bp	Smooth	Before differentiation
Caplonin	1:150	F: GTCTTCCGAACACTTTAACC R: TCAAATCTCCGCTCTTGTTTC	456 bp	Smooth	After differentiation
α- SMA	1:500	F: GATAGGACACGCCATCATCAC R: AGGGAAGAAGAGGAAGCAG	537 bp	Smooth	After differentiation
Pax7	1:150	F: CCGTGTTTCTCATGGTTGTG R: GAGCACTCGGCTAATCGAAC	306 bp	Skeletal	After differentiation
Ng2	1:1000	F: GCACGATGACTCTGAGACCA R: AGCATCGCTGAAGGCTACAT	222 bp	Skeletal	After differentiation
MyoG	1:150	F: ACTACCCACCGTCCATTCAC R: TCGGGGCACTCACTGTCTCT	233 bp	Skeletal	After differentiation

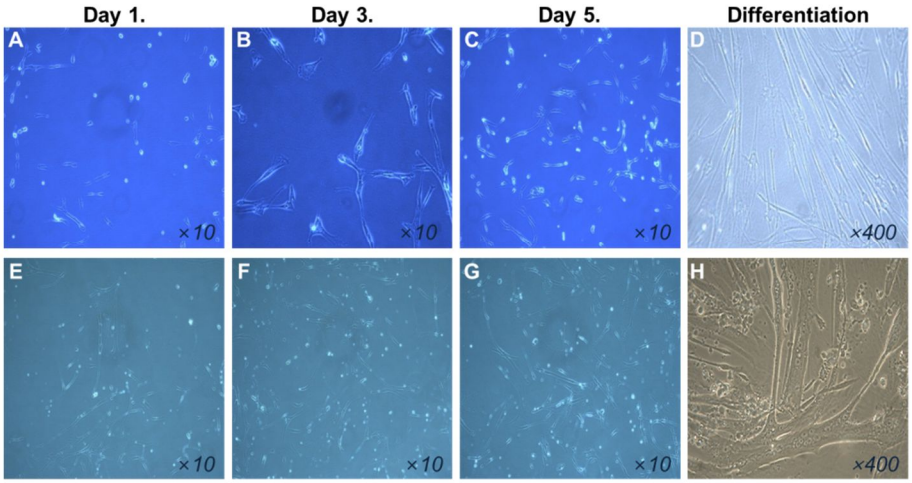
*Antibody dilutions used in the immunofluorescence assay; †primer sequences and sizes used for the RT–PCR analysis; ‡targeted muscle type of progenitor cell before or after differentiation. bp, base pair; NG2, neural-glial antigen 2; MYC, myosin heavy chain; MYOG, myogenic factor 4; α-SMA, alpha-smooth muscle actin

RESULTS

Growth and fusion

The EAS is composed of striated muscle and the IAS is composed of smooth muscle, confirmed with a histological examination ***(Figure 1)***. Severe fibrotic changes were observed in the radiated anal sphincter muscle cells in both the IAS and EAS. The IAS progenitor cells had differentiated into cells with morphological changes indicative of the smooth muscle cell type at 7 days after the replacement of the differentiation medium, and the EAS progenitor cells had differentiated into cells with morphological changes indicative of the skeletal muscle cell type ***(Figure 2)***.

Figure 2 Growth of internal and external anal sphincter progenitor cells harvested from an 80-year-old man with pathologically proven rectal cancer

