封入した後、顕微鏡にて観察した。

（倫理面への配慮）

ヒト材料を用いた実験に関しては、国立長寿医療センター倫理委員会の承認を受けたうえで、説明と同意に関する所定の手続きを行い、注意深く実施した。

C. 研究結果

ヒト筋切片を CD56 抗体で染色すると、緑色で染色された laminin の内側に局在する単核細胞の表面で発現が認められることが分かった (Fig. 1A)。骨格筋幹細胞（筋衛星細胞）は、解剖学的に基底膜の内側で筋細胞膜の外側に存在すると定義されていることから、CD56 は骨格筋幹細胞を認識していると考えられる。さらに、骨格筋幹細胞のマーカーとしてよく知られている Pax7 との二重染色において、Pax7 陽性細胞の表面が染色されることからも (Fig. 1B)、CD56 が骨格筋幹細胞を認識していることを確認した。

また、ヒト筋切片においてアルカリフォスファターゼ活性染色を行ったところ、青紫色の染色で認められるように、小血管壁が陽性であった (Fig. 2)。

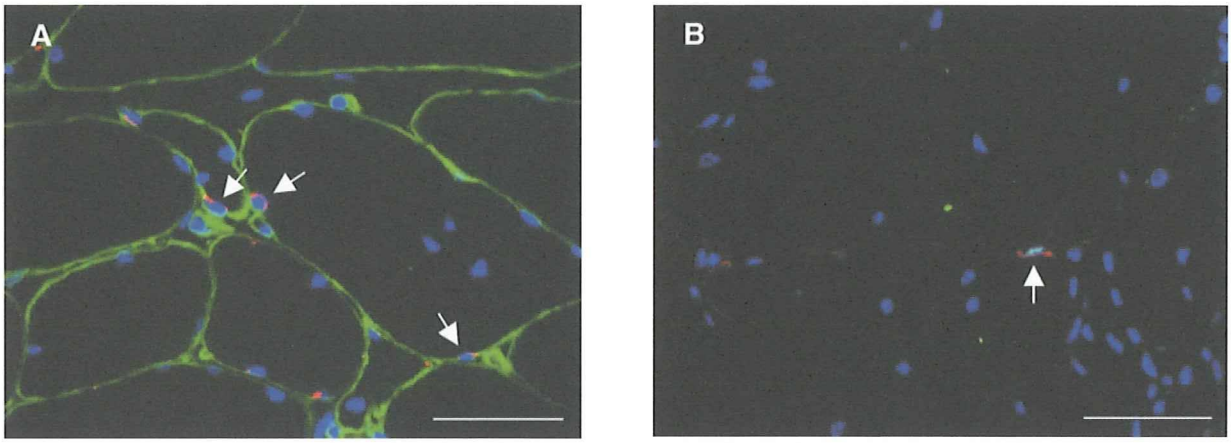

Fig. 1　ヒト筋組織の CD56 抗体染色

A) CD56 (赤), laminin (緑)および核 (青) を三重染色したヒト骨格筋横断切片像。矢印は CD56 陽性の単核細胞を示し、laminin の内側に局在している。
B) CD56 (赤), Pax7 (緑)および核 (青) を三重染色した。CD56 は Pax7 陽性の骨格筋幹細胞（筋衛星細胞）を認識している（矢印）。
Bar = 50 μm

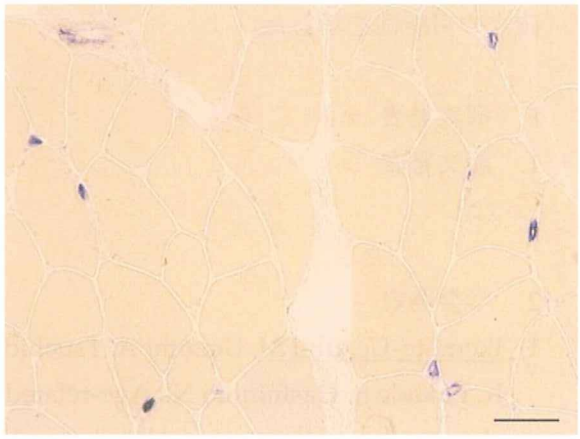

Fig. 2　ヒト筋組織のアルカリフォスファターゼ活性染色小血管壁が青紫色に染色されている
Bar = 50 μm

これらの結果から、CD56 はヒト筋組織から分離し、培養下で増殖させた筋細胞だけでなく、実際の筋組織中においても、骨格筋幹細胞を認識することが示され、CD56 の発現自体が、骨格筋幹細胞としての機能維持に重要である可能性が示唆された。

一方、ヒト骨格筋の血管由来のアルカリフォスファターゼ陽性細胞 (pericyte) が筋再生に寄与し得るという報告がある (Nat Cell Biol. 2007; 9(3): 255-267)。さらに、ヒト骨格筋において、再生初期の筋線維がアルカリフォスファターゼ陽性であると報告されている（臨床のための筋病理、埜中征哉著)。よって、筋再生の過程でアルカリフォスファターゼを発現することが、効率的な筋再生にとって重要であるのかもしれない。

D. 考察

自己骨格筋幹細胞を用いた腹圧性尿失禁に対する細胞移植治療においては、高齢の患者から得られる筋組織は少量であり、単離可能な筋幹細胞の数は限られているため、移植に必要な量の筋細胞を、培養を介さずに確保することはできない。そのため、培養下で増殖させた筋細胞が移植に適した細胞であるか否かを、移植直前に迅速に判定できる方法の確立が不可欠である。CD56 は、培養下で増殖させた筋細胞においてだけでなく、筋組織中においても、骨格筋幹細胞を認識することが確認され、CD56 が移植に適した筋細胞をセレクションするための有力な指標分子の一つになり得ると考えられる。しかしながら、筋細胞集団を CD56 の発現によって品質評価するには、抗体染色やフローサイトメーターを用いた煩雑なセレクション法を取らざるをえない。また、現在、GMP 基準を充たすフローサイトメーターの開発が進んではいるが、安全性の担保という点で、フローサイトメーターを用いて分離した細胞を治療に用いることはまだ現実的ではない。さらに、機器自体が高価であることや操作が複雑であるため、専門のオペレーターが必要になるなどといった問題もある。これらのことから、より安全で簡便な品質評価システムの開発が求められる。

昨年度、我々は培養下で増殖させた筋細胞の大半が CD56 陽性であり、BLK-ALP 陽性細胞の 80%が CD56 陽性であることを示した。この結果は、品質評価の判断基準として、フローサイトメーターによる CD56 の検出を、より簡便な ALP の検出で補える可能性を示唆している。今後さらに、CD56 と ALP の発現率と筋分化能力との相関について判断基準を確立する必要があるが、ALP は生化学的に活性を測定でき、定量化できること、また、検出が迅速で簡便であることから、実際に移植治療の現場で用いる筋細胞の品質評価システムとして早期実現可能な方法であると期待される。

E. 結論

高齢者の筋組織中での CD56 と ALP の発現を調べ、骨格筋幹細胞の維持や増殖・分化能力を反映するマーカーとしての機能的意義について検討した。また、これまでの結果から、移植に適した筋細胞であるか否かを、細胞集団から簡便かつ迅速に判断する評価システム確立の可能性を示した。

F. 研究発表

1. 論文発表

なし

2. 学会発表

1) Ikemoto-Uezumi M, Uezumi A, Tsuchida K, Fukada S, Hashimoto N.: Age-related changes in prospectively isolated muscle satellite cells 第 7 回幹細胞シンポジウム