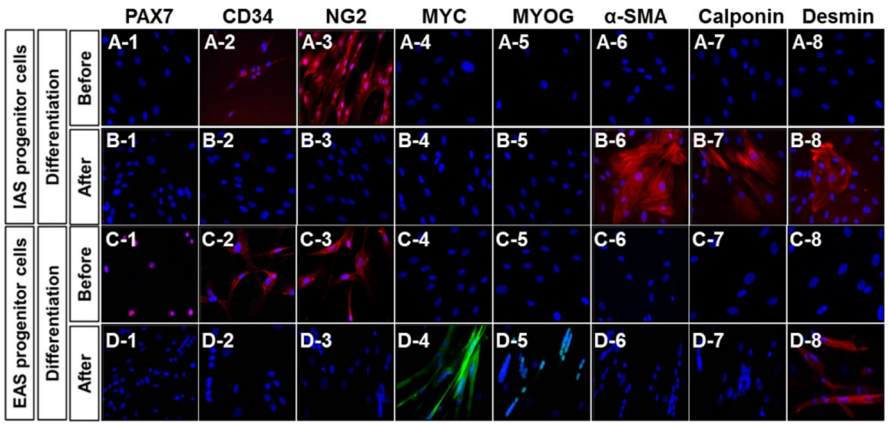


After internal anal sphincter (IAS) and external anal sphincter (EAS) progenitor cells were isolated from patient No. 4 with the preplate technique, the proliferation of the IAS progenitor cells was observed after 1 day **(A)**, 3 days **(B)**, and 5 days **(C)** on culture medium; **(D)** differentiation of IAS progenitor cells into smooth muscle cells on differentiation medium; the proliferation of EAS progenitor cells after 1 day **(E)**, 3 days **(F)**, and 5 days **(G)** of culture on culture medium; **(H)** differentiation of EAS progenitor cells into skeletal muscle cells on differentiation medium.

Characterization of the IAS and EAS

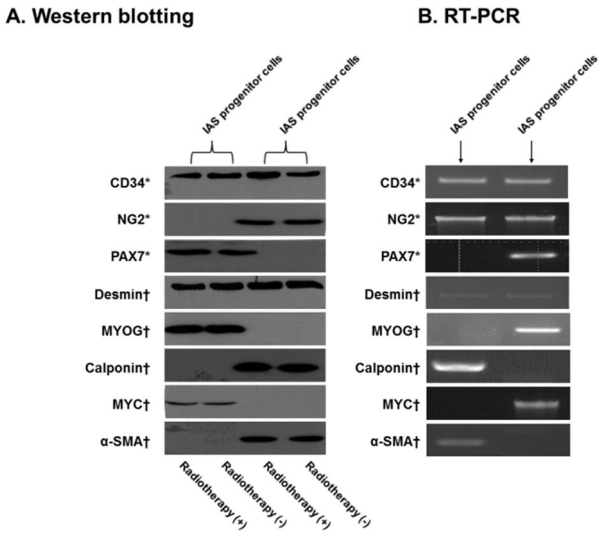
There were differences in the expression profiles according to each anal sphincter muscle type and the differentiation status of the progenitor cells. The immunofluorescence assay before differentiation showed that the IAS progenitor cells expressed CD34 and NG2, whereas the EAS progenitor cells expressed CD34, PAX7, and NG2. After differentiation, the IAS progenitor cells were positive for α-SMA, calponin, and desmin, whereas the EAS progenitor cells were positive for MYC, MYOG, and desmin ***(Figure 3).*** Similar expression profiles were observed with western blotting ***(Figure 4a).*** The IAS progenitor cells expressed CD34 and NG2, whereas the EAS progenitor cells expressed CD34 and PAX7 before differentiation. After differentiation, the expression profiles determined with western blotting were identical to those observed with immunofluorescence. Furthermore, the expression profile of the radiated anal sphincter muscle cells was identical to that of the nonradiated group on western blotting. RT–PCR was used to determine the expression profiles of the IAS and EAS progenitor cells before and after differentiation, and the results were identical to those of the immunofluorescence assay and western blotting, except for NG2 in the EAS progenitor cells before differentiation ***(Figure 4b).***

Figure 3 Characterization of the differentiation of progenitor cells with an immunofluorescence assay



After the isolation of internal anal sphincter (IAS) and external anal sphincter (EAS) progenitor cells from patient No. 4, the muscle types were assessed before and after differentiation using negative control and primary antibodies. Expression profiles of IAS progenitor cells for CD34 **(A–1)** and NG2 **(A–3)** before differentiation and for α-SMA **(B–6)**, calponin **(B–7)**, and desmin **(B–8)** after differentiation. Expression profiles of EAS progenitor cells for PAX7 **(C–1),** CD34 **(C–2)**, and NG2 **(C–3)** before differentiation and for MYC **(D–4)**, MYOG **(D–5)**, and desmin **(D–8)** after differentiation (NG2, neural-glial antigen 2; α-SMA, alpha-smooth muscle actin; MYC, myosin heavy chain; MYOG, myogenic factor 4).