2009, 5/15 - 5/16、東京

2) Madoka Ikemoto-Uezumi, Akiyoshi Uezumi, Kunihiro Tsuchida, So-ichiro Fukada, Naohiro Hashimoto: Age-related Changes in Prospectively Isolated Muscle Satellite Cells Making Muscle in the Embryo and the Adult (A joint meeting of Frontier in Myogenesis and Skeletal Muscle Stem and Satellite Cells) 2009, 5/28 - 6/2, New York, NY, U.S.A

G. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（長寿科学総合研究事業）

研究分担報告書

高齢者腹圧性尿失禁に対する括約筋機能再生治療：移植用筋細胞の安全性検定系の開発と筋増殖・肥大因子の有効性・安全性に関する検討

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研究要旨

高齢者腹圧性尿失禁に対する有力な治療手段の１つとして、ヒト由来骨格筋細胞の移植が考えられる。しかしながら、人体に適応可能な筋幹細胞移植治療を実現するためには、移植細胞の品質（高い筋再生能力と安全性）を確保することが必須である。これまでの研究で、不死化ヒト筋前駆細胞の免疫不全マウスへの移植実験系を用いて、移植細胞の性状を病理組織学的に検討し、発癌性などの異常は検出されず、筋再生過程で正常な筋分化を遂げることを確認してきたが、今年度は、移植した不死化ヒト筋前駆細胞に由来する再生筋線維を顕微鏡下に同定したうえで、laser capture microdissection によって切り出し、そこから抽出した RNA を基に、定量的 PCR によって、細胞増殖にかかわる遺伝子発現を検討した。今回検討した、Ki67 mRNA の発現は、細胞増殖能をもつマウス筋芽細胞 Ric10 の 4.6%であり、対照となる周囲の正常マウス筋組織とは大差ないことが強く示唆された。こうした遺伝子発現の面からも、移植したヒト筋前駆細胞が著しい増殖能を持ち、腫瘍化する危険性は否定的であると判断された。

A. 研究目的

治療手段として用い得る移植細胞の品質を確保するために、実験動物への移植実験系について病理組織学的解析ならびに細胞増殖関連遺伝子の発現検討を行い、筋細胞移植治療の安全性を見極める。

B. 研究方法

1. 実験動物として成熟した雌の NOD/Scid マウスを用い、その両側前脛骨筋に cardiotoxin を注射し、骨格筋再生を誘導した。
2. 注射の翌日に、橋本らによって樹立された Venus（GFP の変異体）遺伝子導入不死化ヒト筋前駆細胞（E18V=E18）を 2 - 2.5x 10^6 個、上記マウスの前脛骨筋に移植した。
3. 移植後 27 日に前脛骨筋を採取し、4% パラホルムアルデヒド（PFA）で固定。
4. 固定された前脛骨筋を O.C.T. コンパウンドに包埋し、凍結。
5. 厚さ 10μ の凍結切片を作成し、顕微鏡下に Venus 遺伝子導入不死化ヒト筋前駆細胞に由来する蛍光を発する再生筋線維を同定し、laser capture microdissection（LCM）によって約 500 個（総面積：約 1.3mm^2）の筋線維を切り出した。対象サンプルとして Venus(-)の筋線維を約 500 個（総面積：約 1.3mm^2）切り出した。
6. 切り出した再生筋線維を Proteinase K 処理し、RNAqueous®-Micro kit（Ambion）を用いて核酸を抽出した。

7. TURBO DNA-free™ kit（Ambion）により混入する genomic DNA を除去し、再度 RNAqeuous®-Micro kit（Ambion）を用いて total RNA を精製した。
8. Message*BOOSTER*™ Whole Transcriptome cDNA Synthesis kit for qPCR（EPICENTRE® Biotechnologies）を用いて精製した total RNA を増幅した。
9. 1 反応（20μl）当たりに得られた cDNA の 1/100 量を用いて real-time PCR を行った。Real-time PCR には SYBR® GreenER™ qPCR SuperMix Universal（Invitrogen）を使用し、Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems)により検出、解析した。
10. Real-time PCR に用いた internal control 遺伝子（Eukaryotic translation initiation factor 2B subunit 1 alpha; *EIF2B1*、E47-like factor 1; *ELF1*、Glucuronidase beta; *GUSB*、Hypoxanthine phosphoribosyl transferase 1; *HPRT*、TATA box binding protein; *TBP*、Transferrin receptor; *TFRC*）と細胞増殖マーカーである *KI67*（Antigen identified by monoclonal antibody Ki-67）と *MCM4*（Minichromosome maintenance complex component 4）、*PCNA*（Proliferating cell nuclear antigen）に対するプライマーセットの種特異性は、ヒトとマウス由来の myogenic cell（それぞれ KD3 と Ric10）から合成した cDNA を用いて、RT-PCR により検討した。
11. 各遺伝子検出用プライマーセットの反応効率を確認するため、KD3 と Ric10 由来の cDNA を用いて検量線を作製し、real-time PCR に使用するプライマーセットを検討した。

（1-4 の行程は国立長寿医療センター研究所　再生再建医学研究部において行われ、本研究では 5 以降の行程を分担研究として行った。）

（倫理面への配慮）

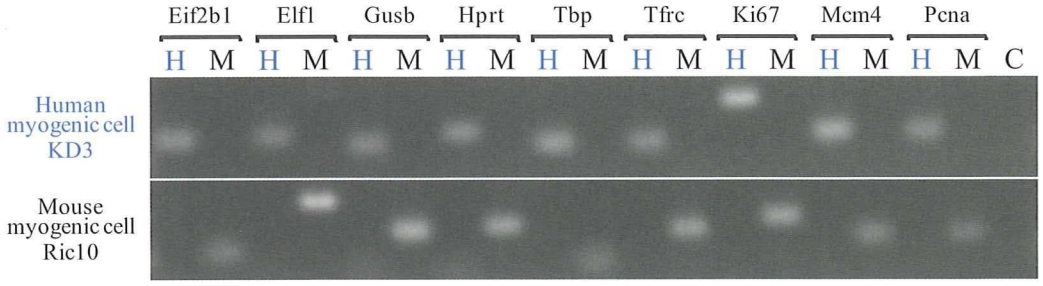
動物およびヒト材料を用いた実験に関しては、国立長寿医療センターの動物実験倫理委員会、倫理委員会の承認を得、規定にしたがって実施した。本研究ではこうした所定の倫理審査を経て得られた検体（不死化ヒト筋前駆細胞を移植されたマウス前脛骨筋）について検討した。

C. 研究結果

1. Real-time PCR 用プライマーの種特異性

今回評価するサンプルは、不死化ヒト筋前駆細胞とマウス内在の筋芽細胞が細胞融合しているため、ヒト由来の遺伝子発現とマウス由来の遺伝子発現を個別に real-time PCR で解析する必要がある。そのため、ヒトとマウスの各遺伝子の塩基配列を比較し、配列の異なる部位にプライマーを設計した。特に、PCR の開始点となるプライマーの 3′末端は、ヒトとマウスで必ず異なる塩基配列になるように工夫した。また、本解析手法では極微量サンプルから mRNA を高感度に検出するため、僅かな genomic DNA の混入があっても解析への影響が懸念される。従って、genomic DNA の混入をイントロンの有無で識別できるように、目的遺伝子に対するセンス、アンチセンスプライマーを隣り合ったエクソンに設計した。さらに、PFA 固定された組織では核酸の断片化も予想されるため、各プライマーにより増幅される配列の長さを 80−150bp とした。各プライマーは相同性検索（UCSC Genome Bioinfomatics; http://genome.ucsc.edu）により、ヒトとマウスのゲノム上で、目的配列以外との相同性が低いことも確認している。これらのプライマーを用いた