Figure 4 Expression profiles of progenitor cells according to their differentiation using western blotting and RT–PCR

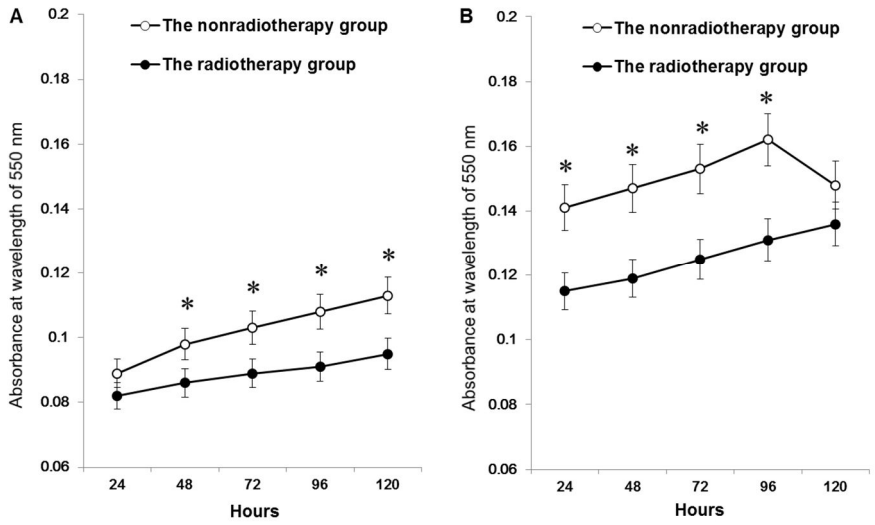


Expression profiles of internal anal sphincter (IAS) and external anal sphincter (EAS) progenitor cells isolated from patient No. 4, using primary antibodies before differentiation* and after differentiation†. **(A)** Western blotting analysis showed that the IAS progenitor cells expressed CD34 and NG2 before differentiation and desmin, calponin, and α-SMA after differentiation; the EAS progenitor cells expressed CD34 and PAX7 before differentiation and desmin, MYOG, and MYC after differentiation. **(B)** RT–PCR detected *CD34* and *NG2* expression in IAS progenitor cells before differentiation; *CD34, NG2*, and *PAX7* in EAS progenitor cells before differentiation; desmin and calponin in IAS progenitor cells after differentiation; and desmin, *MYOG*, and *MYC* in EAS progenitor cells after differentiation.

Viability of progenitor cells

In the MTT assays, the viability of the IAS progenitor cells in the radiotherapy group was lower than that in the nonradiotherapy group at 24 h (14·1% vs 11·5%, p = 0·033), 48 h (14·7% vs 11·9%, p = 0·022), 72 h (15·3% vs 12·5%, p = 0·012), 96 h (16·2% vs 13·1%, p = 0·012), and 120 h (14·8% vs 13·6%, p = 0·536) ***(Figure 5, and Table 3)***. The viability of the EAS progenitor cells in the radiotherapy group was also lower than that in the nonradiotherapy group at 24 h (8·9% vs 8·2%, p = 0·278), 48 h (9·8% vs 8·6, p = 0·001), 72 h (10·3% vs 8·9%, p = 0·001), 96 h (10·8% vs 9·1%, p = 0·003), and 120 h (11·3% vs 9·5%, p = 0·007). The clonogenic assay showed differences in the proliferation of the IAS and EAS progenitor cells with and without radiotherapy ***(Figure6).***

Figure 5 Proliferation of progenitor cells with and without radiotherapy, determined with an MTT assay



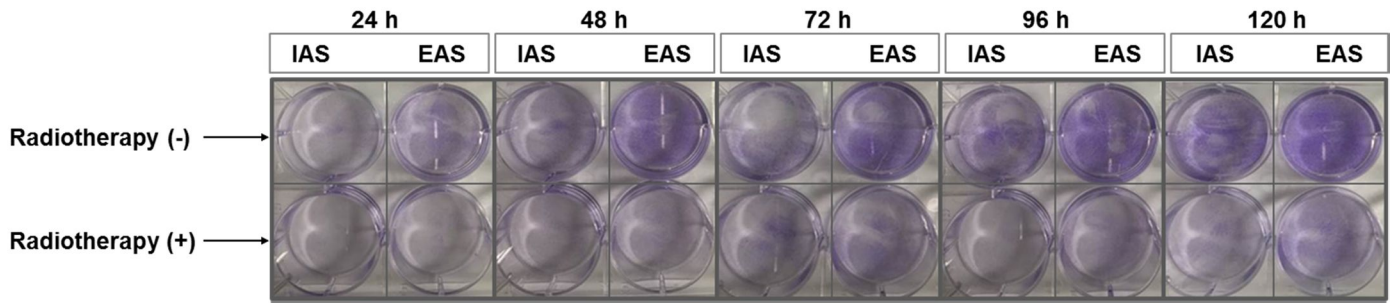
(A) Proliferation rate of EAS progenitor cells differed significantly between the radiated and nonradiated groups at 48, 72, 96, and 120 h. **(B)** Proliferation rate of IAS progenitor cells differed significantly between the radiated and nonradiated groups at 24, 48, 72, and 96 h. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *$P < 0.05$.