RT-PCR の結果、ヒト由来 myogenic cell（KD3）からの cDNA を鋳型として用いた場合では、ヒト発現遺伝子用に設計したプライマーのみで目的産物が増幅され、マウス由来 myogenic cell（Ric10）からの cDNA を鋳型として用いた場合では、マウス発現遺伝子用に設計したプライマーでのみ、目的増幅産物が確認された（Fig. 1）。



Eif2b1、Elf1、Gusb、Hprt、Tbp、Tfrcは用いた内部標準用遺伝子、
Ki67、Mcm4、Pcnaは細胞増殖マーカー蛋白遺伝子を示す。
H、Mはそれぞれヒト、マウス遺伝子mRNA増幅用プライマーを示す。
Cはnegative control（プライマーなしで行ったRT-PCR)を示す。

Fig. 1　RT-PCR による内部標準遺伝子および細胞増殖マーカー蛋白遺伝子mRNA増幅用プライマーの種特異性

2.　各プライマーの反応効率

Real-time PCR による遺伝子発現の定量解析では、相対定量と絶対定量の 2 つの解析手法が一般的に用いられている。本研究では、マウス骨格筋再生段階における不死化ヒト筋前駆細胞移植が持ち得る発癌性の影響を遺伝子発現レベルで解析するため、不死化ヒト筋前駆細胞のマーカーである Venus の蛍光を指標にして、この細胞を移植したマウス筋の組織切片上で、不死化ヒト筋前駆細胞と細胞融合したヒト-マウス筋線維と、それに隣接する、細胞融合をしていないマウス筋線維とを取分け、それらにおける様々な遺伝子の発現量を相対定量により比較検討している。そのため、相対定量解析に重要な、各プライマーの反応効率を測定し、解析可能な internal control 遺伝子と細胞増殖マーカーの組み合わせを同定する必要がある。プライマーの種特異性の確認に用いたヒト由来 KD3 細胞とマウス由来 Ric10 細胞は不死化した増殖性筋芽細胞であり、各細胞増殖マーカー遺伝子の発現も高いため、これらの細胞由来の cDNA を用いて鋳型濃度の希釈系列を作り、各プライマーの検量線を作製してその傾きより、反応効率を計算した（Table 1）。その結果、ヒトとマウスの両方に共通して反応効率が 100%に近いプライマーセットとして、Eif2b1、Tbp、 Tfrc、Ki67、 Mcm4 に対するものを選び、Venus を指標に回収したサンプルの real-time PCR に使用した。

Table 1. 検量線によって評価した個々のプライマーのPCR効率

Human-KD3

Primer	Slope*	%**	R^{2***}
EIF2B1	-3.415	96.3	0.973
ELF1	-3.467	94.3	0.991
GUSB	-2.713	133.7	0.981
HPRT	-2.094	200.3	0.941
TBP	-3.365	98.2	0.977
TFRC	-3.448	95.0	0.988
KI67	-3.444	95.1	0.993
MCM4	-3.502	93.0	0.997
PCNA	-3.724	85.6	0.995

Mouse-Ric10

Primer	Slope	%	R^2
Eif2B1	-3.427	95.8	0.992
Elf1	-3.092	110.6	0.989
Gusb	-3.739	85.1	0.974
Hprt-2	-3.477	93.9	0.990
Tbp	-3.306	100.7	0.983
Tfrc	-3.483	93.7	0.997
Ki67	-3.440	95.3	0.993
Mcm4	-3.477	93.9	0.989
Pcna	-3.768	84.2	0.976

＊Slopeは横軸に初期鋳型DNA濃度の対数をとり、縦軸はCt（Threshold Cycle）値をとったものから計算した。
** %は Slopeから計算した反応効率、*** R^2は検量線の測定値をもとに作製した回帰直線の相関係数の二乗（決定係数）を示す。
赤字はこの検討によって、real-time PCRに使用したプライマーセットを示す。

3. Real-time PCR による検討

Real-time PCR 1 反応（20µl/well）当たり、LCM で回収した Venus(+)または(-)筋線維から合成した cDNA の 1/100 量を用いて real-time PCR を行った。その結果、マウスの発現遺伝子検出用プライマーでは Venus(+)筋線維由来 cDNA を鋳型にした場合、選んだ 5 つ全ての遺伝子が検出され、Venus(-)筋線維由来cDNA を鋳型にした場合では3つの遺伝子（*Eif2b1*、*Tbp*、*Tfrc*）が検出された。一方、ヒトの遺伝子に対する各プライマーではどの遺伝子発現も検出できなかった。これらの反応と同一のプレート（96 well PCR plate）上に、Ric10 細胞から合成した cDNA の希釈系列も作り、その検量線からマウス *Eif2b1* とマウス *Ki67* プライマーの反応効率を測定し、実験の再現性も確認した。両遺伝子のプライマーの反応効率はともにほぼ 100%であり、このプレート（96 well PCR plate）における反応はこれまでの結果を再現していた（Table 2）。従って、このマウス *Eif2b1* とマウス *Ki67* を用いた ΔΔCt 法による相対定量解析を行った。マウス *Eif2b1* とマウス *Ki67* の反応効率は良く相関しており、検量線の各点における ΔCt 値の平均は 2.56（標準偏差; 0.236）であった（Table 3）。一方、これら両遺伝子の発現が認められた Venus(+)筋線維由来 cDNA では同様の ΔCt 値が-1.88 であった（Table 4, 5）。マウス由来増殖性筋芽細胞である Ric10 細胞を基準に求めた ΔΔCt 値は-4.44 であり、反応効率を 100%とすると、Venus(+)筋線維（不死化ヒト筋前駆細胞由来の細胞成分を含む）においてマウス由来の *Ki67* の遺伝子発現量は増殖性 Ric10 細胞の 4.6%（0.046 倍）であった（Table 5）。Venus(-)筋線維（マウス由来筋細胞のみ）では細胞増殖マーカーが検出限界以下であったため、正確な定量は不可能だが、Venu(+)筋線維での反応において Ct 値 35.42 までは検出されていることから、検出限界 Ct 値を 35.5 として計算すると、マウス *Ki67* 遺伝子の発現量は増殖性 Ric10 細胞の 8.2%（0.082 倍）以下であると考えられる（Table 5）。

Table2. マウスEif2b1、Ki67 mRNA検出用プライマーのPCR効率

Mouse-Ric10

Primer	Slope	%	R^2
Eif2B1	-3.368	98.1	0.976
Ki67	-3.302	100.8	0.998

測定の手続きはTable 1の場合と同じ。
Slope、%、R^2の意味は、Table 1の説明を参照

Table 3. マウスEif2b1、Ki67 mRNA検出用プライマーを用いた場合の検量線各点のCt(Threshold Cycle)値とΔCt値

mEif2b1 - Ric10				mKi67 - Ric10				ΔCt: Ct(mEif2b1) - Ct(mKi67)		
Primer	鋳型DNA量	Ct	平均	Primer	鋳型DNA量	Ct	平均	ΔCt	平均	標準偏差
mEif2b1	**10 ng per well**	26.39	26.00	**mKi67**	**10 ng per well**	23.54	23.44	2.56	**2.56**	0.236
		25.95				23.44				
		25.66				23.33				
	2 ng per well	28.36	28.15		**2 ng per well**	25.66	25.56	2.59		
		28.07				25.57				
		28.03				25.45				
	0.4 ng per well	30.61	30.44		**0.4 ng per well**	28.19	28.00	2.44		
		30.42				27.87				
		30.28				27.93				
	0.08 ng per well	32.65	32.70		**0.08 ng per well**	30.18	30.45	2.25		
		32.95				30.68				
		32.49				30.49				
	0.016 ng per well	36.73	35.50		**0.016 ng per well**	32.41	32.53	2.97		
		35.53				32.56				
		34.24				32.63				

Table4. LCMによって採取したVenus (+) あるいは (-) 筋線維中のマウスEif2b1、Ki67 mRNAのreal-time PCRによる検出

検体名	検出遺伝子	Ct	標準偏差	平均
Venus (+) myofibers	mEif2B1	33.77	0.566	33.11
		32.78	0.566	
		32.79	0.566	
	mKi67	34.81	0.376	34.99
		35.42	0.376	
		34.73	0.376	
Venus (-) myofibers	mEif2B1	34.37	0.075	34.45
		34.52	0.075	
		34.45	0.075	

Table 5. Venus(+)、(-)筋線維とRic10細胞におけるKi67 mRNA発現の比較Ct法による相対定量

検体名	mEif2b1 Ct_{Ave}	mKi67 Ct_{Ave}	ΔCt Ct(mEif2b1) - Ct(mKi67)	ΔΔCt ΔCt - ΔCt(Ric10)	$2^{-\Delta\Delta Ct}$
Ric10	*1	*1	2.56	0.00	1.000
Venus (+) myofibers	33.11	34.99	-1.88	-4.44	0.046
Venus (-) myofibers	34.45	35.5*2	-1.05	-3.61	0.082

*1 Table 3を参照。
*2 Ct value 推定値。Table 4を参照。

D. 考察

今回のような固定サンプルからの RNA 回収は一般に困難とされている。しかも、今回は組織切片での所見に基づいて、特定の筋線維のみを laser capture microdissection で切り出し、RNA を回収すると言う、より困難な条件であった。固定組織からの RNA 回収段階で反応温度を 70℃とすることによりアルデヒド架橋を外し、グアニジン塩酸でなく Proteinase K 処理することで回収を試みている報告(Masuda et al., Nucleic Acid Res 27: 4436-4443, 1999)があり、その方法に準拠して、500 の再生筋線維から定量的PCRが可能な量の RNA が得られた。この RNA から合成した cDNA の 1/100 量を用いて real-time PCR を行ったので、理論的には Real-time PCR 1 反応（20μl/well）当たり、厚さ 10μm の組織切片上の 5 個の筋線維からの mRNA が含まれることになるが、チューブやピペットチップへの核酸の吸着による損失や、PFA 固定による mRNA へのダメージを考慮すると、実際には 1 反応当たり 1~2 個程度の筋線維由来の mRNA しか含まれないと推察される。このことからも、本評価方法は超高感度な定量的遺伝子発現解析手法であると考えられる。こうした手法は、様々な筋疾患の病理組織標本から得られる病理組織学的情報と遺伝的情報を総合的に検討していく上で有用な手段となると考えられる。

今回の研究では Venus 蛍光（+）の、移植した不死化ヒト筋前駆細胞に由来する再生筋線維での細胞増殖関連蛋白の mRNA 量を相対定量した結果、増殖マーカー蛋白として頻繁に用いられる Ki67 mRNA（マウス）の発現量は、増殖性を持つマウス由来 myogenic cell である Ric10 細胞の 4.6%であることが判明した。Venus(-)筋線維では細胞増殖マーカーが検出限界以下であり、Venus(+)、(-)筋線維間の直接の比較はできないが、検出限界 Ct 値を 35.5 と仮定した推定では、Venus(-)筋線維でのマウス Ki67 mRNA の発現量は Ric10 細胞の 8.2%以下である。このデータと、我々が以前に得た、Venus(+)筋線維では、Venus (-)筋線維に比べて Mcm4 の免疫活性はむしろ低い(未発表)、子宮頸癌と正常子宮頸部粘膜では Mcm4、Ki67 の発現変化が平行している (Ishimi et al., Eur J Biochem 270: 1089-1101、2003)との免疫組織化学的データを合わせて考えると、Venus(+)、(-)筋線維間で Ki67 の発現に著しい差があるとは考えにくく、この点からは不死化ヒト筋前駆細胞を含む再生筋線維が強い増殖活性を持っていることは否定的である。

再生筋線維では様々な強度の Venus 蛍光が観察され、ヒト由来、マウス由来筋芽細胞が様々な比率で細胞癒合した結果と判定されるが、今回、Venus 蛍光強度が強く、より多くの不死化ヒト筋前駆細胞を含むと思われる

Venus 蛍光強度の強い筋線維においても、ヒト遺伝子の発現は確認できなかった。おそらく、こうした筋線維でも、不死化ヒト筋前駆細胞の癒合比率は多くとも50%未満と推定される。含まれる細胞数が少なくとも、個々の細胞が強い増殖活性を持っていれば、一定の細胞増殖マーカー関連遺伝子の発現が認められることが予想されるが、今回は検出来なかったことから、Venus(+)筋線維における不死化ヒト筋前駆細胞に由来する細胞増殖性は低いものと判断される。今回検討した遺伝子発現と、これまでに行った病理組織学的解析を合わせて、不死化ヒト筋前駆細胞の移植によってできた再生筋線維の増殖性は低く、癌化の危険性はないと考えられる。

E. 結論

1. Venus 遺伝子導入不死化ヒト筋前駆細胞をマウス前脛骨筋に移植した標本について、細胞増殖関連蛋白遺伝子の mRNA 発現を定量的 PCR 法によって行った。
2. Venus 蛍光が強く、不死化ヒト筋前駆細胞を多く含むと思われるヒトーマウス筋細胞癒合再生筋線におけるマウス Ki67 mRNA の発現は、増殖能の強いマウス由来 myogenic cell である Ric10 での発現の 4.6%であった。ヒト Ki67 mRNA の発現は検出限界以下で、検出限界 Ct 値からの推定値では 8.2%以下と考えられた。
3. 以前の病理組織学的検索結果と合わせて、移植したヒト筋前駆細胞が強い増殖活性を持ち、腫瘍化する危険性は極めて考えにくいものと判断される。
4. 今回用いた定量的 PCR 法は、病理組織切片上での遺伝子発現を検討指定いくうえでの有用な方法と見込まれる。

F. 研究発表

1. 論文発表
   なし
2. 学会発表
   なし

G. 知的財産権の出願・登録状況

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

研究成果の刊行に関する一覧表

1. Yamamoto T, Gotoh M, Hattori R, Toriyama K, Kamei Y, Iwaguro H, Matsukawa Y, Funahashi Y: Periurethral injection of autologous adipose-derived stem cells for the treatment of stress urinary incontinence in patients undergoing radical prostatectomy: Report of two initial cases. Int J Urol, 17:75-82, 2010
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Original Article: Clinical Investigation

Periurethral injection of autologous adipose-derived stem cells for the treatment of stress urinary incontinence in patients undergoing radical prostatectomy: Report of two initial cases

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Objectives: To report a novel cell therapy using autologous adipose tissue-derived stem cells (ADSC) for stress urinary incontinence caused by urethral sphincteric deficiency and the outcomes in two initial cases undergoing periurethral injection of stem cells for the treatment of urinary incontinence after radical prostatectomy.