Table 3 Quantitative analysis of the proliferation rates of progenitor cells with and without radiotherapy, with an MTT assay

	External sphincter progenitor cells			**Internal sphincter progenitor cells**		
	Radiotherapy			Radiotherapy		
	No	Yes		No	Yes	
Hours*	Cell viability† (%)		*p*‡	Cell viability† (%)		*p*‡
24	8.9	8.2	0.278	14.1	11.5	0.033
48	9.8	8.6	0.001	14.7	11.9	0.022
72	10.3	8.9	0.001	15.3	12.5	0.016
96	10.8	9.1	0.003	16.2	13.1	0.012
120	11.3	9.5	0.007	14.8	13.6	0.542

*Time after culture; †absorbance at a wavelength of 550 nm; ‡using Student's t test. MTT, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Figure 6 Proliferation of progenitor cells with or without radiotherapy, using a clonogenic assay



Proliferation of IAS and EAS progenitor cells isolated from an 80-year-old man (patient No. 4) treated without radiotherapy and a 59-year-old man (patient No. 6) treated with radiotherapy was assessed with a clonogenic assay. Proliferation rates of IAS and EAS progenitor cells were observed at 24, 48, 72, 96, and 120 h. Progenitor cells stained with 1·25% crystal violet appear as blue colonies. Cells isolated from radiated tissue showed a lower proliferation rate than cells from nonradiated tissue.

DISCUSSION

This is the first study to demonstrate the differential isolation of IAS and EAS progenitor cells from the human anal sphincter. The differential harvest of human anal sphincter cells was supported by confirmation of the different differentiation processes of the IAS and EAS progenitor cells, which displayed different transcription profiles, at both the mRNA and protein levels, before and after differentiation. These outcomes imply that in patients with FI, regenerative medicine using IAS or EAS progenitor cells could facilitate tailored cell therapies based on the sphincter muscle type involved.

Until recently, skeletal-muscle-derived stem or progenitor cells have been used as models of EAS deficiency, including external anal sphincterostomy, obstetric trauma, or external sphincter rupture.[2,3,10–12] Similarly, smooth-muscle-cell-based tissue engineering has targeted the restoration of IAS sufficiency.[4–9] However, these techniques are still limited by the difficulty in scaling these bioengineered IAS constructs to the size of the human anal sphincter.[17] The IAS construct might also be inappropriate for most FI patients requiring treatment for EAS deficiency.[17] Therefore, our challenge was to efficiently isolate homogeneous populations of smooth and skeletal muscle cells from the human IAS and EAS, respectively.

This study has shown that multiple markers, including CD34, PAX7, NG2, desmin, α-SMA, calponin, MYC, and MYOG, define the cellular hierarchy and allow the selection of specific subpopulations of cells, although these cells can potentially differentiate into heterogeneous cells.18 Using

clonogenic and MTT assays, we have also shown that IAS and EAS progenitor cells have different cellular functions during their proliferation, whereas previous studies have been unclear about the final differentiation status of autologous progenitor or derived cells after their isolation.[4,6,10,12] To the best of our knowledge, this is the first study to illustrate the dysfunction of the human anal sphincter at the cellular level, in contrast to previous studies based on the measurement of the anal sphincter pressure or surveillance with questionnaires.[19,20] The quantitative differences in the proliferation rates of these cells means that the capacity of the tissues for restoration might differ if the sphincter has been irradiated, which should be considered in tailored cell therapies.