Methods: Two patients with moderate stress incontinence after radical prostatectomy were enrolled. After liposuction of 250 mL of adipose tissue from the abdomen, we isolated ADSC from this tissue by using the Celution system. Subsequently, the isolated ADSC and a mixture of stem cells and adipose tissue were transurethrally injected into the rhabdosphincter and submucosal space of the urethra, respectively. Short-term outcomes during a 12-week follow-up were assessed by a 24-h pad test, a validated patient questionnaire, urethral pressure profile, transrectal ultrasonography, and magnetic resonance imaging.

Results: Urinary incontinence progressively improved after 2 weeks of injection up to 12 weeks in terms of decreased leakage volume in a 24-h pad test, decreased frequency and amount of incontinence, and improved quality of life as per the questionnaire. In urethral pressure profile, both maximum urethral closing pressure and functional profile length increased. Ultrasonography and magnetic resonance imaging showed sustained presence of the injected adipose tissue. Enhanced ultrasonography showed a progressive increase in the blood flow to the injected area. No significant adverse events were observed peri- and postoperatively.

Conclusion: This preliminary study showed that periurethral injection of the autologous ADSC is a safe and feasible treatment modality for stress urinary incontinence.

Key words: adipose-derived stem cells, injection, radical prostatectomy, stress urinary incontinence, urethra.

Introduction

Prostate cancer is one of the most highly prevalent malignancies among the male population, and radical prostatectomy has been used worldwide as a standard treatment method for localized prostate cancers. Urinary incontinence is a distressing complication of radical prostatectomy and is associated with the functional impairment of the external urethral sphincter. The incidence of urinary incontinence after radical prostatectomy varies greatly across reported literature. Carlson *et al.*[1] summarized the reported rates of postprostatectomy incontinence in a large series and found that it varied from 2% to 90%. The large discrepancies in the reported incidence rates of postprostatectomy incontinence are attributed to a variety of factors such as definitions of continence, method of data collection, and the duration of follow-up; nonetheless, a substantial number of patients are known to suffer from long-lasting moderate to severe incontinence after radical prostatectomy.[2] Although a variety of treatment modalities have been attempted,[3] there has not been any well established treatment for postprostatectomy incontinence. Hence, there is an extreme need to develop a new minimally invasive and effective treatment modality for postprostatectomy incontinence.

Cell therapy for the regeneration of injured tissues has recently been extensively investigated at an experimental level, and its clinical application in a variety of fields has also been in progress. Mesenchymal stem cells (MSC) are multipotent adult stem cells that can proliferate in culture and are able to differentiate into a variety of mesenchymal cell phenotypes.[4–8] Thus far, MSC have been mainly harvested from bone marrow, a tissue source that has many

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limitations. These include donor-site morbidity in the bone marrow, which limits the amount of marrow that can be obtained,[9,10] MSC represent less than 0.01% of all nucleated bone marrow cells in healthy volunteers,[11,12] and an extended culture time is required to obtain therapeutic cell doses of MSC by using the *ex vivo* cell expansion method.

It has recently been shown that adipose tissue contains multipotent cells that are similar to MSC,[13,14] and the abundance of stem cells in the adipose tissue is 100-fold higher than that in the bone marrow. This finding has generated major interest because, unlike bone marrow cells, adipose tissue can be easily and safely harvested in large quantities with minimal morbidity, making it an appealing source for cell therapy. It has been shown that adipose-derived stem cells (ADSC) are multipotent and differentiate into several cell types[4,7,8,15] such as bone, cartilage, fat, nerves, blood vessels, and contractile cells with striated muscle cell[6] or cardiomyocyte features.[6,16] In addition, it has been shown that cultured ADSC secrete a variety of angiogenesis-related cytokines, such as hepatocyte growth factor and vascular endothelial growth factor.[17]

The Celution system (Cytori Therapeutics, San Diego, USA) is a commercially available device that allows the isolation of ADSC fractions from human adipose tissue in a short time.[18] This instrument allows the isolation of therapeutic doses of autologous ADSC after liposuction, obviating the need for culture. We developed a novel cell therapy for stress urinary incontinence (SUI) caused by urethral sphincter deficiency; the cell therapy included periurethal injection of autologous ADSC. In the present study, we report our experience of two initial patients with SUI undergoing this therapy after radical prostatectomy and focused on the procedure and short-term outcomes.

Methods

The present study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine and written informed consent was obtained from both the patients.

Patients

In the present study, two patients with SUI after radical prostatectomy were enrolled. The inclusion criteria for the patients was as follows: persistence of moderate to severe urinary incontinence for more than 2 years after surgery and no evidence of recurrence or metastasis of prostate cancer, with undetectable levels of prostate-specific antigen (<0.008 ng/mL). The first patient (Case 1) was 75 years-of-age, had undergone radical prostatectomy 3 years earlier with a pathological stage of T2N0M0 and had undetectable levels of prostate-specific antigen at enrolment. He had moderate SUI without urgency. The second patient (Case 2) was 83 years-of-age, had undergone radical prostatectomy 7 years earlier with a pathological stage of T1cN0M0 and had undetectable levels of prostate-specific antigen at enrolment. He had moderate SUI without urgency and had undergone periurethral injection of collagen (Contigen, Bard, Covington, GA, USA) 3 years earlier, which was uneventful. Both patients had no pharmacologic treatment for SUI before and after the present treatment.

Harvesting adipose tissue (liposuction)

Under general anesthesia, 250 mL of adipose tissue was harvested from the anterior abdominal wall by making two 3-mm incisions. An 18-G Becker cannula with a 50 mL syringe was used as a collecting device; Ringer's lactate was first infused in the subcutaneous layer, then the adipose tissue was harvested. The suctioned adipose tissue contained in the saline was allowed to stand for settling the blood and cellular debris; adipose tissue floated to the top of the mixture.

Isolation of ADSC

ADSC were isolated from the harvested adipose tissue by using the Celution system[18]. Briefly, adipose tissue was introduced into the Celution cell-processing device, which automatically and aseptically extracts and concentrates the mononuclear fraction of adipose tissue and removes unwarranted or deleterious cells, cell and matrix fragments. It required around 1 h to process 250 mL of liposuction tissue. The final concentrated cell output collected using the Celution system was counted using a NucleoCounter (Chemometec, Allerod, Denmark), which exclusively detected nucleated cells. By using the Celution system, we could finally obtain a 5 mL solution containing concentrated ADSC.

Periurethral injection of ADSC

Subsequent to liposuction and isolation of ADSC, transurethral endoscopic injection of ADSC was carried out. For periurethral injection of ADSC, two distinct formulations were produced: 1 mL of the isolated ADSC fraction alone was preserved for direct injection and another 4 mL of the fraction was mixed with intact autologous adipose, producing a total of 20 mL of this combined solution.

A 22-Fr rigid nephroscope was used for injecting the processed ADSC solution. The nephroscope was inserted into the urethra. Under endoscopic vision, a puncture needle was passed through the nephroscope into the urethra at the region of the external urethral sphincter. The puncture needle with a thickness of 18 gauge, a length of 35 cm and graduated in centimeters was specially ordered. After puncturing the urethra at the region of the external urethral

sphincter under endoscopic vision, the ADSC solution was injected. Initially, a 1 mL solution was injected at a depth of 5 mm into the rhabdosphincter at 5 and 7 o'clock positions. Subsequently, 20 mL of the formulation containing ADSC and adipose tissue was equally injected into the submucosal spaces at 4, 6, and 8 o'clock positions to facilitate complete coaptation of the urethral mucosa by the bulking effect. After the solution was injected, a 6-Fr urethral balloon catheter was placed and was removed the next day to allow micturition.

Outcome measures

The amount of incontinence was evaluated by a 24-h pad test. The amount of leakage was calculated by subtracting the dry weight of the pad from that of the wet pad. The total daily leakage amount was calculated. The 24-h pad test was consecutively repeated for 4 days for each evaluation period. The subjective symptoms and quality of life were evaluated using a validated disease-specific questionnaire – the International Consultation on Incontinence Questionnaire-Short Form (ICIQ-SF)[19,20] (Appendix). In the ICIQ-SF, the therapeutic effects in terms of frequency of urinary incontinence (0–5 point scores), amount of leakage (0–6 point scores) and impact on everyday life (0–10 point scores) were examined, and the total score ranging from 0 to 21 points was calculated. A high score indicated an unfavorable condition. These parameters were assessed at baseline and repeated 2, 4, 8, and 12 weeks after treatment.

The urethral sphincter function was objectively assessed by measuring the urethral pressure profile, using urodynamic equipment (MMS, Enschede, the Netherlands). Maximum urethral closing pressure (MUCP) and functional profile length (FPL) were measured at baseline, and 2 and 12 weeks after treatment.

The condition of the urethra after the administration of ADSC was monitored by transrectal ultrasonography. In addition, the blood flow to the area where ADSC were injected was assessed by contrast-enhanced transrectal ultrasonography by intravenously injecting perflubutane.[21] The morphological condition of the injected area was monitored by magnetic resonance imaging (MRI). These imaging examinations were carried out at 4 days, and 4 and 12 weeks after treatment.

Results

Liposuction from the abdomen was carried out without significant morbidity and 250 mL of adipose tissues could be harvested. The isolated adipose tissue solution contained 2.38×10^7ADSC (2.18×10^7 viable cells) and 3.20×10^7 (2.87×10^7 viable cells) in cases 1 and 2, respectively.

The urethral lumen at the region of the external urethral sphincter remained open, as observed by endoscopy, whereas the urethral lumen completely closed after the periurethral injection (Fig. 1).

Urinary incontinence disappeared within 1 week after receiving the injection, but it deteriorated thereafter. However, urinary incontinence progressively improved after 2 weeks of injection up to 12 weeks. This improvement in urinary incontinence was observed in both the cases (Tables 1,2). At 12 weeks after injection, urinary incontinence improved in terms of leakage volume measured by a 24-h pad test. Assessment of subjective symptoms and quality of life on the basis of the ICIQ-SF questionnaire showed similar improvement. Sphincteric function of the urethra was improved in both the cases, in terms of increased MUCP and FPL (Tables 1,2).

After the urethral catheter was removed, both the patients could void without significant residual urine. Neither of the patients complained of voiding symptoms. Uroflowmetry did not show significant voiding dysfunction or an increase in the amount of residual urine (Tables 1,2).

Transrectal ultrasonography morphologically showed the presence of the injected adipose tissue in the lateral and posterior regions of the urethral lumen; this tissue could also be located at 12 weeks after injection. In addition, enhanced ultrasonography showed a sequential increase in the blood

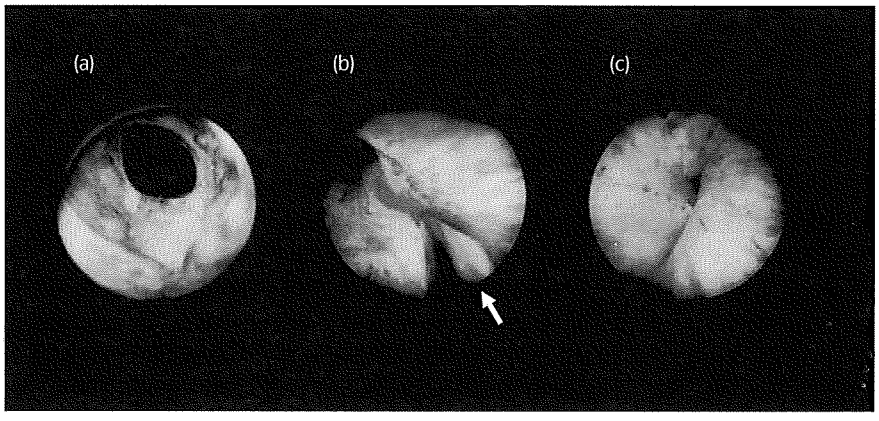

Fig. 1 Endoscopic findings of periurethral injection of adipose-derived stem cells in Case 1. (a) Before injection, the urethral lumen at the region of the external urethral sphincter was open. (b) An 18-G needle was injected into the urethra at 4 o'clock of the region of the external urethral sphincter (the arrow shows a puncture needle). (c) After completing the injection, the urethral lumen was closed and complete coaptation of the urethral mucosa was obtained.

Table 1 Clinical outcome in Case 1

	Baseline	2 Weeks	4 Weeks	8 Weeks	12 Weeks
24-h pad test (g) during 4 days (mean)	40/35/25/40 (35.0)	39/21/10/28 (24.5)	14/0/0/6 (5.0)	15/0/0/15 (7.5)	9/0/0/6 (3.8)
MUCP (cmH_2O)	28	25	–	–	43
FPL (mm)	14	20	–	–	32
ICIQSF (frequency of leakage)	4	4	3	2	2
ICIQSF (amount of leakage)	4	2	2	2	2
ICIQSF (QOL)	4	3	3	3	3
ICIQSF (total score)	12	9	8	7	7
Qmax	17.8	8.0	–	–	20.5
Postvoid residue (mL)	30	38	–	–	0

FPL, functional profile length; ICIQSF, International Consultation on Incontinence Questionnaire-Short Form; MUCP, maximum urethral closing pressure; Qmax, maximum flow rate; QOL, quality of life.

Table 2 Clinical outcome in Case 2

	Baseline	2 Weeks	4 Weeks	8 Weeks	12 Weeks
24-h pad test (g) during 4 days (mean)	76/22/40/38 (44.0)	30/20/20/23 (23.3)	30/25/25/15 (23.8)	23/18/16/19 (19.0)	20/19/18/16 (18.3)
MUCP (cmH_2O)	21	23	–	–	36
FPL (mm)	17	22	–	–	27
ICIQSF (frequency of leakage)	5	2	3	3	3
ICIQSF (amount of leakage)	4	2	2	2	2
ICIQSF (QOL)	10	3	3	3	3
ICIQSF (total score)	19	7	8	8	8
Qmax	9.7	13	–	–	8.1
Postvoid residue (mL)	20	27	–	–	10

FPL, functional profile length; ICIQSF, International Consultation on Incontinence Questionnaire-Short Form; MUCP, maximum urethral closing pressure; Qmax, maximum flow rate; QOL, quality of life.

flow to the area where ADSC were injected, which was maintained during the entire follow-up period (Fig. 2). MRI showed a bulking effect at the site of adipose tissue injection, which persisted even at 12 weeks after injection (Fig. 3).

No significant adverse event was noted throughout the liposuction and ADSC injection procedures. No severe side-effects such as pelvic pain, inflammation, or de novo urgency were observed after the operation in both the cases during the postoperative follow-ups.