In this study, we considered the future potential utility of allogeneic implantation using human IAS or EAS progenitor cells. The transplantation of allogeneic IAS and EAS progenitor cells has never been attempted for FI in humans, although the allogeneic transplantation of several organs, including the liver, kidney, and pancreas, is widely performed. Our concept, using allogeneic progenitor cells, might be limited by immunological barriers, although a previous study demonstrated the survival of muscle-derived progenitor cells with minimal inflammation in a rat urethra model without immunosuppressive agents because the expression of MHC class I in the progenitor cells was downregulated.[21,22] These hypoimmunogenic properties of progenitor cells may reduce the development of apoptosis after transplantation, enhancing graft survival.[23] Several studies have investigated the applicability of immunosuppressive drugs as a regime for preventing cell

rejection, with adjustments to reduce the risk of drug toxicity and any negative effects on cell viability, proliferation, differentiation, and migration in the graft.[24–26] Therefore, our concept of allogeneic cell transplantation for the treatment of FI, extrapolated from previous autologous cell transplantation, might be feasible in the future with a widely used immunosuppressive protocol.

Current regenerative cell therapies are inefficient, probably because implanted cells are lost when cell attachment, migration, or differentiation fails.[27,28] Moreover, cell therapy alone for FI is limited to the restoration of human-sized anal sphincter defects.[29] In previous studies, we have demonstrated the feasibility of basic fibroblast growth factor (bFGF)-loaded biocompatible polycaprolactone (PCL) beads for the functional improvement of anal sphincter defects in an animal model of FI.[29,30] However, a tailored cell therapy using injectable biomaterial (PCL) beads warrants discussion in the future.

In conclusion, this is the first study to show the differential harvest of IAS and EAS progenitor cells from humans. We suggest that IAS or EAS progenitor cells can be used in tailored cell therapies for FI. The feasibility of allogeneic progenitor cell implantation, based on immunotherapeutic strategies, should be evaluated both in vivo and in an experimental study in the future.

Acknowledgments

The study was presented in part as the Best 6 presentation at the European Society of Coloprotology (ESCP) 2015 10th Anniversary Meeting, Dublin, Ireland

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국 문 초 록

서론: 대변 실금의 세포 치료 영역에서 생체 항문 내외괄약근을 분리 동정하는 연구는 아직까지 없었다. 본 연구에서는 대변 실금 환자의 맞춤형 세포 치료를 위한 내외괄약근의 전구세포를 분리 동정하고자 하였다.

방법: 10명의 직장암 환자로부터 구득된 항문 괄약근으로부터 modified preplate technique를 이용하여 내외항문괄약근 전구세포를 분리 동정하였다. 최종 분화 상태는 면역화학염색, Western blotting 및 RT-PCR를 시행하여 확인하였다. 전구 세포의 증식 능력은 방사선 치료 여부에 따라 두 군으로 나누어, clonogenic assay 및 MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay를 이용하여 확인하였다.

결과: 분화 전 면역화학염색에서 CD34, neural-glial antigen 2 (NG2)이 내괄약근 전구세포에서 발현되었으며, CD34, Pax7, 및 NG2가 외괄약근 전구세포에서 발현되었다. 분화 후 내괄약근 전구세포는 α-SMA, calponin, 및 desmin을 발현하였으며, 외괄약근 전구세포는 MHC, MyoG, 및 desmin을 발현하였다. Western

blotting 및 RT-PCR의 양성 표지자 발현 결과는 면역화학염색의 결과와 동일하였다. 내괄약근 전구세포의 MTT assay에서는 방사선 치료 군이 대조 군에 비해 유의하게 적은 것으로 조사되었다 [24 h (14.1% vs. 11.5%, p = 0.033), 48 h (14.7% vs. 11.9%, p = 0.022), 72 h (15.3% vs. 12.5%, p = 0.012), 96 h (16.2% vs. 13.1%, p = 0.012), and 120 h (14.8% vs. 13.6%, p = 0.536)]. 이는 외괄약근 전구세포에도 유사하였다.

결론: 본 연구는 최초로 인간의 내외괄약근 전구세포의 분리 동정 하였다. 향후 내외괄약근 전구세포는 대변 실금의 맞춤형 세포치료로 사용될 수 있을 것으로 판단된다.

주요어: 대변 실금, 항문 괄약근 전구세포, 맞춤형 세포 치료

학 번: 2014-25035