Discussion

ADSC have been successfully used in a variety of indications in humans, including the treatment of Crohn's disease-associated fistulas,[22] Osteogenesis imperfecta,[23] and for breast augmentation and reconstruction after partial mastectomy.[24] Characterization and safety of ADSC isolated by the Celution system were reported in a basic investigation.[18] To explore the safety and feasibility of ADSC transplantation in patients with myocardial infarction, the first-in-man randomized controlled trial is currently in progress in the Netherlands.[16] The cell therapy in the present study is the first attempt to use ADSC for treating SUI.

Before applying this new therapeutic technique to humans, we carried out several animal experiments using rats to confirm the effect of the periurethral injection of ADSC on the urethral resistance and sequential changes of the injected rat ADSCs.[25] Cultured rat ADSC were injected into the proximal urethra of rats after bilateral transection of the pelvic nerves. Bladder leak point pressure was measured 4 weeks after injection of ADSC, GAX-collagen or vehicle. Leak point pressure was significantly higher in the rats undergoing ADSC injection as compared with those undergoing injection of collagen or vehicle. Additionally, green fluorescent protein (GFP)-expressing cultured ADSC

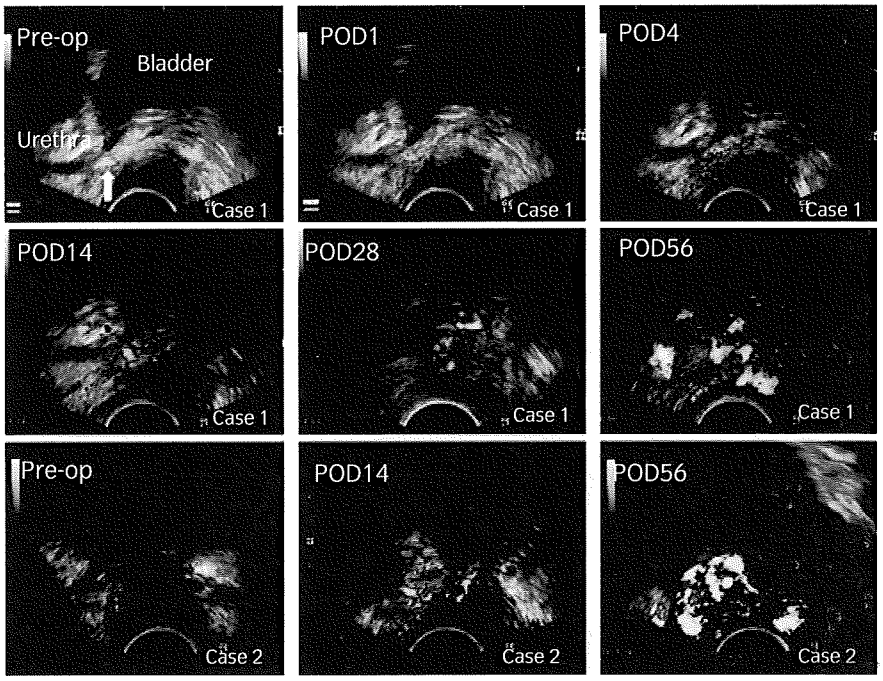

Fig. 2 Contrast-enhanced transrectal ultrasonography to assess the blood flow of the periurethral area after adipose-derived stem cells (ADSC) injection. The bladder and urethra were visualized as a sagittal section. The blood flow around the injected area, visualized as orange color, was progressively increased in both Case 1 and 2 after the injection of ADSC. POD, postoperative day.

obtained from male GFP rats were injected into the urethra of female nude rats. Four weeks after the injection, anti-GFP antibody-positive cells were abundantly stained at the region of ADSC injection. Furthermore, 12 weeks after the injection, although in a small proportion, alpha smooth muscle actin (SMA)-positive cells were stained in the merged distribution with the GFP expressing ADSC, suggesting possible differentiation of ADSC into smooth muscle cells. The results of these preliminary animal experiments support the present clinical outcomes, such as progressive improvement of sphincteric function and incontinence. Zeng *et al.*[26] also reported the feasibility of ADSC use as an improvement in leak point pressure and urethral function of SUI when animals were injected with ADSC in conjunction with hepatocyte growth factor-impregnated microspheres.

Recently, cell therapy using autologous adult muscle-derived stem cells has been developed for treating SUI. A small muscle biopsy sample was obtained from the upper arm and cultured to harvest two types of autologous muscle-derived cells: myoblasts and fibroblasts. The muscle-derived stem cells were injected transurethrally into the urethra under continuous monitoring. Carr *et al.*[27] reported the outcomes of 1-year follow-up of autologous muscle-derived stem cell injection to treat eight women with stress incontinence. With a mean follow-up period of 16.5 months, SUI improved in five patients, with one achieving total continence, and no serious adverse events were noted. The present treatment strategy has an important advantage over the use of muscle-derived stem cells. Because adipose tissue contains abundant multipotent stem cells, as well as key mature cells and progenitor cells, therapeutic levels of ADSC can be obtained rapidly using the Celution system. In the present study, a sufficient number of stem cells could be isolated from each patient using this system. Unlike other cell therapy strategies, the treatment is totally autologous, requires no cell culture and is carried out in the context of a single surgical procedure.

Periurethral injection of autologous fat was previously investigated in female patients with SUI; adipose tissue harvested from the abdomen was transurethrally injected into the submucosal layer under endoscopic vision. Although the injected adipose tissue could sustain a bulking effect, its efficacy was reported to be poor. In a randomized controlled trial that compared the efficacy of fat injection with that of a placebo injection (saline), the researchers found that the improvement rate after fat injection was poor (22%) at 3 months, with no difference than that produced by the placebo.[28] These findings suggested that mature adipocytes were unable to survive at the injected site.

In the periurethral injection of ADSC, it has been suspected that a variety of mechanisms are involved in the ADSC-mediated improvement of the sphincteric function. A similar clinical course in both the cases implies the involvement of specific factors that can suggest the mechanisms underlying the treatment strategy, such as urinary incontinence that disappeared within a week after injection then deteriorating subsequently, progressively improved thereafter up to 12 weeks after the injection. A bulking effect produced by the injected adipose tissue fraction mixed with ADSC is of primary importance. The injected adipose tissue fraction, which was processed to isolate ADSC, contained 30% of lactated Ringer's solution. Absorption of the

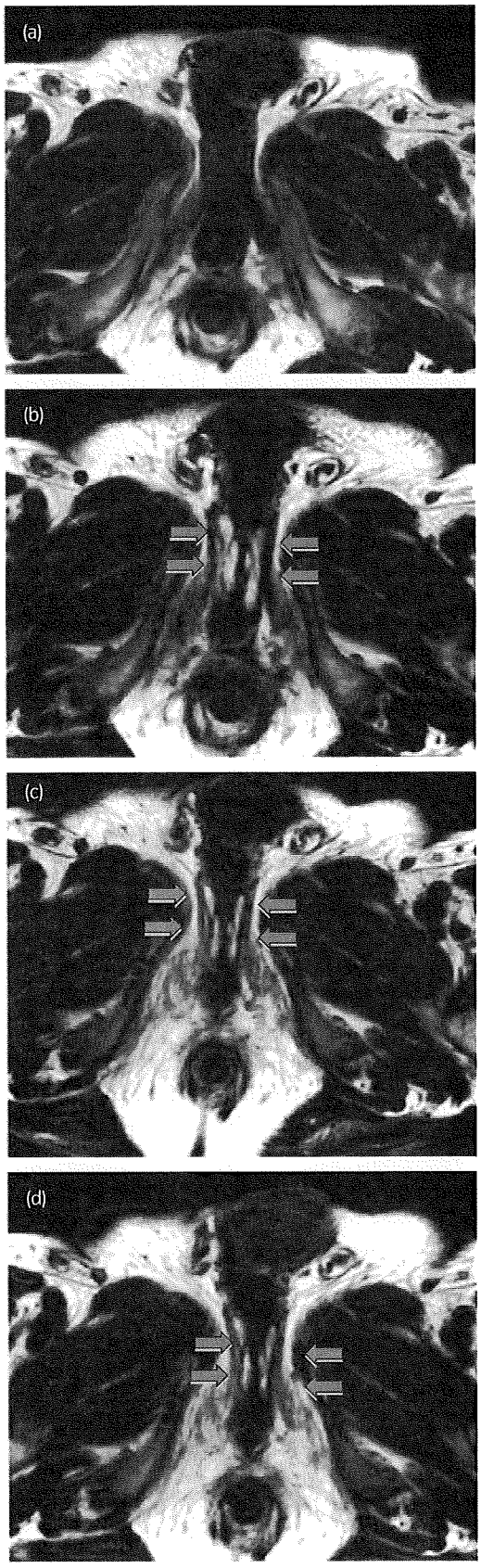

Fig. 3 Magnetic resonance imaging around the urethra before and after the adipose-derived stem cells (ADSC) injection. The urethra in Case 1 was visualized as a cross-section on magnetic resonance imaging. (a) Before injection, (b) 4 days after injection, (c) 4 weeks after injection, and (d) 12 weeks after injection. The arrows show the adipose tissue injected with ADSC. At 4 weeks after injection, the volume of injected adipose tissues had decreased compared with that after 4 days after injection. However, the volume did not change thereafter and persisted at 12 weeks after injection.

solution could be responsible for the temporary deterioration in the patient's condition during the initial week. ADSC might have contributed to the progressive improvement in sphincteric function, which was shown in terms of increased MUCP and FPL, as well as decreased frequency and amount of urinary incontinence. Persistent bulking effect indicates the survival and growth of the injected adipose tissue, which could also be attributed to the presence of ADSC. ADSC might differentiate into mature adipose tissue and, possibly, into contractile cells. Previous studies on rats showed that cultured adipose-derived stem cells injected into the injured urethra differentiated into contractile cells with smooth muscle cell features.[29] Indirect effects of the injected ADSC might also be responsible for the improvement in the sphincteric function. Cultured ADSCs\ are known to secrete a large number of angiogenesis-related cytokines.[17] In the present study, increased blood flow to the injected area was confirmed by ultrasonography. The increased blood flow was not temporary, but was maintained throughout the follow-up period; this increased blood flow could be the result of the angiogenesis effect of the cytokines secreted by the injected ADSC. The increased blood flow might have a positive effect on the regeneration of the injected adipose tissue and impaired intrinsic sphincteric function.

Conclusions

The present preliminary study showed that periurethral injection of the autologous adipose-derived stem cells is a safe and feasible treatment modality for stress urinary incontinence. We could establish the clinical course and obtained excellent short-time outcomes in the two initial cases who underwent this cell therapy. Hence, we intend to increase the number of patients and confirm the long-term outcomes of this treatment modality.

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Appendix

ICIQ-SF

1. How often do you leak urine? *(Tick one box)*		
Never	☐	0
About once a week or less often	☐	1
Two or three times a week	☐	2
About once a day	☐	3
Several times a day	☐	4
All the time	☐	5

We would like to know how much you think leaks.

2. How much urine do you usually leak (whether you wear protection or not)?

(Tick one box)

None	☐	0
A small amount	☐	2
A moderate amount	☐	4
A large amount	☐	6

3. Overall, how much does leaking urine interfere with your everyday life?

Please ring a number between 0 (not at all) and 10 (a great deal)

0	1	2	3	4	5	6	7	8	9	10
Not at all										A great deal

ICIQ score: sum scores 1 + 2 + 3 ☐

Change in Contralateral Renal Parenchymal Volume 1 Week After Unilateral Nephrectomy

Yasuhito Funahashi, Ryohei Hattori, Tokunori Yamamoto, Osamu Kamihira, Yoshie Moriya, and Momokazu Gotoh

OBJECTIVES To measure the contralateral renal parenchymal volume (RPV) before and after nephrectomy and investigate the factors influencing compensatory hypertrophy. Unilateral nephrectomy induces compensatory hypertrophy in the contralateral kidney.

METHODS From December 2003 to January 2008, 142 patients undergoing nephrectomy were enrolled in this study. All patients underwent preoperative technetium-99m dimercaptosuccinic acid renal scintigraphy. The percentage of technetium-99m dimercaptosuccinic acid uptake in the resected kidney was 37.2% ± 15.3%. Contrast-enhanced computed tomography was performed preoperatively and 1 week and 6 months postoperatively, and RPV was calculated as the normally functioning tissue, excluding tumors or nonenhanced areas.

RESULTS The mean RPV of the remaining kidney was 164.2 cm^3 preoperatively and 184.1 and 178.8 cm^3 at 1 week and 6 months postoperatively, respectively. Multivariate regression analysis revealed that the increase in RPV was positively associated with the percentage of technetium-99m dimercaptosuccinic acid uptake in the resected kidney ($P < .001$) and negatively associated with patient age ($P = .008$). Logistic regression analysis showed that the group with an RPV increase of <15% had a 4.1-fold increased risk of a 10% decrease in the glomerular filtration rate during the next 6 postoperative months compared with the risk in the group with an RPV increase of ≥15% ($P = .004$).

CONCLUSIONS The change in contralateral RPV occurred during the first week after nephrectomy and remained stable for ≥6 months. The change in RPV increased when the removed kidney had greater function and decreased with increasing patient age. The risk of progression to renal insufficiency can be predicted according to the change in RPV. UROLOGY 74: 708–712, 2009. © 2009 Elsevier Inc.

The number of patients undergoing unilateral nephrectomy for renal malignancy has increased because of the aging population and the widespread use of various imaging modalities such as ultrasonography, computed tomography (CT), and magnetic resonance imaging. Because these patients tend to be elderly, it is important to predict the postoperative renal function when selecting the appropriate surgical procedure, whether radical or partial nephrectomy. Many reports have described the functional adaptation of the remaining kidney after unilateral nephrectomy. The glomerular filtration rate (GFR) increases to 60%-80% of the preoperative level within several weeks postoperatively and then stabilizes or increases very slightly for >10 years after nephrectomy.[1,2] Advanced age, female sex, hypertension, and proteinuria have been reported as negative factors for functional adaptation.[3-5] Not only the function, but also the size of the remaining kidney is known to be a factor in the ability to compensate for the loss of functioning nephrons. Most of the renal growth occurs in the cortex, particularly in the proximal tubules, and appears to be of a hypertrophic (an increase in cell size), rather than a hyperplasic (an increase in cell number) nature.[6] However, few reports have described the sequential changes in renal volume after unilateral nephrectomy or the correlation between hypertrophy and renal function. The evaluation of contiguous CT slices is a reliable, objective, and reproducible method of assessing the renal volume.[7] The kidney volume has been shown to correlate well, although indirectly, with the number of functioning nephrons found in autopsy studies,[8] and the correlation between the renal parenchymal volume (RPV) calculated using CT images and the single-kidney GFR is significant.[9] In the present study, we measured normally functioning renal tissue, excluding tumors, cystic lesions, and low-density areas,

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